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Current Trends in Biotechnology and Pharmacy
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Personalized Medicine for Cancer in the Developing World

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Abstract

Personalized Medicine evolved from the genomics era and allows disease prediction based on genetic mutation, and development of individualized health care, both preventive and responsive, as a consequence of a patient's own genetic features. Personalized medicine for cancer is similarly proactive and individualized, based on genetic information and used to manage cancer risk and disease for solid and lymphoproliferative cancers, those with a hereditary basis and those arising spontaneously. In the developing world, cancers now kill more people than infectious disease and while many resource-limited countries still lack basic facilities to care for cancer patients, middle-income countries, such as Panama, are beginning to make simple applications of personalized medicine for cancer diagnosis and treatment. These applications focus on cancers which affect the greatest number of people and those for which proven tests and therapies already exist. Three such cancers are lung, breast and colorectal cancers, which have similarities in the biochemical dysfunctions at their foundations. A limited and affordable portfolio of genetic tests could be established by developing world diagnostic laboratories to aid oncologists in risk assessment, diagnosis, prognosis and pharmaceutical choice in the personalized management of cancer. The establishment and clinical use of these tests in developing world nations will require innovative models of financing and strengthening of human resources and technical and legislative infrastructure.

Keywords: Panama, Individualized Medicine, Genetic Testing, Medical Oncology, Developing World

Introduction

Personalized Medicine is an advance in patient care arising from genomics. At its foundation is our capacity to read and analyze an individual's genome, allowing identification of changes in the genetic code that correlate with a phenotype of increased disease risk. While practicing medicine has always been "personal" in nature, with a physician responding to the specific medical needs of a specific patient in a specific way which is standard for the disease condition presented, personalized medicine is distinguished from traditional care in two ways. Firstly, it precisely predicts disease occurrence based on changes to an individual's genetic code, rather than relying on presentation of clinical symptoms to identify a disease condition. In this way, it is a proactive, not reactive form of care. Secondly, it uses the genetic information about disease risk to develop customized preventive and responsive health care for individual patients, based on genetic features.

Analysis of an individual's disease risk based on their genetic background was a possibility which evolved with the completion of

the Human Genome Project in 2000. For the first time, scientists and physicians could read the entire human genetic code and begin to correlate genotype with phenotype, for disease conditions and other traits. Since 2000, when one genome was sequenced for approximately 3 billion USD (1), decreasing sequencing costs have led to the very real prospect of sequencing a human genome for less than 1000 USD (2, 3). A handful of high profile artists, scientists and religious figures have already had their own genomes sequenced to publicize various health issues and initiate discussion on genomic technology and its ethical implications (4). Some genomicists predict we will have the capacity to sequence every human genome at birth within the next 10 years.

Until the time that genome sequencing for disease prediction is routine in patient management, however, personalized medicine must take a broader definition. The first major textbook on genomics and personalized medicine suggests it is characterized by "the use of predictive tools to develop a new model of health care based on health planning that is proactive and preventive", compared to traditional medicine, which is "reactive, episodic and geared towards acute crisis intervention once disease is already manifest and largely irreversible" (5). The National Cancer Institute recognizes personalized medicine as "a form of medicine that uses information about a person's genes, proteins and environment to prevent, diagnose and treat disease" (6) and the Jackson Laboratory notes that personalized medicine "shifts the emphasis in medicine from reaction to prevention; predicts susceptibility to disease, improves disease detection and preempts disease progression; customizes disease-prevention strategies; prescribes more effective drugs and avoids prescribing drugs with predictable side effects; reduces the time, cost, and failure rate of pharmaceutical clinical trials, and eliminates trial-and-error inefficiencies that inflate health care costs and undermine patient care" (7).

Personalized medicine for cancer : Personalized medicine for cancer is similarly based on the foundations of prediction and prevention. By using information on personal genetics and environment, management of cancer risk and disease can be proactive and individualized. Cancers with underlying hereditary or infectious cause and those arising sporadically will all benefit from applying personalized medicine in the form of genetic and genomic testing, followed by clinical surveillance or therapy specifically appropriate to the individual's genetic information. Standard community-based screening and diagnostic programs, such as those for bowel, cervical, breast and prostate cancer, can now be complemented by such gene-based tests, which are available for pre-symptomatic risk assessment of cancer, as well as diagnosis, prognosis and treatment optimization for a number of solid and lymphoproliferative malignancies (7).

Cancer in the developing world : Of the 12.6 million new cases of cancer and 7.5 million cancer deaths, every year, world-wide (8), 56% of cases and 64% of deaths occur in low- and middle-income countries (9). Not only is the burden of cancer mortality and morbidity higher in developing countries (Table 1), but individual cancer risk, currently higher in the developed world, is also increasing in the developing world (10).

In low- and middle-income countries, increased risk of cancer and cancer death arises from exposure to carcinogens including alcohol, cigarette and fire smoke, and oncogenic infectious agents, thought to cause 20% of cancer in these nations (11); the presence of specific racial groups with higher cancer susceptibility due to genetic background (discussed below); increasing longevity from improved nutrition, infectious disease control, maternal-child health programs, and economic growth and political stability; and perhaps most significantly, decreased and delayed diagnosis and treatment options (12).

In at least thirty low- and middle-income countries, cancer diagnosis and treatment are retarded due to the absence of basic resources, such as tools to analyze Pap smears, radiation therapy machines and trained technicians and oncologists (12). In these countries, a primary goal in caring for cancer patients has been to establish essential physical and human infrastructure, a task being supported by the IAEA's Programme of Action for Cancer Therapy (13). A second goal is ensuring that basic programs for cancer prevention are established and promoted: vaccination campaigns against Hepatitis B and Human Papillomavirus; public education campaigns against smoking and excessive alcohol consumption; control of malaria, co-implicated with Epstein-Barr virus as the underlying cause of African Burkitt's lymphoma (14); and screening programs for cervical and breast cancer, as well as for *Helicobacter pylori* and liver fluke, significant contributors in some regions to gastric and liver cancer, respectively (14). In these nations, furthermore, while cancer causes more deaths than malaria, tuberculosis and HIV/AIDS combined, health care priorities may justifiably be focused elsewhere than cancer for some years to come (15).

In middle-income countries, however, where basic laboratory, clinical and public health infrastructure and cancer-care services already exist, there is clearer justification for investing in personalized medicine for cancer. One such country is Panama, a country of approximately 3 million people in Central America whose rates of cancer death and incidence and the likelihood of survival from cancer still more closely resemble the developing world than the developed world (Table 1). The application of personalized medicine for cancer in Panama will be our case study in this paper.

Priorities for personalized medicine for cancer in the developing world : Personalized medicine for cancer in resource-poor countries needs to be considered relative to other health and cancer goals and applied on three criteria. The first concerns the impact of the cancer on society, focusing personalized medical efforts on cancers with the highest incidence of morbidity and mortality, those predominantly affecting young people and cancers for which survival rates are lower in the developing world than in the developed world. The second prioritizes cancers for which genetic testing has been proven reliable and effective, which is based on high-penetrance genes, and whose application in the developing world can be justified on the basis of improving patient survival and quality of life for the greatest number of people, with small initial investments in technical infrastructure to establish their use. The third prioritizes cancers for which therapy will be reliably and affordably available in the long-term.

Considering the first criterion, it is relatively simple to prioritize cancers which demand attention based on statistics of incidence and death. In the developing world, cancers with the highest incidence of death overall, and for men specifically, are lung/bronchial, liver, stomach, esophagus and colorectal cancers, with breast and cervical cancers also being important causes of cancer death for developing world women (Table 2). In the middle-income country of our case study, Panama, other adult cancers of significance for their rates of incidence or mortality are prostate cancer, leukemia, Non-Hodgkin lymphoma and brain and nervous system cancers. In children of less than 14 years, significant causes of cancer in low- and middle-income countries are leukemia, Non-Hodgkin lymphoma, brain and nervous system cancers, kidney cancer and Hodgkin lymphoma (16). The likelihood of surviving cancer in the developing world is decreased for Hodgkin and non-Hodgkin lymphoma; multiple myeloma; thyroid, prostate and testicular cancer, or if you are less than 14 years of age (Table 3). Survival rates for other, high incidence cancers of the developing world, such as lung and liver, are similar to rates for the developed world, for reasons explained below (Table 3).

The second criterion which prioritizes genetic tests and personalized therapeutics

Table 1. Cumulative burden (%) of cancer deaths and incidence, and likelihood of survival by age group compared for less and more developed countries. Raw data taken from IARC (16) and comparisons were performed using the GLOBOCAN Online Analysis - Age-Specific Tables tools, using Population as Less Developed, More Developed or Panama; Sex as Both Sexes; Data Type as Incidence or Mortality; and Statistic as Numbers. 'Deaths' and 'Incidence' expressed as the cumulative percentage of deaths (or incidences) occurring due to cancer in defined age groups. 'Crude likelihood of survival' calculated as [1-total number of deaths/total number of incidences]. Relative likelihood of survival calculated as [Crude likelihood of survival for Less developed countries or Panama/Crude likelihood of survival for More developed countries].

	Deaths			**Incidence**			**Crude likelihood of survival**			**Relative likelihood of survival**	
Age	*Less developed*	*More developed*	*Panama*	*Less developed*	*More developed*	*Panama*	*Less developed*	*More developed*	*Panama*	*Less developed*	*Panama*
0-14	1.9	0.2	1.9	2.1	0.5	2.9	0.36	0.80	0.53	0.45	0.66
15-39	9.4	1.8	8.3	12.1	4.5	12.0	0.47	0.79	0.52	0.59	0.66
40-44	14.1	3.2	11.2	18.3	7.3	16.2	0.46	0.72	0.52	0.64	0.72
45-49	20.4	6.3	15.2	26.1	11.9	21.0	0.42	0.66	0.42	0.64	0.64
50-54	29.5	11.6	20.8	36.5	18.9	27.1	0.38	0.60	0.37	0.63	0.62
55-59	40.8	19.9	29.2	48.7	28.8	36.0	0.34	0.56	0.35	0.61	0.63
60-64	52.0	29.7	38.7	60.1	40.3	46.1	0.30	0.55	0.35	0.55	0.64
65-69	64.3	42.2	50.2	71.8	53.5	58.3	0.25	0.51	0.35	0.49	0.69
70-74	77.6	57.2	63.3	83.3	68.2	71.5	0.18	0.46	0.31	0.39	0.67
75+	100.0	100.0	100.0	100.0	100.0	100.0	0.05	0.29	0.11	0.17	0.38

Table 2. Leading causes of cancer cases and deaths compared between less developed and more developed countries. Data summarized from IARC (16).

Cancer type	Less developed country deaths			Less developed country cases			More developed country deaths			More developed country cases		
	Overall	*Men*	*Women*	*Overall*	*Men*	*Women*	*Overall*	*Men*	*Women*	*Overall*	*Men*	*Women*
All sites but skin	4,810,100	2,697,500	2,112,600	7,107,600	3,654,000	3,453,600	2,751,400	1,528,200	1,223,200	5,560,000	2,975,200	2,584,800
Lung & bronchus	778,000	539,000	239,000	884,500	612,500	272,000	600,400	412,000	188,400	724,400	482,600	241,800
Liver	580,600	402,900	177,700	626,700	440,700	186,000	115,300	75,400	39,900	122,000	81,700	40,300
Stomach	556,400	353,500	202,900	713,900	466,900	247,000	181,700	110,900	70,800	275,700	173,700	102,000
Esophagus	338,900	223,000	115,900	400,500	262,600	137,900	67,700	52,900	14,800	81,300	63,600	17,700
Colon & rectum	288,500	154,400	134,100	506,400	274,000	232,400	320,100	166,200	153,900	727,400	389,700	337,700
Breast	268,900	0	268,900	691,300	0	691,300	189,500	0	189,500	692,200	0	692,200
Cervix uteri	242,000	0	242,000	453,300	0	453,300	32,900	0	32,900	76,700	0	76,700
Leukemia	170,200	95,100	75,100	209,900	116,500	93,400	87,300	48,600	38,700	140,700	79,000	61,700
Prostate	121,900	121,900	0	255,000	255,000	0	136,500	136,500	0	648,000	648,000	0
Non-Hodgkin lymphoma	120,100	71,600	48,500	175,200	103,800	71,400	71,400	37,900	33,500	180,500	95,700	84,800
Brain, nervous system	114,000	63,700	50,300	152,400	81,200	71,200	61,000	33,600	27,400	85,500	45,600	39,900
Pancreas	104,300	55,500	48,800	112,500	60,200	52,300	161,800	82,700	79,100	165,100	84,200	80,900
Oral cavity	96,900	61,200	35,700	171,800	107,700	64,100	30,700	21,900	8,800	91,200	62,800	28,400
Ovary	75,700	0	75,700	125,200	0	125,200	64,500	0	64,500	100,300	0	100,300
Urinary bladder	75,700	57,200	18,500	155,100	119,500	35,600	74,500	55,000	19,500	230,500	177,800	52,700
Kidney	47,800	29,000	18,800	92,100	56,900	35,200	68,600	43,000	25,600	180,300	111,100	69,200
Corpus uteri	41,165	0	41,165	144,900	0	144,900	32,700	0	32,700	143,500	0	143,500
Melanoma of skin	14,900	7,900	7000	30,600	15,700	14,900	31,500	18,000	13,500	167,700	86,100	81,600

Table 3. Relative likelihood of death from specific types of cancer among specific age groups in less developed countries compared to developed countries. Raw data taken from IARC (16) and comparisons were performed using the GLOBOCAN Online Analysis - Age-Specific Tables tools, using Population as Less Developed or More Developed; Sex as Both Sexes, or Male or Female for sex-specific cancers (breast, cervix uteri, corpus uteri, prostate and testis); Data Type as Incidence or Mortality; and Statistic as Numbers.

		Age groups									
Cancer type	**Total**	*0-14*	*15-39*	*40-44*	*45-49*	*50-54*	*55-59*	*60-64*	*65-69*	*70-74*	*75+*
All cancers excl. non-melanoma skin cancer	1.37	3.07	2.45	1.89	1.71	1.59	1.56	1.65	1.60	1.60	1.36
Bladder	1.49	5.57	2.12	1.79	1.95	1.95	1.97	1.99	1.87	1.86	1.58
Brain, nervous system	1.05	2.06	1.40	1.05	1.08	1.03	0.99	1.06	1.05	1.10	1.16
Breast	1.42	2.95	1.86	1.81	1.85	1.96	1.88	2.04	1.91	1.71	1.49
Cervix uteri	1.24	3.25	1.78	1.41	1.41	1.30	1.23	1.28	1.29	1.40	1.18
Colorectum	0.32	4.13	1.80	1.50	1.50	1.41	1.35	1.43	1.43	1.50	1.47
Corpus uteri	1.25	-	2.04	2.21	1.92	1.84	1.86	1.94	1.77	1.75	1.65
Gallbladder	1.05	3.85	1.80	1.46	1.42	1.22	1.04	1.11	1.16	1.18	1.14
Hodgkin lymphoma	2.63	15.26	4.36	3.59	2.90	2.84	2.57	2.38	2.19	2.05	1.61
Kaposi sarcoma	-	-	-	-	-	-	-	-	-	-	-
Kidney	1.37	6.12	3.29	1.91	2.00	1.70	1.43	1.50	1.52	1.47	1.30
Larynx	1.33	0.68	2.10	1.94	1.46	1.27	1.25	1.36	1.24	1.49	1.48
Leukaemia	1.31	3.72	1.85	1.76	1.87	1.92	1.70	1.63	1.45	1.36	1.12
Lip, oral cavity	1.68	2.97	2.03	2.16	1.73	1.63	1.61	1.73	1.84	2.01	1.97
Liver	0.99	2.12	1.28	1.37	1.23	1.10	1.06	1.09	1.06	1.07	0.98
Lung	1.06	1.32	1.30	1.09	1.08	1.06	1.04	1.10	1.10	1.16	1.10
Melanoma of skin	2.61	5.16	4.32	3.42	3.05	3.18	2.63	2.71	2.31	2.69	2.20
Multiple myeloma	1.30	18.98	3.73	2.54	2.33	1.83	1.73	1.63	1.49	1.44	1.16
Nasopharynx	1.35	12.00	1.72	1.79	1.44	1.46	1.20	1.35	1.54	1.72	1.65
Non-Hodgkin lymphoma	1.73	7.41	3.25	3.12	2.95	2.58	2.46	2.28	1.98	1.85	1.44
Oesophagus	1.02	-	1.39	1.02	0.96	0.91	0.91	1.00	1.07	1.18	1.12
Other pharynx	1.60	5.96	3.95	2.80	2.00	1.65	1.51	1.49	1.40	1.67	1.39
Pancreas	0.95	6.17	1.13	0.92	1.01	0.99	0.96	0.97	0.97	1.00	0.97
Prostate	2.26	15.68	16.58	7.85	4.93	5.36	4.33	4.49	3.49	2.74	1.50
Stomach	1.18	2.41	1.18	1.08	1.08	1.09	1.14	1.28	1.24	1.35	1.32
Testis	4.53	5.86	6.31	4.89	3.55	3.42	2.42	2.34	1.22	1.20	1.39
Thyroid	2.27	59.44	8.03	7.82	6.07	4.28	4.79	4.07	3.13	2.56	1.67

which are well-established will be reviewed and their value to low- and middle-income countries discussed in the following section. Three cancers of importance in the developing world will be considered: lung, colorectal and breast cancer. These cancers share some similarity in the biochemical dysfunctions at their foundations, which will assist in rationalizing technical resources for genetic testing and analysis in developing world laboratories. Gastrointestinal stromal tumors, of special interest in Panama, will also be reviewed. Relatively simple genotyping tests which enhance risk assessment, prognosis or pharmaceutical choice in the management of these cancers will be referenced and their implementation in low- and middle-income countries will be evaluated. It should be noted that well-established options for personalized medicine for those cancers with higher relative morbidity and mortality in the developing world, such as lymphoma, multiple myeloma and cancers of the thyroid, prostate and testis are lacking at this time. Nonetheless, with the recent and complete sequencing of prostate cancers reported by Berger *et al.* (17), there are new insights into the molecular mechanisms giving rise to this group of cancers (18), the potential of targeting these pathways with new therapeutics (19) and of using their components as prognostic markers of clinical outcome, important in identifying men with aggressive forms of the disease who will benefit most from therapy (18, 20).

The third criterion is beyond the scope of this review, but rationalizes applications of personalized medicine that are sustainably affordable for developing world economies. The genetic tests used in personalized medicine and the chemo- and biologic therapies indicated by the results of these tests are expensive. Tests and therapies will either be strictly limited for specific cancers or patient groups, or innovative models of funding for personalized medicine will need to be developed.

Lung and bronchial cancer : Lung cancers are the leading cause of cancer death worldwide (Table 3; 16), with five-year survival rates of only 10% in most countries (21), a consequence of the advanced stage at which many are diagnosed (22). There is little difference in the likelihood of survival between patients in developed and developing nations (Table 3).

Lung cancers are classified morphologically according to the 2004 WHO scheme which identifies four major types: small-cell carcinoma, and the non-small cell carcinomas (NSCLCs), which are classified further into squamous cell carcinoma, large-cell carcinoma and adenocarcinoma, the predominant histological type in women and patients of Asian descent (21). NSCLCs comprise about 80% of lung cancers and patient age, gender and smoking history influence tumor histology (21, 23). Molecular classification confirms morphological classification and describes subtypes, especially for adenocarcinomas, although heterogeneity among the four major groups exists and cellular transitions between subtypes are observed, making absolute classification difficult (15, 24).

There are two well-defined applications of personalized medicine in managing lung cancer. The first application is genetic typing of *EGFR* (also known as *Her1* and *erb-b1*) in lung cancers to identify patients who will respond to tyrosine kinase inhibitors (TKIs), which act on EGFR, and which are demonstrated to prolong disease-free survival for some patients with advanced lung cancer.

EGFR is a cell membrane receptor responsible for relaying, via phosphorylation cascades, gene activation signals which mobilize cells, progress cell cycle progression and angiogenesis. Several cancer types, including 43 to 83% of NSCLCs (25), show somatic *EGFR* mutations which result in constitutive activation of the receptor, downstream phosphorylation events and uninhibited cell proliferation as a consequence.

Activating *EGFR* mutations are observed in about 10% of all lung cancers. More than 18 mutations of phenotypic significance are found

in the intracellular tyrosine kinase domain of the receptor involving exons 18 to 21, for which exon length ranges from 99bp (exon 19) to 186bp (exon 20) (26, 27, reviewed in 28). EGFR with activating mutations in these exons are sensitive to tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, both approved by the US FDA. These competitively bind to the ATP-binding cleft of the tyrosine kinase domain and their use significantly improves progression-free survival in patients with *EGFR*-mutant tumors, but not those with unmutated *EGFR* (29).

Genotyping of exons 18, 19 and 21 of *EGFR* in patients with advanced lung adenocarcinomas, especially patients who are non-smokers, women and those with Asian ancestry (27, 29-31), will identify patients sensitive to TKIs and may improve quality of life and survival among lung cancer sufferers in the developing world through the selective use of TKIs after the failure of standard first-line chemotherapies. This test will be especially useful in China, Japan and south-east Asia, and those low- and middle-incomes countries which have significant numbers of migrants from these regions, such as Panama (32).

The second application of personalized medicine to lung cancer management is the genotyping of *EGFR* and *KRAS* for mutations that predict the development of resistance to TKIs. In exon 20 of *EGFR*, the presence of the T790M substitution is associated with gefitinib resistance (33), and is a valuable predictor of patients who will show primary resistance to TKIs, as well as a molecular marker for patients formerly responsive to TKIs, but demonstrating therapeutic failure.

KRAS is one of the first GTPases in the phosphorylation cascade activated by EGFR. Activating mutations in *KRAS* codon 12 are observed in about 30% of NSCLCs (34) and most commonly in patients with a history of smoking (35). Activating mutations also observed in codon 13 and rarely in codons 59 and 61 (34, 36). Codon 12 and 13 mutations are associated with primary resistance to gefitinib and erlotinib (35), and as such, identify another group of patients who will not respond to TKIs.

Unfortunately, there is no reliable pre-symptomatic testing for lung cancer, which would significantly reduce the proportion of patients presenting with advanced cancers and increase the likelihood of survival through early detection and treatment. Similarly, genes with reliable prognostic value are limited: since 2001, a large number of studies have surveyed lung cancers for genetic markers which will predict disease outcome, refining the number from over 800 (15) to five (37). However, much debate remains about the utility of specific genetic markers, no clinical test is in routine use, and the application of such tests in resource-poor settings is some way off. Until reliable biomarkers for early detection and prognosis are identified, the best methods for preventing the development of advanced lung tumors will remain reduction of lung cancer risk factors through community education, and traditional radiographic screening for early-stage cancers. Nonetheless, value remains in genotypying lung cancer biopsies to assess sensitivity to TKIs.

Colorectal cancer : Colorectal cancers are one of the top five causes of cancer and cancer death worldwide (Table 2), and may arise sporadically or be caused by inherited mutations. Many begin with the mutational inactivation of the *Adenomatous Polyposis Coli* (*APC*) gene, a tumor suppressor which leads to dysregulated gene transcription, inactivation of other tumor suppressor genes and activation of proto-oncogenes (38). There is a small decrease in the likelihood of survival for patients with colorectal cancer in the developing world compared to developed countries (Table 3). Reliable applications for personalized medicine in the management of these cancers include pre-symptomatic risk assessment, diagnosis and tumor typing for drug selection.

Approximately 10% to 30% of all colon cancers occur with some heritable basis (39) and

mutations in a number of genes, including *APC* and others, are associated with familial colorectal cancer syndromes. Individuals and families at risk from heritable colon cancer may be identified with pre-symptomatic assessments following the US National Comprehensive Cancer Network guidelines for colorectal cancer screening, a document which could be adapted by countries of the developing world (40).

Pre-symptomatic and diagnostic tests for two of the most common heritable cancers of the colon are described below, Hereditary Non-Polyposis Colorectal Cancer (HNPCC) and Familial Adenomatous Polyposis (FAP), together causing about 5% of all colorectal cancer. A number of other cancer syndromes with well-defined genetic foundations make a small contribution to the remaining 20-25% of colorectal cancers with familial bases, but the majority of this group is thought to be caused by low-penetrance, poorly-characterized susceptibility loci, for which genetic markers are currently unavailable (41). Personalized pre-symptomatic risk assessment and diagnosis in these cases is not yet possible.

HNPCC, also known as Lynch Syndrome, is an autosomal dominant disease, causing 2-4% of all colon cancers (39-42). No studies on the incidence and significance of HNPCC in Panama have been performed, although data from Colombia (43-45) and Mexico (46, 47) may be a useful basis for regional epidemiological studies. HNPCC manifests as colorectal carcinoma, endometrial carcinoma or cancers of the small intestine, ureter or renal pelvis and lifetime risk of developing colorectal cancer if diagnosed with HNPCC is 50-80% (41). To be assessed as having HNPCC, families should fulfill the Amersterdam I criteria, Amsterdam II criteria or Bethesda guidelines; the introduction of the two latter tests have increased the sensitivity of detection of families with HNPCC to above 50% (reviewed in 41).

The underlying genetic basis for HNPCC is reasonably clear: 70-90% of families show germline mutations in mismatch repair genes: *MSH2* accounts for about 30-60% of cases, (41, 48)*;MLH1* accounts for about 30% of cases, (48); and *MSH6* or *PMS2* are largely responsible for the remainder (38, 40). Some debate remains about the importance of mutations in *EXO1* (40, 49); *PMS1* (40, 50); *EpCAM*, a gene directly upstream of *MSH2* (41, 51) and *MLH3* (40, 52), while up to 30% of HNPCC families have no genetic mutations detectable in the main mismatch repair genes described above (48). New and unique mutations in these genes continue to be reported from specific populations and those noted in Colombian patients (43-45) may be of particular interest in Panama. While genotypic studies have not yet been performed to confirm that genetic homogeneity exists between the populations of these countries, Panama and Colombia share common anthropological histories of colonization and settlement by European, African and Amerindian groups. We expect, therefore, that modest extrapolations of Colombian data may be relevant to Panama.

HNPCC families can be identified after assessment of an index case with colorectal cancer. Biopsied tissues are analyzed by immunohistochemistry for mutations in MLH1, MSH2, MSH6 and PSM2 (40). Tumor tissues can also be analyzed for microsatellite instability (MSI), which arises as a consequence of mutations in mismatch repair genes. Around 80-90% of colorectal cancers have MSI (40) and a panel of five microsatellites (BAT25, BAT26, D5S346, D2S123, D17S250) has been recommended for use in characterizing HNPCC tumors (38, 53). Sixty percent of HNPCC adenomas have high MSI (38), which is associated with a lower frequency of metastasis and better prognosis (54). It should be noted, however, that 10-15% of sporadic colorectal cancer also demonstrate MSI and positive MSI tests on tissue biopsies must be followed by pedigree analysis and genetic testing for mutations in mismatch repair genes before HNPCC can be defined (41, 42). If HNPCC is

suspected, genetic counseling and further testing is then recommended, according to guidelines, for the index case and asymptomatic family members (40).

FAP is another autosomal dominant disorder characterized by numerous adenomatous colorectal polyps with a tendency to form adenocarcinoma; penetrance by 40 years of age is almost complete (38, 55). Clinical variations on FAP include attenuated FAP, Gardner and Turcot syndromes, but all forms are associated with germline mutations in *APC* (55). FAP is responsible for less than 1% of colorectal cancer cases and 25-50% of FAP individuals will have *de novo APC* mutations with no family history of the condition, with 20% of these patients also showing somatic mosacism (38, 41). Nonetheless, there is value in the genetic screening of families of index cases as prophylactic endoscopic screening, initiated before 20 years of age, and prophylactic colectomy strongly reduces risk of colorectal cancer (38, 41).

APC is a gene of 160kb with up to 21 exons and many alternative transcripts (38, 56). Polakis *et al.* (57) provides a summary of the somatic and germline mutations common in *APC* and a database of mutations and polymorphisms in *APC* is maintained by the International Society for Gastrointestinal Hereditary Tumours (58). (This database also tracks mutations and polymorphisms in HNPCC-associated genes.) Correlations exist between the location of these mutations in *APC* and the clinical presentation of FAP (55).

The second main application of personalized medicine in managing colon cancer is typing of colorectal tumors to improve therapeutic choice. A number of examples exist where genetic testing of tumors will guide drug use.

First-line chemotherapies for colon cancer include fluoropyrimidines (5-fluorouracil (5-FU) and capecitabine, the prodrug of 5-FU), oxaliplatin and irinotecan (59). 5-FU is metabolized by rate-limiting enzyme dihydropyrimidine dehydrogenase (DPD) and a deficiency of this enzyme has been associated with severe 5-FU toxicity (60). The *DPD* gene is well-characterized and a number of single-nucleotide polymorphisms (SNPs) have been associated with reduced DPD activity. Genetic screening of *DPD* before administering 5-FU to patients may be of value in avoiding toxicity and enabling more appropriate chemotherapeutic choices, although no regulatory steps dictate testing is yet mandatory (60, 61). A number of screening strategies for *DPD* mutations are available (62).

One biologic therapy for colon cancer targets EGFR, important in metastatic colon cancers, and also identified as significant in some lung cancers (above). Drugs which target EGFR in the treatment of colon cancer belong to a class of inhibitor acting against EGFR's extracellular domain. These inhibitors take the form of monoclonal antibodies which block the binding of EGFR's natural ligands, promote receptor internalization and degradation and prevent activation of downstream phosphorylation cascades (63) . Monoclonal antibody products used in treating colon cancer include cetuximab and panitumumab, approved by the US FDA for use alone or in combination with other first-line chemotherapies (63, 64). The US FDA requires that all patients be tested immunohistochemically for EGFR expression before initiation of cetuximab and panitumumab therapy (65), although cetuximab has proven efficacious in colorectal cancer patients whose tumors do not express EGFR detectable by immunohistochemistry (66).

However, in a similar situation to that observed for lung cancers, colon cancers with *KRAS* mutations in codons 12, 13 and rarely 61, show resistance to cetuximab and panitumumab (67-69). A Provisional Clinical Opinion from the American Society of Clinical Oncology (ASCO) therefore states that *KRAS* genotyping should be another mandatory procedure before monoclonal antibody therapy can be prescribed

for colorectal cancer treatment (70); approximately 40% of patients who will not respond to cetuximab or panitumumab will have mutated *KRAS* (71).

Chemorefactory colon cancers may otherwise involve mutations in exon 15 of *BRAF* which encodes a protein acting downstream of KRAS (10% of resistance cases); *PI-3K*, encoding a protein kinase activated by EGFR as an alternative to KRAS (15-20% of resistance cases); and *PTEN*, whose product acts in the phosphorylation cascade downstream of PI-3K (25% of resistance cases) (reviewed in 71). A large body of data suggests screening of colorectal cancers for *PI-3K* and *BRAF*, as well as *KRAS* could better identify patients likely to benefit from monoclonal antibody therapy, but official statements from bodies such as ASCO have not yet been made regarding these tests. An US-FDA approved test for the resistance-conferring V600E mutation in *BRAF* is available, but currently only indicated for non-colorectal cancer (melanoma) (72). Clinicians should, however, consider *BRAF*, *PI-3K* or *PTEN* mutations in cases of cetuximab or panitumumab therapy failure.

Breast cancer : Breast cancer is one of the top five cancer killers in both the developed and developing worlds, and is the most significant sex-specific cancer in terms of morbidity and mortality (Table 2). Likelihood of death from breast cancer in the developing world is nearly twice as high as in the developed world for women aged 50 to 69, a possible consequence of the absence of mammography programs in these countries (Table 4; 73). Community-based education programs promoting and making available mammograms for women from specific demographic groups and regular breast checks are important in the early detection of breast cancers, but there are also applications of personalized medicine that could be used by the developing world, including pre-symptomatic risk assessment, prognosis, diagnosis and pharmacogenomic testing.

Up to 20% of breast cancers are hereditary (74) and about 40-60% of these are due to autosomal dominant gene mutations in the *BRCA1* and *BRCA2* tumor suppressor genes, which repair DNA or dictate to cells in which DNA cannot be repaired that the cell should undergo apoptosis (73, 74). Of the 80% of breast cancers arising sporadically and without family history, 5% will also show mutations in one of these genes (75): a total of 5-10% of all breast cancer patients will have mutated *BRCA1* or *BRCA2.* Patients with these mutations have a 40-80% chance of developing breast cancer and analysis of these genes in families with a history of breast cancer is a valuable pre-cancer risk assessment tool (73, 76). Such families in the USA are being identified and genotyped using detailed guidelines on familial breast and ovarian cancer published by the National Comprehensive Cancer Network, a document similar to that for colorectal cancer screening, which could also be adopted by other countries (76). Identifying mutations in these genes is not only a risk assessment tool for families and individuals, but indicates that the patient may benefit from therapy with PARP inhibitors, particularly effective against breast cancers with mutated *BRCA1* and, or, *BRCA2* (77, 78).

Testing for mutations in a number of other high-penetrance genes associated with breast cancer is also of clinical value, and those genes include tumor suppressor genes *TP53* and *PTEN*, and *CDH1,* respectively associated with Li-Fraumeni, Cowden and Hereditary Diffuse Gastric Cancer Syndromes, three hereditary conditions with an increased risk of breast cancer (73, 76). It should be noted that Li-Fraumeni Syndrome is also associated with increased risk of colorectal cancer (38) and that *MLH1* and *MSH2* mutations, associated with familial HNPCC colorectal cancer, are also implicated in hereditary breast cancer (73). A number of other, low- and moderate-penetrance genes which may contribute to breast cancer continue to be investigated and may be of clinical utility for pre-symptomatic risk assessment in the future (73).

Genetic profiling of tumors is the second major application of personalized medicine for breast cancer and assists in prognosis and choice of chemotherapies. Based on gene profiling, histology and immunohistochemistry, five subtypes of breast cancer have been identified and essentially distinguished by the expression of three genes: the *Estrogen Receptor* (*ER*) and *Progesterone Receptor* (*PR*), two nuclear hormone receptors; and *HER2* (also known as *Neu* and *erb-b2*), an extracellular receptor and member of the EGFR family (reviewed in 73).

HER2-like tumors over-express HER2; luminal A and luminal B types are ER-positive; basal-like tumors, also known as "triple negative" or "hormone unresponsive" tumors, are ER-negative, PR-negative and HER2-negative; and normal-like tumors resemble normal breast tissue (79). Luminal subtypes have best prognosis, while HER2-like and basal-like tumors have traditionally had poor prognosis (73). There is considerable variability in the incidence of subtypes observed among women of different racial backgrounds (73), which will be important for laboratories which serve patients of diverse racial backgrounds, as is the case in Panama, a country which has experienced migration waves from Europe, Africa, the Caribbean, North America and Asia. Testing continues on the sensitivity and specificity of two prognostic kits, available commercially, Oncotype Dx and MammaPrint, which profile 21 and 70 genes associated with breast cancer respectively, and which are designed to identify patients with higher likelihoods of relapse and the need for additional therapies (79).

Differences in prognostic outcomes are partly due to the roles that ER, HER2 and PR play, and how effectively they can be targeted by drugs. Patients with ER-positive tumors can be treated with drugs such as estradiol and tamoxifen, which lead to down-regulated HER2 expression. Patients with HER2-positive tumors can be targeted by trastuzumab, a monoclonal antibody which down-regulates cell proliferation, and lapatinib, a TKI acting on the tyrosine kinase domains of both HER2 and EGFR. Both therapies are US FDA approved. The use of both trastuzumab and lapatinib is strictly limited to patients with HER2-positive cancers, and the latter only for women on a regimen of complementary drugs. Immunohistochemistry and fluorescence *in situ* hybridization are two techniques used to assess HER2 expression on biopsy samples.

Gastrointestinal stromal tumors : Gastrointestinal stromal tumors (GISTs) are observed at a global incidence of 10-20 per million people each year and present as tumors of the stomach (50-60% of cases), small intestine (30%-40%), colon and rectum (5-10%) and esophagus (5%) (80) with a malignancy rate of 20-30% (81-83). Most GISTs arise as a consequence of somatic mutation, but familial GIST also exists with nearly 100% penetrance resulting in all affected family members manifesting multiple GISTs (reviewed in 84).

Biochemically, GISTs are associated with activating mutations in the *c-kit* and *pdgfra* genes, respectively encoding the KIT and PDGFRA oncoproteins, two membrane receptors with tyrosine kinase activity which are involved in cellular signaling pathways promoting cell growth and proliferation (85-91). Nearly 85% of GISTs express constitutively activated KIT and about 5% constitutively activated PDGFRA (92). Constitutive activation is associated with exon 11 in *c-kit*, and at a lesser incidence in exons 9, 13, 14 and 17, while activating mutations are most commonly associated with exons 12, 14 and 18 in *pdgfra* (84, 92). Familial GISTs most commonly arise from *c-kit* mutations in exons 8, 11, 13 and 17 and *pdgfra* mutations in exon 12 (84).

Imatinib is an US FDA-approved TKI used in the treatment of KIT-positive GISTs. Its clinical efficacy correlates with the specific *c-kit* or *pdgfra* mutation present, with *c-kit* exon 11 mutations responding most favorably (93). Immunohistochemistry for KIT expression and

genotyping GISTs for the *c-kit* and *pdgfra* mutations present are tools in determining the utility of imatinib for GIST patients.

However, resistance to imatinib occurs in 14% of the patients after 6 months of treatment and 50% of patients after 2 years of treatment (94, 95). Resistance mechanisms include the activation of alternative downstream signaling pathways, activation of other tyrosine kinase receptors, the loss of KIT expression and the development of secondary mutations in *c-kit* or *pdgfra* (96). Patients with treatment failure after initial success may demonstrate tumors which have developed second, activating mutations in exons 13, 14 and 17 of *c-kit*, or very rarely, in *pdgfra* (97). Therapeutic failure together with the presence of such secondary mutations allows identification of patients which may benefit from an alternative TKI therapy, sunitinib, which inhibits KIT, PDGFRA and the angiogenic vascular endothelial growth factor receptors (VEGFRs) (98).

Our group recently published one of few Latin American studies to evaluate the mutational status of the KIT/PDGFRA oncoproteins in clinical samples (99). We examined the histopathologic features of paraffin-embedded tumor tissues and the mutations in *c-kit* and *pdgfra* genes from 39 archived Panamanian cases, 1994-2004. The highest frequency of mutations was in exon 11 of the *c-kit* gene (70%), while mutations at a lower frequency were found in *c-kit* exon 9 and *pdgfra* exon 18. The results obtained in that study for *c-kit* and *pdfgra* validated our laboratory's developing program of personalized mutational analysis, which also involves using *EGFR* and *KRAS* for lung and colon cancers, respectively.

Applying personalized medicine for cancer in the developing world : Cancers of importance in the developing world are those with high incidences of morbidity and mortality, those affecting young people and cancers for which survival rates are relatively lower. Three cancers with high incidence in low- and middle-income countries are lung, colorectal and breast cancers and in the previous section, applications of personalized medicine were examined for these. It was noted that these cancers not only share a similar significance for their impact on developing world patients, but similarity in the genetic mutations and biochemical dysfunctions that are at their basis.

These commonalities may guide diagnostic laboratories with limited resources to develop a concise portfolio of genetic tests that will provide maximum benefit for the greatest number of cancer patients, including:

1. *EGFR* genotyping of exons 18-20 for lung cancer patients, and immunohistochemical analysis of EGFR for colorectal cancer;
2. *KRAS* genotyping of codons 12 and 13 for lung and colorectal cancers;
3. *MSH2, MLH1* and *APC* genotyping; microsatellite instability tests using BAT25, BAT26, D5S346, D2S123 and D17S250 markers; and immunohistochemical tests for MLH1, MSH2, MSH6 and PSM2 for colorectal cancer patients;
4. *BRCA1* and *BRCA2* screening and *MSH2* and *MLH1* genotyping for breast cancer patients and their families;
5. HER2 immunohistochemistry or FISH analysis for breast cancer patients; and,
6. KIT immunohistochemistry and genotyping of *c-kit* (exons 9, 11, 13, 14 and 17) and *pdgfra* (exons 12, 14 and 18) for GIST patients.

In this section, the technical aspects of implementing such tests are briefly reviewed. A second group of tests for the genes *BRAF*, *PI-3K* or *PTEN, TP53* and *CDH1* or their products could also be introduced, once standardized tests have been developed for these genes, and after the primary tests proposed above are established in laboratories.

***EGFR testing*:** Protocols for PCR and Sanger sequencing of *EGFR* for lung cancer patients from genomic DNA are available in the

supplementary material of Paez *et al.* (27) and Pao *et al.* (100). The relevant exons for lung tumor analysis, 18-20, are short, ranging from 99bp (exon 19) to 186bp (exon 20) (26, 27, reviewed in 28), and these protocols can be performed using standard PCR and sequencing equipment. More advanced techniques involving real-time PCR, high-resolution melting analyses and pyrosequencing are under development (36).

Evidence of EGFR expression by immunohistochemistry of biopsy samples is requisite in the US before cetuximab and panitumumab therapy is prescribed for metastatic colorectal cancer (65). For this analysis, the Dako EGFR pharmDx® kit is recommended (65). Other anti-EGFR antibodies in common use for analysis of formalin-fixed, paraffin-embedded tissue are the CONFIRM™ anti-EGFR primary antibody and the 31G7 clone (101, 102).

KRAS testing: The assays described as appropriate for *KRAS* genotyping in ASCO's Provisional Clinical Opinion on testing for mutations in codons 12 and 13, are real-time PCR and direct sequencing (70). A number of other techniques have been described to analyze *KRAS* (36, 103, 104), including one which claims to provide results on codon 12 testing in 60 minutes (105). There is no US FDA-approved test (70), but commercial testing kits are available (see below).

MSH2 and MLH1 testing: Several types of analysis are available for mutation testing in *MSH2*, *MLH1* and the mismatch repair genes of lesser importance in their contribution to colorectal cancer, *PMS2* and *MSH6.* The most common involve sequencing of the entire coding region and deletion/duplication analysis, while less common methods involve sequence analysis of select exons, mutation scanning of the entire coding region and targeted mutation analysis (106-109). Hampel *et al.* (110) review and compare several of these methods. Antibodies for immunohistochemistry for mutations in MLH1, MSH2, MSH6 and PSM2 are available commercially from a number of companies, as are kits genetic analyses of *MLH1* and *MSH2* (see below).

APC testing : A number of molecular tests are available for patients suspected of Familial Adenomatous Polyposis. *APC* mutations are most reliably detected by complete sequencing of exons and intron-exon boundaries, which has largely replaced the *in vitro* protein truncation test (55). Some laboratories in the US and elsewhere also offer sequence analysis of select exons, mutation scanning, targeted mutation analysis, linkage analysis and deletion/duplication analysis using Southern blot, multiplex ligation-dependent probe amplification and quantitative PCR (55, 111).

BRCA1 and BRCA2 screening : Cancer-associated mutations in *BRCA1* and *BRCA2* are distributed along the entire coding region and intronic sequences flanking each exon. Identifying such mutations requires examination of the entire gene sequences, a time-consuming and costly analysis, or the use of various scanning techniques, of which a number have been developed and reviewed for sensitivity and specificity, and whose use may be appropriate in diagnostic laboratories of some developing countries (112, 113). Recent advances in pyrosequencing also allow for deep-sequencing of an individual patient's *BRCA1* and *BRCA2* genes with relative ease and speed. The development of mini-pyrosequencers costing less than 200,000 USD and commercially-prepared reagents for *BRCA1/2* analysis may make routine sequencing for families and individuals at increased risk of breast cancer a possibility in middle- to mid-upper income countries.

An alternative approach is to analyze *BRCA1* and *BRCA2* for only specific mutations. In certain populations in the Philippines (114), Pakistan (115), Colombia (116) and other developed and developing nations, founding effects make certain *BRCA1* or *BRCA2* mutations more common than others and these may serve as particular targets for simplified

genotyping tests for families of certain geographical or ethnic origins (112). Of interest to Panama are the mutations observed in high frequency in the Hispanic populations of Colombia, well as those observed among Afro-American women, as nearly10% of Panama's population identifies as being of African descent (117).

HER2 testing : Several US-FDA approved tests exist for analysis of HER2 expression in breast cancer biopsies, demonstration of which is a necessary step before women can be prescribed trastuzumab or lapatinib. These tests are based on detection of the HER2 protein or quantification of *HER2* gene copy number and include the Dako Anti-HER2 IHC System (the Hercep Test) and the Ventana Medical Systems Inform Dual ISH test.

KIT testing and c-kit and pdgfra genotyping : Somatic mutations in exons 9, 11, 13 and 17 of *c-kit* can be evaluated using PCR-based assays and direct cycle sequencing of the PCR product. Patient samples with non-mutated *c-kit* can then be evaluated for *pdgfra* gene mutations in exons 12 and 18 (97, 118). Immunohistochemistry for KIT expression can be performed using the DakoCytomation c-Kit pharmDx ™ product (US-FDA approved) and other commercially available anti-KIT monoclonal antibodies.

Commercial kits : Several companies offer products for genetic analysis and pyrosequencing, the major ones being QIAGEN (genetic analysis for *KRAS, EGFR, BRAF, PIK3CA* and others, and pyrosequencing for *BRAF, KRAS* and *EGFR*) and Roche Molecular Diagnostics (genetic analysis for *KRAS*, *EGFR* and *BRAF*). Wang *et al.* (119) review several commercial products for *KRAS* mutation testing and note the importance of identifying a gold-standard assay to determine reference standards. MLC Holland sells multiplex ligation-dependent probe amplication kits for *BRCA1, BRCA2, CHD1, PTEN, MLH1, MSH2, APC, MSH6* and *DPD* analysis. The SNaPshot(R) Multiplex System from Applied Biosystems has been applied for SNP analysis of *MLH1* and *MSH2* (120) and *KRAS* mutations in codons 12 and 13 (103).

Other resources : The National Center for Biotechnology Information (NCBI) hosts a useful database called GeneTests (http://www.ncbi.nlm.nih.gov/sites/GeneTests/). Its main feature is the listing of tests offered in the USA and globally for a large number of genetic disorders. It is organized by genetic disorder and describes laboratories which offer testing and the method of testing employed, if a variety of protocols are available. It can be searched by disease or gene name and also has links to GeneReviews, which are peer-reviewed descriptions of genetic diseases.

Conclusion

Cancer has overtaken infectious disease as the leading cause of death in the developing world. Of significance are lung/bronchial, liver, stomach, esophageal, colorectal, breast and cervical cancers in adults, and leukemia, Non-Hodgkin lymphoma, brain and nervous system cancers, kidney cancer and Hodgkin lymphoma in children (16), with the likelihood of surviving cancer being decreased for the developing world for Hodgkin and non-Hodgkin lymphoma; multiple myeloma; thyroid, prostate and testicular cancer, or if you are less than 14 years of age.

Many low- and middle-income countries lack even basic resources for diagnosing and treating cancer, but others with better developed medical and scientific infrastructure, such as Panama in Central America, are beginning to apply personalized medicine for pre-symptomatic risk assessment, diagnosis, prognosis and treatment of solid tumors and lymphoproliferative malignancies. In Figure 1, we list a number of initiatives to expand the existing program of personalized medicine in that country.

Even in regions with better scientific and medical resources, however, the application of personalized medicine for cancer is being

balanced against other health concerns and prioritized for cancers that affect the largest numbers of people, the young, or those for which there is a significant relative decrease in life expectancy compared to wealthier nations. In this review, we propose newly developing diagnostic laboratories focus initially on a group of cancers which have common biochemical dysfunctions at their origins, and the development of a small portfolio of tests, based on hereditary cancers with high-penetrance genes and biomarkers which have high value to physicians making treatment choices.

In both resource-rich and resource-poor settings, the clinical use of personalized medicine cannot occur without building the necessary technical, legislative, administrative and human infrastructure, (Figure 2). Most developing countries will have to establish, expand or strengthen this infrastructure, a process which will involve cooperation among many different groups. Even in medically and scientifically advanced nations, there are considerable challenges to translating basic cellular and molecular research to effective patient care for personalized medicine in cancer (121) and the developing world has the opportunity to learn from what is emerging in other places, or to create new solutions that serve their local community best.

Finally, the utility of certain tests and therapies for cancer care is unquestioned, but concerns about the processes validating others still remain (122). As Offit (7) comments, patients

Fig. 1. Priorities for establishing personalized oncology care in Panama

1. Implement technologies and tests described in this review:
   - Identify funding for their implementation
   - Optimize tests by conducting retrospective epidemiological surveys using archived tissues of colorectal, lung and breast tumors to analyze the types and incidence of specific genetic mutations, and from these and patient case files, infer the historical prevalence of familial cancer syndromes in Panama;
   - Offer and perform genetic analyses for patients with cancer of the lung, colorectum, breast and GIST, and their families, to aid physicians in therapeutic choices and to identify families with hereditary cancer risk;
2. Develop clinical infrastructure to match and respond to new genetic technologies available:
   - Educational resources for oncologists and allied health professionals;
   - Participation from peak medical bodies to support new initiatives in personalized medicine;
   - Funding for routine screening programs, including endoscopy and mammography, for families identified as having hereditary colorectal or breast cancer syndromes;
   - Funding and legalization of chemotherapies indicated by genetic analyses;
3. Develop post-graduate degree programs in genetic counseling, medical genetics, molecular medicine and biotechnology;
4. Publication education:
   - Improve participation in cancer prevention and screening programs by promoting HPV and HBV vaccines, Pap smears, mammography, prostate cancer screening, lung cancer screening, and lifestyles which include healthy diets, exercise, responsible alcohol consumption and smoking reduction;
   - Improve genetic literacy in schools and through public education programs.

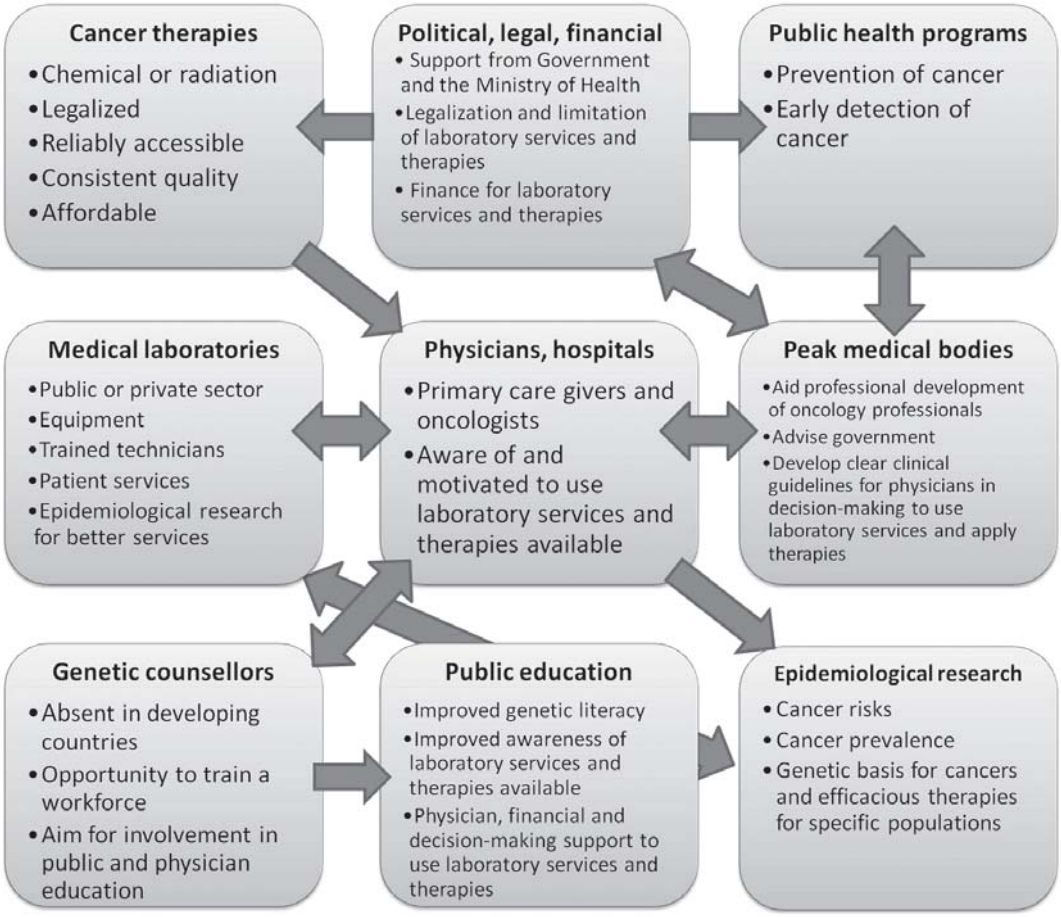

Fig. 2. Using personalized medicine to improve quality of life for the developing world oncology patient.

and their families must be protected from premature translation of research findings. The inequities in health care experienced by resource-poor nations should not be addressed by transforming them into testing grounds for new personalized therapies and tests, but by ensuring that legal, financial, technical and medical barriers are lowered to allow transfer and uptake of the best technologies available elsewhere.

The lowering of these barriers will take considerable cooperation, but is vital for the success of programs of personalized medicine in the developing world. The tests and therapies discussed in this review are costly, which limits their availability even to patients of relative affluence (121). Making them available to the developing world will require innovative models of funding, possibly along the lines of the International Finance Facility for Immunisation's vaccine bonds, and will require considerable negotiation, financial support and good will from drug manufacturers, international health agencies, philanthropic organizations and national governments.

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Advances in Production and Characteristic Features of Microbial Tannases: An Overview

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Abstract

The enzyme tannase (tannin acyl hydrolase, E.C.3.1.1.20) has diverse applications, but are currently limited due to inadequate information on the basic characteristics such as physicochemical and catalytic properties, regulation mechanisms and high production cost. Tannases are gaining more scientific relevance because of their hydrolytic as well as synthetic capability in suitable solvent systems. Available art on tannases is either superficial or forms a part of other information. This review attempts to present a state-of-art and detailed information on various aspects of tannases, exploring scientific and technological facets, emphasizing on substrates, production, physicochemical properties, effect of inhibitors and purification strategies.

Keywords: Tannin acyl hydrolase, Tannins, Purification, Characterization

Introduction

Tannin acyl hydrolases (E.C.3.1.1.20) commonly referred as tannases are inducible enzymes, catalyzing the hydrolysis of ester and depside linkages of gallotannins to give gallic acid and glucose (1, 2), and other tannins viz. catechins and ellagicatechins to give respective products (3, 4). 'Tannase' is a generic term that groups a number of enzymes from different organisms that are all able to hydrolyze tannins (5). Tannases are mostly produced by microorganisms viz. fungi, mainly *Aspergillus* and *Penicillium* species (6-11), yeasts (12) and bacteria (13-18), but they have also been described in plants (19) and animals (20, 21). Gallotannin-degrading esterase and depsidase from leaves of pedunculate oak *(Quercus ruber)* or fruit pods of divi-divi (*Caesalpinia coriaria*) are classified as plant tannase which closely resemble the properties of fungal tannases (19).

Tannases are either membrane-bound or extracellular depending upon source and mode of cultivation, finding extensive use in food, feed, beverage, brewing, pharmaceutical and chemical industries for production of gallic acid, pyrogallol, propyl gallate, methyl gallate, tea and in clarification of beer and fruit juices (1, 22, 23) and animal feed manufacture. Tannase also has potential in the manufacture of coffee flavored soft drinks, improvement in the flavor of grape wine, as an analytical probe for determining the structures of naturally occurring gallic acid esters (24) and in treatment of industrial effluents containing tannins such as olive mill (25) and leather industry waste (26).

Our objectives in this review are to highlight the current findings on tannase in terms of production, purification and also to showcase the salient features and findings of tannases as evidenced from a growing number of reports. Interestingly, during the last five years, the number of publications is almost equal to the total

number prior to 2006, a pointer to the increasing interest for tannases.

Recombinant Tannases : The tannases of wild microbial sources do not satisfy all requirements for optimal versatility in industrial processes. They exhibit rather limited substrate spectra and are relatively expensive to purify because they are secreted only at low levels by their microbial producers. Obviously, the high-yield production of fully active recombinant tannases is an attractive goal, both for basic research and for industrial purposes. There is therefore an ongoing search for new sources of tannases and recombinant technology to produce the same with more desirable properties for commercial applications (27). Over-expression of TAN genes in different hosts seems to be relatively difficult. Hatamoto *et al.* (28) cloned and sequenced the gene-encoding tannase from *A. oryzae*. Later, the *Aspergillus* tannase gene was heterologously expressed in *Saccharomyces cerevisiae*, although with a low yield of protein production. Conversely, large quantities of enzyme were obtained when tannase gene was cloned in *Pichia pastoris* (29). In addition, *A. adeninivorans* is thermo and osmotolerant and can be cultured at temperatures up to 48 ºC in media containing up to 20% NaCl. These unusual properties make *A. adeninivorans* an ideal host for heterologous gene expression as well as a useful source of the same genes for biotechnological significance (27).

The secreted *Arxula* tannase was glycosylated with a carbohydrate content of 31.2%, higher than that of the *A. oryzae* tannase (22.7%) but close to the level found in the recombinant tannase of the *A. oryzae* produced in *P. pastoris* (29). Tannases are often post-translationally modified. In *A. oryzae*, the product of the tannase gene is translated as a single polypeptide and then cleaved into two subunits linked by disulphide bonds (28, 29). In contrast to the case in *A. oryzae*, the *A. adeninivorans* tannase subunit Atan1p does not undergo post-translational cleavage (except for the removal of the secretion signal). This may be one of the reasons why the tannase from *Arxula* exhibits a higher specific activity than its counterpart from *A. oryzae* (27).

Zhong *et al.* (29) have described the successful expression of an *A. oryzae* TAN gene in the methylotrophic yeast *P. pastoris*. For this purpose the gene was fused to the open reading frame (ORF) coding for the α-mating-type-specific genes (MAT-α) sequence and placed under the control of the methanol-inducible alcohol peroxidase 1 (AOX1) promoter from *P. pastoris*. Recombinant tannase was successfully secreted by *P. pastoris* and the productivity of recombinant tannase was found to be approximately 3.5-fold higher compared to that of solid-state fermentation with wild strain (29). These features will enhance future acceptance of the transformants as tannase producers in industrial applications. In another report the identification and cloning of a gene (tanLpl) encoding tannase from *Lactobacillus plantarum* ATCC 14917 and subsequent expression in *P. pastoris* was carried out (30). Curiel *et al.* (15) have reported the production and characterization of recombinant tannase from *L. plantarum.* The physicochemical characteristics exhibited by *L. plantarum* recombinant tannase make it an adequate alternative to the currently used fungal tannases (15).

Substrates for Tannase production : There are two main categories of tannase substrates reported i.e. natural and synthetic. Propyl and methyl gallate are synthetic while tannins are naturally occurring substrates. Tannins are plant secondary metabolites and also the second most abundant group of plant phenolics after lignin (9). They are defined as naturally occurring water-soluble polyphenols of varying molecular weight ranging from 500 to 3000 Da and used as substrates for tannase production by microbial fermentation (3, 31).

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Tannins are widely distributed in different parts (barks, needles, heartwood, grasses, seeds and flowers) of vascular plants (32) and they are further classified into two major groups: hydrolysable and condensed tannins (33). The hydrolysable tannins (Fig. 1) are constituted by several molecules of organic acids such as gallic, ellagic, digallic and chebulic acids, esterified to a core molecule of glucose. Also molecules with a core of quinic acid instead of glucose also considered as hydrolysable tannins. Condensed tannins or proanthocyanidins are complex compounds constituted by flavonoid groups (2 to 50) which are considered not to be hydrolysable (Fig. 2). Their major constituents are cyanidin and delphinidin which are also responsible for the astringent taste of fruit and wines (26, 34).

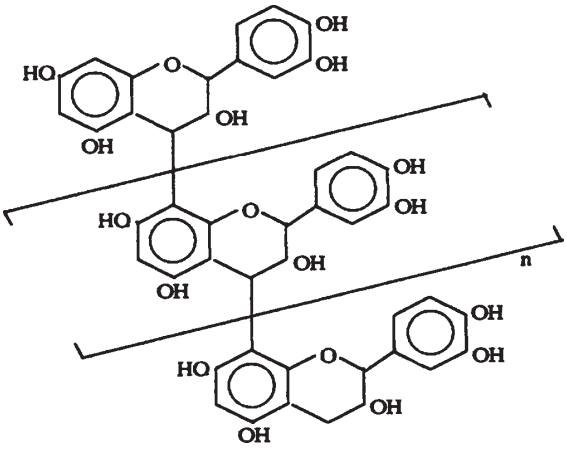

Fig. 2. Structure of a typical condensed tannins. Condensed tannins are all oligomeric and polymeric proanthocyanidins. 'n' denotes number of flavonoid groups (2 to 50).

The selection of a substrate for tannase production by fermentation depends on several factors viz., cost, availability and suitability of the substrate for obtaining the desired yield of tannase, and thus requires screening of several agro-industrial and forest residues (35, 36) Mukherjee and Banerjee (35) evaluated the various substrates for production of tannase and gallic acid on the basis of their tannin contents as 30-41% in case of myrobalan fruit (*Terminalia chebula*), 10-14.1% for tea leaf and 40-52% in case of teri pod (*Caesalpinia digyna*) cover powder (37). Optimum tannase activity and yield were reported with a mixed substrate of myrobalan and teri pod powder at a fixed ratio (4:6) with maximum gallic acid production.

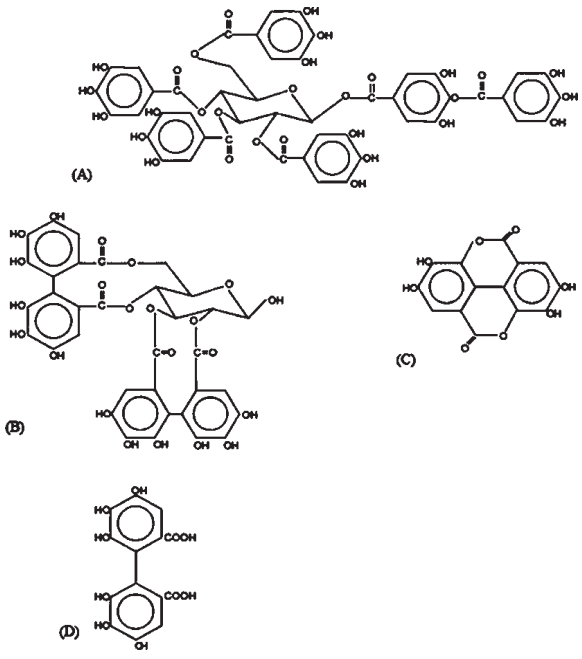

Fig. 1. Structure of hydrolysable tannins [Gallotannin (A), Ellagitannins (B), Ellagic acid (C), Hexahydroxyphenic acid (D).

Microbial degradation of condensed tannins is less documented than that of gallotannins; however, it is known that the selective hydrolysis of galloyl groups of the ellagitannins is catalyzed by tannase (4). Ellagic acid production from cranberry pomace (*Vaccinium microcarpum*) by SSF using a fungus *Lentinus edodes* has been reported, attributing the catalysis to the enzyme β-glucosidase (38). A review work of Li *et al.* (3), presents information on tannase production by species of *Lactobacillus*, *Leuconostoc*, *Oenococcus,* and *Pediococcus,* using substrate

such as gallotannins, ellagitannins and condensed tannins present in muscadine grapes.

Production of Tannases : Tannases are conventionally produced mainly from microorganisms because of high yield and easy mode of cultivation in comparison to plant and animal sources (Table 1). Filamentous fungi of the *Aspergillus genus* have been widely used for tannase production (39). *Aspergillus* can tolerate tannic acid concentrations as high as 20% (w/v) with reasonable yields. Depending on the strain and the culture conditions, the enzymes can be

Table 1. Various sources and mode of cultivation of tannases

Sources of tannases	Mode of cultivation	References
Yeast		
Arxula adeninivorans	SmF	Boer *et al.* (27)
Candida sp.	SmF	Aoki *et al.* (41)
Candida utilis	SmF	Shi *et al.* (42)
Debaryomyces hansenii, Pichia sp.	SmF	Deschamps *et al.* (43)
Mycotorula japonica	SmF	Belmares *et al.* (26)
Saccharomyces cerevisiae	SmF	Zhong *et al.* (29)
Filamentous fungi		
Aspergillus aculeatus	SmF, SSF	Banerjee *et al.* (44)
Aspergillus niger	SSF	Sabu *et al.* (2, 45)
Aspergillus aureus, Aspergillus fischeri, Aspergillus terreus, Aureobasidium pullulans	SmF	Bajpai and Patil (46)
Aspergillus awamori	SmF	Beena *et al.* (47)
Aspergillus candidus	SmF	Murugan and Al-Sohaibani (48)
Aspergillus ficuum	SmF	Lu and Chen (49)
Aspergillus flavus, Aspergillus sojae, Aspergillus usamii, Aspergillus ustus, Penicillium expansum, Penicillium javanicum, Penicillium oxalicum		Yamada *et al.* (50)
Aspergillus foetidus	SmF	Naidu *et al.* (51)
Aspergillus caespitosum, Aspergillus alliaceus, Aspergillus fumigates, Aspergillus versicolor, Penicillium crustosum, Penicillium restrictum, Penicillium variable	SmF	Batra and Saxena (52)
Aspergillus gallomyces , Mucor sp.	SmF	Belmares *et al.* (26)
Aspergillus heteromorphus	SmF	Chhokar *et al.* (11)
Aspergillus japonicas, Aspergillus oryzae Aspergillus niger, Aspergillus rugulosus, Cunnighamella sp., Fusarium oxysporium, Fusarium solani, Heliocostylum sp., Neurospora crassa, Penicillium acrellanum, Penicillium carylophilum, Penicillium charlessi, Penicillium chrysogenum, Penicillium citrinum Penicillium digitatum, Syncephalastrum racemosum,		

Trichoderma hamatum, Trichoderma harzianum, Trichoderma viride	SmF	Bradoo *et al.* (53)
Aspergillus ornatus, Aspergillus rugulosa, Aspergillus terricola, Penicillium commune	SmF	Cruz-Hernández *et al.* (54)
Aspergillus ruber	SSF	Kumar *et al.* (36)
Aspergillus tamarii	SmF	Costa *et al.* (7)
Cryphonectria parasitica	SmF	Farias *et al.* (55)
Cylindrocladiella peruviana, Doratomyces stemonitis, Penicillium concentricum, Trichoderma atroviride, Mariannaea camptospora	SmF, SSF	Peterson *et al.* (56)
Fusarium subglutinans	SmF	Hamdy (57)
Hyalopus sp.	SSF & SmF	Mahapatra and Banerjee (58)
Neosartorya fischeri	SSF & SmF	Aguilar *et al.* (59)
Paecilomyces variotii	SSF	Battestin and Macedo (60)
Penicillium atramentosum	SSF	Selwal *et al.* (61)
Penicillium canescens, Penicillium purpurogenum, Penicillium spinulosum, Penicillium zacinthae, Penicillium frequentans	SmF	Sariozlu and Kivanc (2009)
Penicillium glabrum	SSF	Lagemaat and Pyle (62)
Penicillium glaucum	SmF	Lekha and Lonsane (40)
Penicillium islandyicum, Penicillium notatum	SmF	Ganga *et al.* (63)
Rhizopus oryzae	SSF, MSSF	Kar *et al.* (64)
Verticillium sp.	SmF	Kasieczka-Burnecka *et al.* (65)
Bacteria		
Bacillus cereus	SmF	Mondal *et al.* (66)
Bacillus licheniformis	SmF	Mondal *et al.* (13)
Bacillus polymyxa, Bacillus pumilus, Corynebacterium sp., Klebisella planticola, Klebisella pneumonia, Paenibacillus polymyxa, Pseudomonas solanaceanum	SmF	Deschamps *et al.* (43)
Bacillus sphaericus	SmF	Raghuwanshi *et al.* (67)
Citrobacter freundii	SSF	Murugan *et al.* (68)
Enterobacter sp.	SmF	Sharma *et al.* (69)
Lactobacillus acidophilus, Lactobacillus animalis, Lactobacillus murinus, Lactobacillus paraplantarum, Lactobacillus pentosus, Pediococcus acidilactici, Pediococcus pentosaceus	SmF	Nishitani *et al.* (70)
Lactobacillus apodemi	SmF	Osawa *et al.* (71)
Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus casei Lactobacillus fermentum, Lactobacillus helveticus, Lactobacillus hilgardii, Oenococcus oeni, Pediococcus acidilacti Pediococcus pentosaceus,	SmF	Matthews *et al.*(72)
Lactobacillus plantarum,Leuconostoc fallax, Leuconostoc mesenteroides	SmF	Kostinek *et al.* (73)

Lonepinella koalarum	SmF	Osawa *et al.* (74)
Microbacterium terregens, Providencia rettgeri,		
Serratia ficaria, Serratia marcescens	SmF	Belur *et al.* (75)
Pantonea agglomerans	SmF	Zeida *et al.* (76)
Pantonea sp., Serratia sp.,	SSF	Pepi *et al.* (77)
Selenomonas ruminantium	SmF	Skene and Brooker, (78)
Staphylococcus lugdunensis	SmF	Noguchi *et al.* (79)
Streptococcus bovis	SmF	Belmares *et al.* (26)
Streptococcus gallolyticus	SmF	Sasaki *et al.* (80)
Plant sources		
Myrobolan (*Terminalia chebula*)	Fruits	Madhavakrishna *et al.* (81)
Divi-divi (*Caesalpinia coriaria*)	Pods	
Dhawa (*Anogeissus latifolia*)	Leaves	
Konnam (*Cassia fistula*)	Bark	
Babul (*Acacia arabica*)	Bark	
Avaram (*Cassia auriculata*)	Bark	
Pedunculate oak (*Quercus ruber*)	Leaves	Niehaus and Gross (19)
Pods of divi-divi (*Caesalpinia coriaria*)	Fruits	
Animals sources		
Cattle	Rumen mucosa	Begovic and Duzic (20)
Bovine	Mucosal membrane	Begovic and Duzic (21)

constitutive or inducible, showing different production patterns (7). Phenolic compounds such as gallic acid, pyrogallol, methyl gallate and tannic acid are inducers of tannase synthesis (40).

Tannases are produced and optimized by various techniques such as submerged fermentation (7, 82), solid state fermentation (83) and liquid surface fermentation (1, 40). All the above processes have their own advantages and disadvantages over other fermentation methods.

Submerged Fermentation (SmF) of Tannase: The industrial production of enzymes is mainly performed under SmF. Use of SmF is advantageous because of ease of sterilization and easier process control during fermentation (36). Tannic acid has been reported as an inducer for tannase synthesis under SmF and SSF, but at higher concentrations, it repressed tannase synthesis under SmF (59). An example of tannase production by SmF with *A. niger* HA37 on four-fold diluted olive mill waste (OMW) water as substrate was studied by Aissam *et al.* (84). Lekha and Lonsane (1) mentioned that *A. niger* has the ability to produce both extra and intracellular tannase in semisolid medium and the higher intracellular enzyme accumulation is due to the non-fragile nature of the cell wall or synthesis of enzyme occurring as integral membrane protein (85).

Tannic acid acts as sole carbon and energy source, so the concentration of tannic acid in production medium is a crucial factor for microbial growth and tannase induction (13, 51). Maximum intracellular and extracellular enzyme synthesis occurred below 1% (w/v) of glucose in medium containing tannic acid but higher concentration of glucose repressed enzyme production (82). It has been reported that tannase is produced during the primary phase of growth in SmF and

thereafter production declined. The decline in enzyme production may be due to gallic acid production which showed end-product repression (6).

Recently, SmF for production of tannase has been reported by Enemuor and Odibo (10), from *A. tamari* IMI388810 with maximum yield at 144 h. Mondal and Pati (13) reported tannase production by SmF in different types of media using *B. licheniformis* KBR 6. The strain *B. licheniformis* KBR 6 produces tannase in the presence of tannic acid showing its inducible nature (13, 16). Addition of glucose, lactose or sucrose at higher concentrations repressed tannase production though low concentrations of glucose or lactose were not repressive (13).

Solid State Fermentation (SSF) of Tannases: SSF can be defined as microbial growth on a moist solid material or as a fermentation process that takes place on solid or semisolid substrates or on an inert support in the presence of continuous gas phase and absence of free flowing water. Recent literature on SSF for tannase production (8, 59, 86, 87), claim advantages of extracellular nature and high-production titres (3 to 6 times higher than SmF) (59). Also, in SSF the tannase produced, exhibits good stability parameters and higher tolerance to a wide range of pH and temperature (5, 88). Initial moisture content of the solid substrate is an important factor which dictates the growth of the organism and enzyme production; in the case of fungi a wider moisture range (20-70%) supports better growth and metabolic activities, but for bacteria only higher moisture content of the solid matrix can yield better performance (89).

Substrates used for tannase production under SSF are wheat bran, coffee husk (45, 88, 90) paddy straw (91), jamun leaves (36), pomegranate residues, creosote bush and tar bush (86, 87). Hydrolyzable tannins are present in most of the residues from higher plants can be suitably used for tannase production under SSF. Tamarind seed powder (TSP) obtained after removal of the fruit pulp from tamarind fruit pod was tested for the production of tannase under solid-state fermentation using *A. niger* ATCC 16620 (2).

Studies have indicated tannase production by SSF rather than SmF as more advantageous. However, a large quantity of heat is generated in fermenting solids due to the microbial metabolic activity in SSF leading to rapid rise in temperature of the fermenting solid bed. The poor heat transfer through the solid substrate bed and absence of sufficient heat-exchange surface result in large moisture losses and drying of the solid substrate. Understanding the interplay of transport phenomena and biochemical reaction in various reactor configurations is important for the design, monitoring and control of SSF processes in batch systems (89, 92). In another study (93) indicated the need for more research in a continuous SSF (CSSF) strategy especially the understanding of the microbial mechanisms, the experimental system, and their interaction. The CSSF concept was translated to a laboratory-scale prototype reactor, which was built with the aim of providing adequate mixing and tested with operating times of 2-3 weeks for the production of tannase from a tannin-containing model substrate with *Penicillium glabrum* (94).

Liquid-Surface Fermentation (LSF) of Tannase: LSF involves the growth of culture on the surface of a liquid medium at a shallow depth and held in a suitable container (40). Only few preliminary reports (63) are available on the production of tannase by liquid-surface fermentation. Production of tannase is not much advantageous and preferable in comparison to SmF and SSF as evidenced from the published reports . Tannase production by *A. niger* PKL 104 in the three different fermentation systems revealed that enzyme production is 2.5 and 4.8 times higher in the SSF system, as compared to those in SmF and LSF, respectively. Tannase produced by *A. niger* PKL 104 is exclusively

intracellular in the SmF and LSF processes during the first 48 h of fermentation. Subsequently, a larger portion of the enzyme is excreted in the SmF and LSF processes and the ratio of intracellular to extracellular tannase is about 1:6 in SmF and 1: 1 in LSF at the peak enzyme titre levels at 144 h (1).

The results obtained in three different fermentation conditions for extracellular tannase production by *P. Variotii* shows a significant difference in tannase yield between fermentation processes. Maximum (167 ± 3.6 U/ml) tannase production was obtained by SSF at a relatively short incubation time (60 h) followed by SmF (123 ± 3.6 U/ml) at 72 h and LSF (102 ± 4.2 U/ml) at 96 h, respectively. In another report of Rana and Bhat, (88) tannase-producing efficiency of LSF and SSF vis-à-vis SmF was investigated in a strain of *Aspergillus niger*, besides finding out if there was a change in the activity pattern of tannase in these fermentation processes. The studies on the physicochemical properties were confined to intracellular tannase as only this form of enzyme was produced by *A. niger* in all three fermentation processes. In LSF and SmF, the maximum production of tannase was observed by 120 h, whereas in SSF its activity peaked at 96 h of growth (88).

Purification and Immobilization Techniques

Purification : Battestin *et al.* (60) reported fractional precipitation of tannase with 80% ammonium sulphate saturation that removed some of the non-enzymatic proteins at lower concentration with about 34% recovery. Further the elution profile of the tannase obtained from the diethylaminoethyl (DEAE)-sepharose column showed five protein peaks, but tannase activity was reported only in two peaks with 10-fold purification and 3% yield. In another approach, Bhardwaj *et al.* (95) reported a two-step purification procedure for a fungal tannase. In the first step, contaminating proteins precipitated from broth supernatants by ammonium sulphate at 60% (w/v) saturation, were pelleted by centrifugation, discarded and tannase precipitated from the supernatant at 80% saturation. While in the second, it was purified by column chromatography using a DEAE-cellulose column to homogeneity. Mahapatra et al. (96) partially purified the tannase by acetone precipitation and further by gel filtration chromatography (GFC) using Sephadex G-100 column. High performance liquid chromatography (HPLC using GF-250 column) analysis showed a single major peak with the elution time of 6.8 min. Aqueous two phase separation (ATPS) is yet another useful technique for purification of enzymes (97). Tannase from *A. heteromorphus* was partially purified using ultrafiltration (30 kDa membrane) and ATPS but the recovery was not very significant in case of ATPS (98).

Two extracellular tannin acyl hydrolases (TAH I and TAH II) produced by *Verticillium sp*. were purified to homogeneity (7.9 and 10.5 fold with a yield of 1.6 and 0.9%, respectively) by Kasieczka-Burnecka *et al.* (65). Tannase from *P. variable* IARI 2031 was purified by a two-step purification strategy comprising of ultrafiltration using 100 kDa molecular weight cut-off membrane and gel-filtration using sephadex G-200. Also HPLC analysis of the purified tannase showed that the enzyme eluted as a single peak with retention time at 6.31 min (99). A similar strategy of ultrafiltration for partial purification and concentration of *A. niger* LCF 8 tannase was reported by using a 200 kDa cut off membrane. Permeate obtained was again filtered through 100 kDa cut off membrane to eliminate impurities of lower molecular masse that resulted in 80% recovery with a 14.9 purification fold (85).

Mahendran *et al.* (23) attempted to purify tannase from *Paecilomyces variotii*. The dark brown extracellular extract was treated with 1% (w/v) activated carbon that removed more than 50% of the coloured impurities. Further fractional precipitation with 50% saturation of ammonium sulphate removed some of the non-enzymatic

proteins, and tannase was precipitated at 70% saturation with 78.7% recovery. Homogeneity achieved with DEAE-cellulose column chromatography followed by gel filtration led to an overall purification of 30.5-fold with a yield of 17.6%. A recombinant *Aspergillus oryzae* tannase in *Pichia pastoris* was purified to homogeneity from cultured broth supernatants by a simple procedure on DEAE-sepharose. In most of the cases as discussed above, a combination of gel filtration and ion exchange chromatography seems to be more suitable to purify the tannase to homogeneity.

Immobilization : Once the tannase activity is concentrated and eventually purified, it can be immobilized on polymer matrix or solid supports by various immobilization techniques (22). There are several methods reported on enzyme immobilization, microencapsulation being one of the best, creates artificial vesicles with permeable polymer membrane, which like much of living cells, can control the size of molecules transported into or out of the cell. One of the advantages of microencapsulation over regular enzyme entrapment is the high surface area possible per unit of enzyme immobilized, allowing high effectiveness and high concentration of enzyme in the original solution. Microencapsulated *A. niger* tannase on chitosan-alginate complex coacervate membrane was used for synthesis of propyl gallate (100).

Sharma and Gupta (23) immobilized tannase on celite-545 to synthesize propyl gallate. Agarose, chitosan, alginate and different derivatives of siliceous materials were used for immobilization of tannase from *P. variable* by microencapsulation (32). Tannase from *A. oryzae* was also immobilized on various carriers; however tannase immobilization on chitosan glutaraldeheyde showed the highest activity (101). Microencapsulated tannase showed higher synthetic activity than free enzyme and retained about 20.3% of original specific activity. Immobilization of *A. niger* tannase on eupergit-C substantially increased the esterification activity and was used in galloylation (esterification with gallic acid) of catechin at room temperature in ionic liquids. On the other hand Sharma *et al.* (102) immobilized tannase from *A. niger* on concavalin A-sepharose via bioaffinity interaction.

The immobilized preparations are quite stable to reuse, it retained about 81% activity even after the sixth cycle of operation. Ester hydrolysis was also studied using the immobilized enzyme led to a 40% conversion into gallic acid as compared with 30% obtained with the free enzyme (26). So it is noticed that tannase immobilization was found to be beneficial in both the ways for synthesis as well as hydrolysis.

Characteristic Features of Tannases

Molecular Mass : Tannases are known to be high molecular weight proteins and reported to vary from 186 to 300 kDa, mostly polypeptide in nature, depending on the source and type of the microorganisms (40). The molecular weight of *A. niger* MTCC 2425 tannase has been reported of 185 kDa with two polypeptide chains of apparent molecular weights of 102 and 83 kDa (95). The same was in the case of a commercial tannase (Kikkoman, Japan) which separated into two different polypeptides of dissimilar molecular size (87 and 56 kDa) with a total of 143 kDa. Gel filtration of the native enzymes on a calibrated Sepharose Cl-6B column revealed that the molecular mass of the tannases, TAH I and TAH II was 154.5 kDa (65).

Gel-filtration experiments with a calibrated Sephadex G-200 column show an apparent molecular weight of 300 kDa while HPLC on a GPC-diol column was indicative of molecular weight of only 150 kDa. Moreover, PAGE of native purified tannase revealed two bands on silver staining (19). In another report, the native tannase from *P.variotti* showed a single protein band in PAGE corresponding to a molecular mass of 149.8 kDa (23), but when the enzyme sample was treated with SDS and

mercaptoethanol, a single protein band of 45 kDa was found that resembles in a monomeric form of tannase.

However, Hatamoto *et al.* (28) have reported multimeric recombinant tannases from *A. oryzae*. Tannases from *P. variable* showed a single band on urea SDS-PAGE with a molecular weight of 158 ± 2 kDa while the native molecular weight estimated by gel-filtration chromatography was 310 kDa, indicating that *P. variable* tannase probably may be a dimer of two subunits of 158 kDa (78). Similarly *Cryophonectrica parasitica* tannase had molecular weight of 240 kDa as determined by gel-filtration chromatography and it was suggested that the enzyme was a tetramer comprising of four subunits of 58 kDa (55). A tannase of 225 kDa, consisting of 50, 75, and 100 kDa subunits from a xerophilic strain of *A. niger* GH1. Chokar *et al.* (11) have reported a monomeric tannase of 101 kDa from *A. awamori*. Table 2 summarizes details of some tannases along with their molecular masses.

Effect of pH and temperature: Protein structures are influenced by change in pH and a decline in enzyme activity beyond the optimum pH could be due to structural changes, enzyme inactivation or its instability. Tannases are acidic proteins in general and mostly act optimally in acidic range (40). Amongst *Penicillia*, tannase produced from *P. restrictum* showed 100% activity at pH 6.0 and this enzyme was moderately active at an alkaline pH of 8.0 (retaining 31% activity) and at an acidic pH of 5.0 (48% activity) (52). Tannase from *P. charlesii* and *P. crustosum* showed 100% activity at pH 5.0 but no tannase activity was detected at pH 7.0 and subsequent alkaline pH (52). Similar results have been published for *A. niger* LCF 8 (85).

The functional temperature range of the tannases produced from *A. flavus*, *A. fumigatus*, *A. versicolor* and *P. variable*, whereas *A. caespitosum*, *P. charlesii*, *P. crustosum* was 30-70 ºC with optima at 60 ºC and *P. restrictum* had an optimum activity at 40 ºC (52). These results were also in accord with the previous reports of *A. niger* van Tieghem (32). However, lower temperature optima of 30 ºC have also been reported for the tannase from *A. oryzae*, *A. niger* LCF8 (85) and *P. chrysogenum* (106). Tannase from *A. fumigatus* showed maximum stability at 60 ºC, whereas 86% and 13% residual activity were observed at 30 and 70 ºC. Amongst *Penicillii*, none of the tannases was stable at higher temperature of 80 ºC, whereas *P. crustosum* and *P. variable* tannase retained 99 and 95% activity respectively at 60 ºC. Also

Table 2. Molecular mass of some tannases

Sources of tannases	**Molecular mass**(kDa)	**References**
P. variotti	149.8	Mahendran *et al.* (23)
Quercus robur	150.0	Niehaus and Gross (19)
Verticillium sp.	155.0	Monika *et al.* (103)
A. niger	168.0	Sabu *et al.* (104)
A. niger	186.0	Barthomeuf *et al.* (85)
A. niger	205	Marco *et al.* (105)
Candida sp.	250	Aoki *et al.* (41)
A.oryzae	290.0	Hatamoto *et al.* (28)
Quercus robur	300.0	Niehaus and Gross (19)
P. variable	310.0	Sharma *et al.* (99)
A. oryzae	310.0	Hatamoto *et al.* (28)

tannase from *P. charlesii* and *P. crustosum* retained 100% activity at 40 and 50 ºC, respectively (52).

Mondal *et al*. (66) observed that the partially purified tannase was active over a pH range 3.5 to 6.0 and showed an optimum activity at pH 5.7 and found active over a temperature range of 20 to 70 ºC with optimum activity at 60 ºC. Mahapatra *et al.* (96) discussed the effect of pH on purified tannase in the range of 3.5 to 6.0, and the enzyme was active at acidic pH while activity decreased as the pH approached the alkaline range and optimum activity was recorded at pH 5.0. There are several reports mentioning the optimum pH to be 5.5 as in the case of tannase obtained from *A. flavus*, *A. oryzae*, and pH 6.0 in the case of tannase obtained from *P. chrysogenum* (106) and *A. niger* (85).

The optimum pH for the tannase activity from *P. variotii* ranged from 5.0 to 7.0 (23). Also optimum temperature of 40 °C for tannase activity and stability was similar to those reported from *A. niger* (85). According to Sharma *et al.* (99), tannase from *P. variable* was found to be active in the temperature range of 25-80 ºC in three enzyme formulations (crude, purified and immobilized) with temperature optima at 50 ºC. In many fungi, temperature optima for tannase activity have been reported to be in the range of 30-40 ºC (104). However, tannases from *A. niger* van Tieghem (83) and *Bacillus cereus* KBR 9 (66) have been reported to have a temperature optima between 45 and 60 ºC and also temperature optimum of 60-70 ºC has been reported for *A. niger* tannase (88). There was no change in the temperature optima when tannase of *P. variable* was immobilized (99) as against the findings where the temperature optima of immobilized and free *A. niger* tannase has been reported at 40 ºC and 30 ºC, respectively (102). This result is supported by the findings where thermal stability of *A. niger* tannase is significantly improved by the immobilization process (101). Also functional pH range (3.0-10.0) was found broader in all the three forms and more than 80% relative activity at pH 3.0 was observed. In many reports, the pH optimum has been reported in the range of pH 3.0 - 6.0 (106, 107). Two pH optima peaks at 4.0 and 6.0 have been reported for *A. niger* van Tieghem tannase (88). Also, stability of *C. parasitica* tannase was reported over a pH range of 4.0 - 7.5 for 12 h (55) and *A. niger* PKL tannase in a narrow pH range of 4.5 to 5.5 (1).

The crude tannase produced by *Paecilomyces variotii* showed optimum activity at pH 6.5, whereas purified tannase showed pH optima at 5.5 (108). The crude tannase from *Paecilomyces variotii* was stable in a temperature range from 20-70 ºC, where it retained 96% activity at 20 ºC (108). A comparable summary of pH optima and stability is given in table 3.

Substrate Specificity: There is no consistency reported on substrate specificity for tannase activity. *P. variable* tannase showed broad substrate specificity with more affinity for tannic acid having a K_m of 32 milliMolar (mM) followed by methyl gallate (14 mM) and propyl gallate (12 mM). The V_{max}/K_m ratio for tannic acid is almost four times higher than that for methyl gallate and 1.2 times for propyl gallate (65). Other report on K_m for tannic acid was of 0.28 mM for *A. niger* MTCC 2425 tannase (95). A commercial grade (Sigma, USA) tannase from *A. oryzae* was found to have a K_m of 0.42 M with tannic acid (99). Battestin and Macedo, (60), studied the effect of substrate concentration on tannase activity and the graphical analysis of the effect of substrate concentration on tannase activity yielded K_m of 0.61 ìM and V_{max} of 0.55 U/mL proteins. The K_m values for tannase from *C. parasitica* using tannic acid and methyl gallate as substrate were reported as 0.94 mM and 7.49 mM respectively (55).

Effect of Metal Ions: More than 75% of enzymes require metallic ions as cofactors to demonstrate their maximal catalytic capacities. At low

Table 3. Temperature optima and stability alongwith pH optima and stability of tannases from various sources

Sources	Temperature optima, range (ºC)	Temperature stability, (ºC)	pH optima, range (pH)	pH stability	Reference
A. foetidus, R. oryzae	40	5-55	5.0	3.0-6.0	Mukherjee and Banerjee (35)
Paecilomyces variotii	50, 20-80	90	4.5, 4.5-6.5	3.5-8.5	Battestin and Macedo (60)
A. niger	30, 20-70	20-70	5.0, 5.0-6.5	3.0-9.0	Sabu *et al.* (104)
Selenomonas ruminantium	30-40	60	7.0	—	Skene and Brooker (78)
A. niger	35, 20-45	4-50	6.0	3.5-8.0	Barthomeuf *et al.* (85)
P. variable	50	25-80	5.0	3.0-8.0	Sharma et al. (99)
A oryzae	40, 25-75	50	5.5	4.5-6.0	Abdel-Naby *et al.* (101)
A. niger	55	30-90	6.0	3.5-7.0	Ramirez-Coronel *et al.* (38)
Verticillium sp.	20-25	40-50	—	4.5-7.5	Kar et al. (109)
L.plantarum	30	20-60	5.0	4.5-6.5	Rodriguez *et al.* (107)
A. niger	70	40-60	5.5	2.0-8.0	Lekha and Lonsane (1)
A.flavus P. restrictum P. charlesii	40	30-50	5.0-6.0	3.0-8.0	Batra and Saxena (52)
Bacillus cereus	40	40	4.5	3.0-7.0	Mondal *et al.* (66)
Quercus robur	35-40	55	4.3-5.0	3.8-7.8	Niehaus and Gross (19)
P. variotti	30-50	30-50	5.0-7.0	4.0-8.0	Manjit *et al.* (8)

concentrations some metals act as cofactors enhancing the enzymatic activity, but at high concentrations the effect is inhibitory (9). Therefore, the effect of metals and ions on purified and partially purified tannase was evaluated at concentrations ranging from 0.5 to 2.0 mM by several researchers. Mg^{++} and Hg^{++} stimulated maximum tannase activity at 1.0 mM, but Ba^{++}, Ca^{++}, Zn^{++}, and Ag^{+} inhibited it slightly whereas Fe^{+++}, Co^{++} completely inhibited tannase activity at the same concentration (109). Also tannase from *A. niger* GH1 was highly inhibited by Fe^{+++}, mildly inhibited by Cu^{++} and Zn^{++} while the same concentration of Co^{++} enhanced the enzyme activity (9). Kasieczka-Burnecka *et al.* (65) also reported similar findings for TAH I and TAH II, where only Mg^{++} ions activated both the tannases and the other metal ions (Zn^{++}, Cu^{++}, K^{+}, Cd^{++}, Ag^{+}, Fe^{+++}, Mn^{++}, Co^{++}, Hg^{++}, Pb^{++} and Sn^{++}) acted as inhibitors.

In another report of Kar et al. (109), Br^{-} and $S_2O_3^{-}$ stimulated tannase whereas, CO_3^{-}, OH^{-}, inhibited the activity of the enzyme. Mg^{++} acts as an activator on tannase produced by co-culture of *R. oryzae* and *A. foetidus* (37). Metal ions like K^{+}, Ca^{++} and Zn^{++} did not affect *L. plantarum* tannase activity but Mg^{++}, Hg^{++} partially inhibited at 1mM (107). Similarly in the report of Barthomeuf *et al.* (85) tannase from *A. niger* was

not inhibited by Ca^{++}, Co^{++}, Mg^{++}, Mn^{++}, Ni^{++} even at 100 mM.

Effect of Denaturants and Surfactants: The effects of chemical substances on the activity of an enzyme are often precise and specific. Surfactants are reported to play key roles in the catalytic activity of the enzymes. Urea acts as denaturing agent at about 6 - 8 M (23), by breaking all hydrogen bonds present in the protein structure. Although urea is known to inhibit enzyme activity, it was reported to enhance maximum tannase activity at a concentration of 1.5 M (109).

Tannase activity was reported at various concentrations (0.1 - 2.0% w/v) of sodium lauryl sulphate (SLS) that gradually decreased with increasing percentage of SLS (23). This inhibition may be the result of the reduction in the hydrophobic interactions that play a crucial role in holding together the protein tertiary structure and the direct interactions with the protein molecules. Tween 80 is predominantly composed of oleic acid (70%) and Tween 20 of lauric acid (52%) so due to the predominance of oleic acid and lauric acid, they cause a decrease in tannase activity. Triton X-100 also caused a decrease in tannase activity at concentrations of 0.5 and 1.0% (v/v) as reported by Battestin and Macedo (60). In a similar report, Kar *et al.* (109), Tween 60 at 0.05-1.0% (v/v) and SLS, at 0.05-0.7%, caused inhibition of tannase activity. The extent of stimulation and inhibition by surfactants varies for the different enzymes, needs to be studied thoroughly.

Effect of Chelators and Inhibitors: The chelators viz., ethylene diamine tetra acetic acid (EDTA) disodium salt and 1, 10-*o*-phenanthrolein at a concentration of 1.0 mM (60) inhibited the tannase activity. Also the effect of EDTA (1 to 10 mM) on tannase activity was studied and a concentration of 5 mM was reported completely inhibitory for tannase activity (23). Tannase from *A. niger* and *A. oryzae* was also inactivated by EDTA, whereas no inhibition was observed in the case of the tannase from *A. flavus* (109). The decreased enzyme activity reported in the presence of EDTA, could be due to its influence on the interfacial area between the substrate and enzyme.

In industrial enzymology, the main importance of inhibitors is that they reduce the efficiency of enzyme reaction by altering the active sites. Studies on *P. variable* tannase showed that the enzyme was inhibited by PMSF and *b*-mercaptomethanol. Moreover, N-ethylmaleimide showed strong inhibition while 1,10-*o*-phenanthroline was a mild inhibitor (99). Similarly *R. oryzae* tannase was inhibited by DMSO, *b*-mercaptoethanol and 1, 10-*o*-phenanthrolien (109) and *A. niger* LCF 8 tannase was inactivated by *b*-mercaptoethanol (85).

Inhibition studies primarily provide an insight into the nature of the enzyme, its cofactor requirements and the nature of the active enzyme. Tannase activity was found to be inhibited by sodium bisulphite, iodoacetamide, 2-mercaptoethanol, 4-aminobenzoic acid, sodium azide, n-bromosuccinimide and cysteine at a concentration of 1 mM. When added to the reaction medium, cysteine inhibited the tannase activity of *Paecilomyces variotii* also the inhibition of tannase activity by cysteine and 2-mercaptoethanol suggests the presence of sulphur containing amino acids at the active site of the enzyme (60).

Stability of Tannase in Non-aqueous Solvents: Organic solvent stability of tannase is a very important parameter for its synthesis activity in non-aqueous media. Synthesis of various gallic acid esters by tannase takes place only in non-aqueous solvents. *P. variable* tannase (99) showed different degrees of stability in various organic solvents. More than 60% residual activity in 20% (v/v) of carbon tetrachloride, heptane, petroleum ether and toluene after 60 min of incubation, reflects its stability, but in the

case of acetone and formaldehyde only 49 and 12% of residual activity was retained. The effect of different solvents at two levels (20 and 60% v/v) was tested (9) and it was found that at 60% of concentration, ethanol and acetone completely inhibited the tannase activity, whereas tetrahydrofuran and formaldehyde exhibited inhibitory effect only at 20% (v/v). Propanol acts as an activator in the range of 3.6 - 7.3% (v/v), but higher concentration inhibits the propyl gallate synthesis by denaturation of tannase (100). Stability in organic solvents suggests suitability of tannase for synthetic reactions, and opens new path to synthesize novel compounds of pharmaceutical interest.

Conclusions

This review focuses on the recent advances on the scientific and technological aspects of tannase fermentation techniques (SmF, SSF and LSF) and downstream processing. Recent advances in submerged and solid state fermentation of tannase bioprocessing have been explained thoroughly. Additionally this review includes a list of agro-industrial residues used for high yield cost effective fermentation of tannase. A detailed depiction of physicochemical characterization like pH and temperature stability, stability in non-aqueous solvents, effects of metals and ions, surfactants and denaturants including special features represents a comparative and unique collection of information for future researchers and industrial need. Moreover this review gives clear idea of future perspectives on bioprocessing strategies to reduce the production cost of tannase by using agro-industrial residues and increased feasibility of scale-up studies towards commercialization.

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Delivery Strategies to Improve *In Vivo* Stability of Immunogenic Peptide PADRE

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Abstract

PADRE, a peptide with potential in breast cancer immunotherapy exhibits low *in vivo* efficacy due to poor tumor uptake and significant enzymatic degradation. In the present work, novel delivery based approaches were utilized to improving the efficacy of PADRE. The utility of these approaches in the delivery of PADRE was tested on *in vitro* cell based models. In the *in vitro* systems, PADRE was not internalized in Caco-2 cells to a significant extent as observed from the concentrations in cell pellet, which were always lower than 5%. Also, 90% of PADRE externally associated with the Caco-2 cells was degradated within 4 hours, possibly due to the breakdown by ecto-peptidases associated with tumor cells. Enzyme inhibitors, antitrypsin reduced the PADRE degradation in Caco-2 cells (20%) as compared to untreated cells (55%). The lipoprotein based systems were formulated using with and without 0.1% sodium lauryl sulfate that yielded 45 and 53% loading of PADRE, respectively. The efficacy of PADRE lipoprotein-based systems was determined by performing CD4 proliferation assay. The lipoprotein based systems with 20 µg/mL treatment with and without 0.1% sodium lauryl sulfate showed 3.2 and 2.8 times CD4 proliferation observed as compared to control. The CD4 proliferation on treatment with native PADRE was 4.3 times compared to a no treatment control, however this was not statistically significant ($p>0.05$) when compared to the PADRE activity from lipoprotein systems indicating that thelipoprotein-based approach was suitable delivering PADRE as it retained the immunogenic activity. The strategies studied to deliver PADRE were successful *in vitro* and showed potential for improving its immunogenic efficacy *in vivo* by improving its stability.

Keywords: PADRE delivery, peptidase inhibitors, lipoprotein delivery system

Introduction

Breast cancer is the most common cancer detected amongst women worldwide and also the second most leading cause of cancer related deaths amongst women in the world. According to statistics released by the National Cancer Institute, there were 2,30,480 cases of breast cancer in females in the United States alone reported in the year 2011 and an estimated 39,520 deaths. Breast cancer treatment involves radiation and surgery to locally treat and remove the primary tumor followed by adjuvant therapy to combat the metastasized tumor cells. Adjuvant therapy includes treatment with chemotherapeutic drugs, hormonal therapy and immunotherapy. Chemotherapy is effective but non-specific and therefore suffers from several adverse effects. Immunotherapy is gaining importance due to the selective recognition and destruction of cancer cells, thereby sparing the normal cells and minimizing the adverse effects.

Trastuzumab, a monoclonal antibody targeting the HER2 receptor, is a leading immunogenic therapy for breast cancers that exhibit HER2 over expression. However treatment with Trastuzumab is limited to around 25% of total breast cancer cases showing HER2 overexpression and the treatment also suffers from adverse effects like cardiotoxicity (1).

Breast cancer cells show expression of various MHC II antigens, which makes them suitable targets for treatment with immunogenic peptides. Several tumor associated antigens (TAA's) including HER2, p53, MUC1, BRCA2, survivin, IGFBP2 etc., have been identified in breast cancer patients and autoantibodies to these TAA's have been detected in the serum samples which gives further evidence of their immunogenic potential (2). This immuno-genic response has been exploited for early diagnosis of these tumors and also for treatment of the tumors by targeted delivery of tumor specific immunogenic peptides. The immunogenic peptides mark the tumor cells for recognition by the immune system and eventually destroy them (3). Some immunogenic peptides exhibit strong immune response *in vitro* or when coated on to tumor surfaces before innoculation *in vivo* indicating that the peptides are recognized by the T lymphocytes. The physiological barriers and the metabolic enzymes may be responsible for the poor uptake and degradation of the peptides respectively, which prevents the same immune response to be observed when the peptides are injected systemically.

Pan DR Reactive Epitope (PADRE) is a 13 amino acid peptide sequence, aK(X)VAAWTLKAAa, which effectively binds to several MHC allelic variants and produces potent immunogenic responses in *in vitro* systems like T cell proliferation assays. PADRE is not effective on systemic administration, which could possibly be due to the enzymatic degradation of the peptide by the proteases and peptidases secreted by the tumor cells and also the physical and physiological barriers presented by the tumor cells. Wenning *et al.* developed a model for relating immunotoxin toxicity to cellular trafficking in a single cell and extrapolating with diffusive transport of immunotoxin in a solid tumor sphere. Immunotoxins were found to be less effective against multi-cell tumor spheroids (MTS) than monolayer cells under equivalent conditions. The poor efficacy was traced to either heterogeneous receptor distribution in MTS or significant barrier(s) to the penetration of the immunotoxin into the spheroid (4). In our previous publication, we reported the degradation of PADRE in biological matrices like intact tumor, tumor homogenates and plasma (5).

Improving the stability of PADRE and make them available for systemic and tumor uptake of PADRE using various delivery approaches is the objective of the current study. Two different approaches were investigated to improve the stability and *in vivo* immunogenic potential of PADRE, namely, use of enzyme inhibitors and use of lipoprotein based drug delivery systems. Based on previous studies, co-administration of peptidase inhibitors with proteins and peptides like insulin and nonapeptide PHPFHLFVF (a renin inhibitor) was found to improve their stability and absorption (6,7). We investigated the utility of this approach by selecting inhibitors of specific peptidases, which could possibly metabolize PADRE on the basis of its structure. The second approach investigated was the development of a lipoprotein based drug delivery system of PADRE to specifically target the low-density lipoprotein (LDL) receptors overexpressed on the tumors (8). The lipoprotein carriers have two major advantages over liposomes, mainly the smaller size and longer half lives of 3-5 days. Also, because lipoproteins are natural endogenous substances, they are non immunogenic and escape the reticuloendothelial system and will not interfere with PADRE immunogenicity. The LDL and HDL based drug delivery systems have been designed for the

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delivery of some hydrophobic cytotoxic drugs to solid tumors. In a previous report, the LDL-drug complex was prepared for selective delivery of the cytostatic agent to tumors. However rapid dissociation of the complex in plasma resulted in stability issues preventing further development of these systems (9). In our study we explored lipoprotein approach for delivery of PADRE to tumors by physical entrapment of the peptide within the lipoprotein systems.

Materials and Methods

The peptide PADRE was synthesized by Genemed Synthesis Inc., San Francisco, USA. Low-density lipoproteins (LDL) and enzyme inhibitors were obtained from Sigma, USA. Caco-2 cells were obtained from ATCC (Manassas, VA, USA). The Caco-2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM,Invitrogen Co., Carlsbad, CA) containing 10% heat inactivated fetal bovine serum and 1% penicillin-streptomycin (5000 I.U./ml, Cellgro, Mediatech Inc, VA). The cells were incubated in an environment of 37 °C and 5% CO_2. The cells were grown up to 80-90% confluency before further passage for experiments.

Effect of peptidase inhibitors on stability of PADRE: Caco-2 cells were cultured at a density of 1 x 10^6 cells. The cells were trypsinized and suspended in growth media. For the control studies, 500 ìL of 100 ìg/mL of PADRE was incubated in PBS with Caco-2 cells in culture tubes for 0.5, 1, 2, and 4 hours at 37ºC. The cells were separated from the supernatant by centrifugation at 10,000 rpm for 5 minutes, digested, and the amount of peptide in supernatant and cell pellets was estimated by the HPLC method described previously (5). To determine effect of enzyme inhibitors on the stability, Caco-2 cells were pretreated with enzyme inhibitors (Anti-trypsin – 10 µM, Bestatin – 100 µM, Diprotinin A – 100 µM , Phospharidon –1 µM and Phenanthroline –100 µM) for one hour followed by treatment with PADRE (Table 1). At the end of 2 hour incubation, samples were deproteinated and the concentration of PADRE was analyzed by HPLC.

Preparation of lipoprotein delivery systems: Ten mg of LDL was triple washed with 5 mL of heptane to extract the lipids. The apoprotein B was suspended in 3 mL of PBS using a laboratory

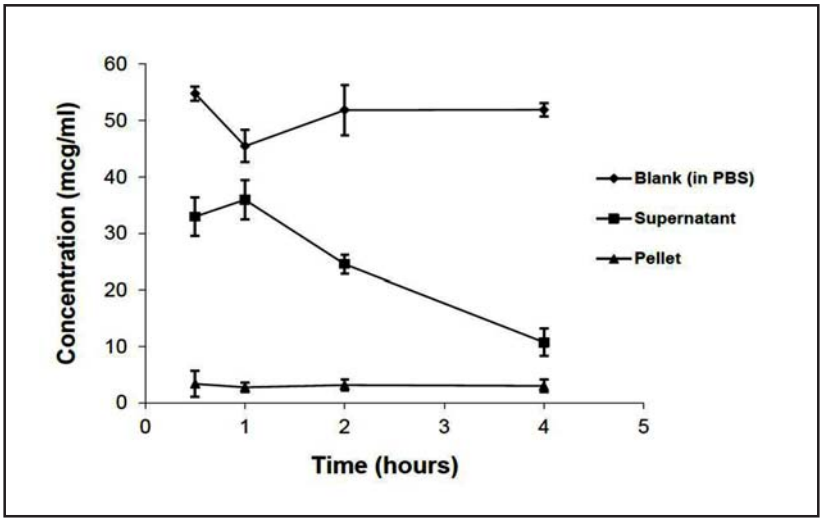

Fig. 1. Stability of native PADRE in Caco-2 cells

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vortex. To this, aqueous solution of peptide at 1 mg/mL was added, vortexed, and lyophilized at –50 °C for 12 hours to remove water. The heptane extract of lipids were then added to the powder mixture of apoprotein B and peptide and vortexed for 5 minutes. Heptane was slowly removed using rotoevaporator at 40 °C for 30 minutes under vacuum. Lipoprotein delivery systems were also prepared using exactly the same protocol except that 0.1% sodium lauryl sulphate was used during the suspension of the peptide in the apoprotein B.

Stability of PADRE-lipoprotein system in Caco-2 cells: Caco-2 cells were cultured at a density of 1 x 10^6 cells. The cells were trypsinized and suspended in growth media. PADRE-lipoprotein equivalent to 50 mcg/mL of peptide, was added to Caco-2 cells in culture tubes and incubated for 4 hours at 37 degrees. The cells were separated from the supernatant by centrifugation at 10,000 rpm for 5 minutes, digested, and the amount of peptide was estimated by the HPLC method. Native PADRE solution of 50 mcg/mL in water was used as control.

CD4 proliferation assay to determine immunogenicity of PADRE-lipoprotein system: CD4 positive T-lymphocytes were derived from immunized mice and subsequently selected via *in vitro* stimulation by peptide and dendritic cells. PADRE specific, CD4 cell proliferation assay was performed by incubating T-lymphocyte cells in triplicate with PADRE 10 and 20 µg/mL or equivalent amount of PADRE incorporated lipoprotein delivery systems, and dendritic cells in the presence of IL2 at 37°C for 48 hours. At the end of 48 hours, the cells were incubated with 3H-Thymidine for 4 hours and harvested. Thymidine incorporation was measured on a scintillation counter. CD4 proliferation was taken as a measure of immunogenicity of PADRE. Naïve lymphocytes served as a negative control.

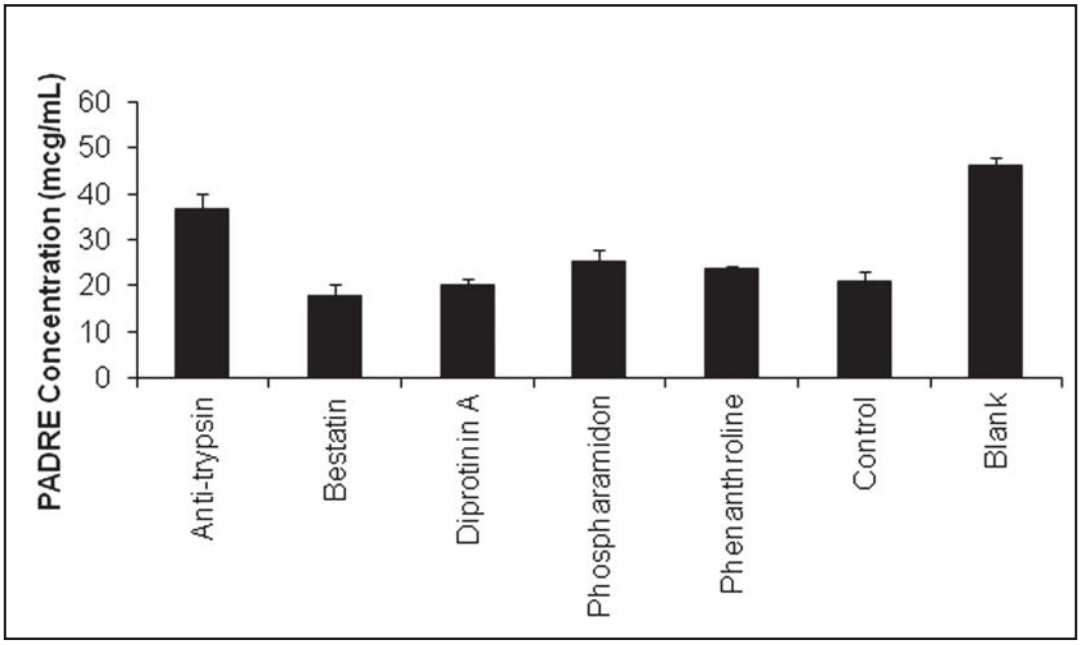

Fig. 2. Improvement in PADRE stability upon pretreatment with peptidase inhibitors determined in Caco-2 cells.

Results and Discussion

As reported in our previous manuscript, PADRE showed considerable degradation in all biological matrices like plasma, tumor and tumor homogenates, with most rapid degradation seen in plasma. PADRE was found to be stable in PBS (control) as shown in figure 1. PADRE stability in Caco-2 cultures was determined by incubating in the culture meda. After Caco-2 cell digestion, the amount of PADRE present in the pellet represented the internalized fraction and amount present in the supernatant represented the PADRE associated with the cells. The amount of PADRE in the pellet at all times was less than 5%. PADRE present in the cell supernatant exhibited rapid degradation. The concentration of the peptide decreased to 50% of the original amount in 2 hours and up to 90% in 4 hours. This data suggested that PADRE is degraded by the ecto peptidases present on the tumor cell surface. The substrate specificity of these peptidases is found to be limited to small peptides (di-, tri-, and oligopetides) up to a maximum of approximately 30 residues (10,11). Based on the possible degradation sites, peptidase inhibitors given in Table 1 were selected for co-delivery with PADRE. In a study by Takaori *et al,* the nonapeptide, PHPFHLFVF, was found to cross rabbit jejunum 90% intact in the presence of phosphoramidon, a metalloproteinase inhibitor (6). In the absence of protease inhibitors, 50% of the peptide was degraded in 5 min and 100% in 30 minutes. Insulin, another large peptide used to study the effect of protease inhibitors, was protected by diisopropyl fluorophosphate against serine proteinases (12), and aprotinin against RNAase (7). The unnatural D- amino acids at the N- and C- termini of the PADRE peptide and the unnatural amino acid cyclohexylalanine at position 3, rendered it more stable than other immunogenic peptides. The data from peptidase inhibitors shown in Figure 2 indicated that antitrypsin offered the maximum protection; only 20% of PADRE was degraded compared to 55% in untreated Caco-2 cells. Phosphoramidon and phenanthroline offered only marginal protection (45% and 49%, respectively).

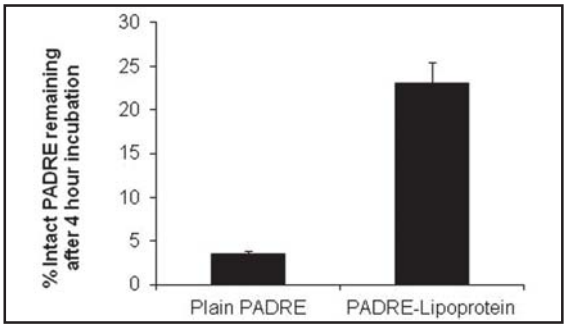

Fig. 3. Stability of PADRE determined in Caco-2 cells after 4 hour treatment with PADRE- lipoprotein delivery system.

To counter the problem of poor tumor barrier penetration, PADRE was formulated into a lipoprotein based drug delivery system. The LDL receptor overexpression on tumor surfaces improves the uptake of the lipoprotein based drug delivery systems (13). The PADRE lipoprotein based system exhibited a yield of 45%. The use of 0.1% sodium lauryl sulfate during the suspension of the PADRE in the apoprotein B, improved the yield to 53%. The stability of PADRE in Caco-2 cells was enhanced when incorporated into lipoprotein systems. The percent PADRE in Caco-2 cells remaining intact upon incubation for 4 hours increased from 3.5±0.4% to 23.1±2.4% when used in the lipoprotein system (Figure 3). The proliferation of CD4 cells when incubated with native peptide and lipoprotein delivery systems (without SLS and with SLS) was higher when compared with incubates without PADRE. As shown in Table 2, CD4 proliferation was increased by 4.3, 3.2, and 2.8 times at 20 µg/mL and 2.5, 2.0, and 2.3 times, at 10 µg/mL equivalents of PADRE for treatment with native peptide and lipoprotein delivery systems (without SLS and with SLS). The CD4 proliferation was not statistically significant amongst the PADRE treated groups ($P>0.05$). This data suggested that the immunogenicity of PADRE was preserved during the preparation of lipoprotein delivery systems and these systems were suitable in offering protection against loss of immunogenicity (Table 2). In previous studies, the lipoprotein based systems containing

Table 1. Different peptidase inhibitors selected for pretreatment with PADRE and test concentrations used.

Peptidase Inhibitor	Enzyme/ Enzyme familyinhibited	Recommended Concentration (micromolar)
Antitrypsin	Serine proteases	10
Betastatin	Aminopeptidase N	100
Diprotinin A	Dipeptidyl peptidase IV	100
Phosphoramidon	Endopeptidase 24.11	1
Phenanthroline	Metallopeptidases	100

Table 2. CD4 proliferation on treatment with native PADRE and PADRE lipoprotein systems.

Control	Native PADRE		Treatment with PADRE-Lipoprotein without surfactant		Treatment with PADRE-Lipoprotein with surfactant	
	20 mcg/mL	10 mcg/mL	20 mcg/mL	10 mcg/mL	20 mcg/mL	10 mcg/mL
X	3.5X	1.7X	3.4X	1.5X	2.8X	1.3X
	9.1X	3.2X	3. OX	2.1X	2.8X	1.3X
	2.7X	2.9X	3.5X	2.1X	2.9X	3.7X
	1.9X	2.3X	2.7X	2.3X	2.5X	2.9X
AVEGARE	4.3X	2.5X	3.2X	2.0X	2.8X	2.3X
SD	3.3X	0.7X	0.4X	0.3X	0.2X	1.2X

anticancer drug Daunorubicin (14), showed higher cytotoxicity in the LDL receptor positive Chinese hamster cells as compared to mutant cells (LDL negative). The lipoprotein-based approach has recently been utilized for siRNA delivery. The delivery of chol-siRNA via lipoproteins, improved the knock-down efficiency from 0% to 38% as compared to the delivery of the native siRNA at a 100 nM treatment in HepG2 cells (15). However due to receptor mediated endocytotic uptake, siRNA remained entrapped in the endo-lysosomal compartments. Disruption of the endosomes by "photochemical internalization" further increased the silencing efficiency to 78% (15). The results with the PADRE containing lipoprotein systems followed this trend, with significant CD4 proliferation observed for treatment with PADRE lipoprotein based systems. Thus, lipoprotein delivery systems have potential in the delivery of immunogenic peptides PADRE to tumor while maintaining their immunogenic potential.

Conclusion

Two approaches were studied for improving the stability and immunogenic potential of PADRE. The use of peptidase inhibitors provided protection against degradation of the PADRE in

the *invitro* systems. Antitrypsin provided significant improvement in stability when compared to other inhibitors of ecto-peptidases. The lipoprotein based drug delivery approach offered better tumor barrier penetration due to the proposed endocytotic uptake and also helped in maintaining the immunogenic potential of the peptide. Thus, the *in vitro* results indicate that these delivery approaches have potential in improving *in vivo* efficacy of PADRE.

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Analysis of Population Structure of *Magnaporthe grisea* Using Genome Specific Microsatellite Markers

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Abstract

In order to understand the pathogen's genetic diversity and dynamics of fungal populations, 34 rice blast isolates collected from various blast endemic areas of India were analyzed using *Magnaporthe grisea* genome specific microsatellite markers (MGM) and repeat element based Pot2 primer. All the blast isolates showed average pair wise similarities in the range of 0.15 to 0.9 and suggested the large variations with in the isolates collected from the different places. To know the genetic relationship among the blast isolates, a dendogram was generated based on analysis of MGM and Pot2 primers separately and in combination using SHAN/ UPGMA program. The cluster analysis grouped all the blast isolates in to different clusters mostly based on the location, from where they were collected. Based on International blast differential lines containing single resistance genes, it was identified that *AVR Pi-k* gene was present predominantly in isolate collected from Nellore in costal Andhra Pradesh; and *AVR Pi-2* and *AVR Pi-4* genes in isolates collected from Mandya (Karnataka). The isolates collected from Almora, Ranchi and Nawagam showed the presence of *AVR Pi-1* and *AVR Pi-4a* genes. The present study helped us to understand the population diversity and their AVR genes spectra in the blast hotspot regions of India, which can be useful in deployment strategies for blast resistance genes in rice improvement programmes.

Key words: *Avr* genes, Blast resistance genes, Cultivars, Pathosystem, Repetitive DNA sequences.

Introduction

Rice blast disease is caused by a heterothallic ascomycetes fungus *Pyricularia grisea* (Teleomorph: *Magnaporthe grisea* (Hebert) Barr). Among all the biotic stresses of rice, blast disease alone causes yield loss of about 50 % (1). The fungus grows in all rice-growing areas across the world and attacks almost all the aerial parts of rice plant typically, leaves and panicles. Sesma and Osbourn (2) revealed that the foliar blast pathogen also invades roots using a typical root-specific pathway. It is estimated that the loss due to this disease is over 70 % in the USA (3). Neck blast is the most destructive form of the disease, which causes the maximum damage to the yield. Though chemicals are available to combat this disease, deployment of resistant cultivars is the best alternative method, which is economically viable and environmentally safe. But, high mutation rate of this fungus can overcome the resistance within a short time after the release of a new cultivar thus making the breeding for resistance to blast a constant challenge. To

understand the rice-*Magnaporthe grisea* pathosystem, knowledge of the pathogen's genetic diversity and the mechanisms that lead to the development of new virulent genotypes are very much required. Population studies of the rice blast pathogen have been studied across world for their phenotypic and genotypic variations (4, 5, 6, 7, 8, 9, 10, 11, 12, 13). The genetic diversity of *Magnaporthe grisea* and its correlation with pathotypes have been studied by various methods, including use of repetitive DNA sequences *i.e., Magnaporthe grisea* repeats (MGR), which have core repetitive sequence of 1,860 bp, with an estimated average of 46 copies per genome (14, 15). Retrotransposon repetitive elements like *grh* (16) and *MAGGY* (17) have also been exploited for diversity analysis. But, assessment of polymorphism at these repetitive element regions has relied on restricted fragment length polymorphism (RFLP) technique, which is often costly and labor intensive. Kachroo *et al.,* (18) described a method that revealed the polymorphism of Pot2, (a unique repetitive element found in the *M. grisea* genome) based on PCR with primers flanking the core repetitive sequence but, the Pot2 marker also targets the repetitive element which is shared by the isolates which infects rice and other hosts. At present, simple sequence repeats (SSR) which are relatively abundant in genomes of eukaryotes have become a method of choice for diversity studies (19, 20, 21). To develop, microsatellite markers, (AG)n microsatellite-enriched genomic DNA library varying from fivefold to 60 repeat motifs depending on the isolates was constructed for *Magnaporthe grisea* and a set of 24 SSR markers were designed out of which three markers were validated (22). In India, a huge diversity of blast pathogen existing since rice is grown in different agro climatic regions (23), but the analysis of diversity was carried out using random and repeat element based markers (24). Therefore development of new and alternative tools is required to analyze the diversity and to monitor the dynamics of fungal populations which will help in designing strategies for disease control in a more advanced and precise manner. Admittedly a PCR based system is much simple, inexpensive, convenient and more accurate than a fingerprint based system. Hence, the present study was aimed to determine the population diversity and the relationship of the blast pathogen races in the blast hotspot regions of India using *Magnaporthe grisea* genome specific microsatellite markers.

Materials and Methods

***Isolation and maintenance of M. grisea cultures*:** Thirty four isolates of *M. grisea* were collected from blast infected rice leaves or panicles from different blast hot spot regions of India (Table 1). The leaf bits having a single, non coalescing spot were selected for fungal isolation. The leaf bits were surface sterilized with 0.1 % mercuric chloride followed by 4 to 5 times repeated washes with sterile distilled water. The infected leaf bits were cultured on leaf extract agar (Leaf decoction 100 g^{-l}, sucrose 20 g^{-l} and agar 20 g^{-l}) and incubated at 27°C for 5 days in dark and 3 days in light for mycelial growth. From the mycelia, spore suspension was prepared in sterile distilled water and plated on to 1 % water agar and incubated for 12 h at 27°C for conidia germination. Single germinating conidium was observed under microscope and subsequently transferred to test tubes containing sterile rice bran agar for culture establishment. The established cultures were kept at 4°C for storage and further investigations.

***DNA extraction*:** Seven day old pre-inoculated *M. grisea* agar block was transferred into sterile 2% Yeast Extract Glucose (YEG) broth and incubated at 28°C for 7 days for mass production of the fungal mycelium. The obtained mycelium (~250 mg) was lyophilized using liquid nitrogen and used for DNA extraction. The DNA was extracted by following CTAB method (25). A working DNA solution was made by diluting DNA stock to approximately 10 – 20 $ng\mu l^{-1}$.

***DNA fingerprinting of M. grisea isolates*:** Polymerase Chain Reaction (PCR) was performed in a final volume of 25 µl consisting of 0.25 mM of each dNTP's, 1 unit of *Taq* DNA polymerase, 15-20 ng of DNA template, 0.3 µM of primer (Integrated DNA Technologies, Iowa, USA), 1 X PCR buffer (containing 1.5 mM $MgCl_2$) and sterile distilled water. The reactions were carried in a Thermal Cycler PT-100 (MJ Research, Watertown, MA). Thermal profile was 94°C for 5 min, followed by 36 cycles of 94°C for 45 sec, 50-60°C for 1 min, 72°C for 1 min with final extension of 72°C for 7 min. The PCR products were electrophoresed at 100 v for 3 h on 3.5 % agarose gels in 0.5 X TBE buffer for MGM primers and on 1 % agarose gel for Pot2 primer. Gels were stained with ethidium bromide and visualized using UV Trans illuminator. Twelve MGM Primers and a Pot2 primer were used for PCR amplification of 34 *M. grisea* isolates.

***Analysis of DNA fingerprints*:** The profiles generated by different MGM and Pot2 primers were compiled to determine the genetic relatedness among the different *M. grisea* isolates. The presence or absence of each band in all the isolates was scored manually by binary data matrix with '1' indicating the presence of the band and '0' indicating the absence. Data were generated separately for each primer. A similarity matrix was generated from the binary data using Jaccard's similarity coefficient in the SIMQUAL program of the NTSYS-pc package. Cluster analysis was performed with the unweighted pair group arithmetic mean method (UPGMA) in the SHAN program of the NTSYS-pc package (26).

Results and Discussion

Thirty four *M. grisea* isolates from various endemic regions in India were selected for the present study to gain insight into the genetic diversity of the fungal population. A set of 12 *Magnaporthe grisea* Microsatellite (MGM) markers and a repeat element specific primer Pot2 were used in the present study. Of these markers, 10 MGM markers *viz.,* MGM - 1, MGM - 2, MGM - 3, MGM - 4, MGM - 5, MGM - 6, MGM - 9, MGM - 10, MGM - 21 and MGM - 24 showed consistent banding pattern. All the MGM markers gave a large number of distinct scorable fragments per primer (Fig. 1). All the bands that could be reliably read within the size range of 100- 800 bp were treated as individual loci. A total of 70 amplicons were obtained from 34 *M. grisea* isolates with 10 MGM markers. The number of loci amplified by each primer pair ranged from 6 to 8 with an average of 7.8 per primer. The maximum number (8) of fragments was amplified by the marker MGM - 9, whereas minimum number (6) of fragments was amplified by MGM - 24. The Pot2 primer has shown consistently 17 amplicons; all the amplicons were polymorphic among the isolates. The PIC values were estimated for all the markers, a high PIC value of 0.60 was observed with MGM - 21 and a low PIC value of 0.24 was observed with MGM - 24, while the Pot2 primer displayed a PIC value of 0.26. Although Brondani *et al.,* (22) identified and designed MGM primers; they could not standardize the amplification conditions for most of the primers, except for MGM - 1, where they observed the presence of 9 alleles. Here, we successfully standardized the amplification conditions all the MGM markers and utilized them in the diversity analysis. Although many previous reports on diversity analysis of blast fungus exists in India (1) but, none employed the PCR based SSR maker system. We found this marker

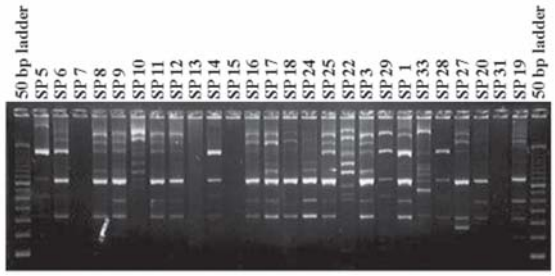

Fig. 1. *MGM*-based polymerase chain reaction fingerprint patterns from genomic DNA of rice-infecting *Magnaporthe grisea* isolates from India analyzed on ethidium bromide stained 3.5 % agarose gel.

system is easy in operation, locus specific and works uniformly with all the isolates. The speed, simplicity and reliability of PCR based approaches make microsatellite analysis on agarose gels an attractive tool for MAS in rice breeding programs aiming at developing durable rice blast resistant cultivars (27), SSRs also the marker choice for genomic analysis for many crop species (28).

Moreover, we observed the higher PIC scores for MGM markers in compared to the Pot2 primers, which indicate that these markers were better suited for understanding the genetic diversity in blast fungus populations.

***MGM based genetic relationship*:** Cluster analysis of the blast isolates revealed the average pair wise similarities in the range of 0.15 to 0.9 suggesting large variations among the isolates of the present study. The observed diversity level was much higher than that of an earlier study (24). This could be because the present study involved isolates collected from different hotspot regions of India, while the earlier study used only the isolates collected from Northwestern Himalayan region of India. All the isolates were grouped into two major groups (Fig. 2). Cluster–I consisted of 29 isolates, which were further divided into two sub clusters (IA and IB). The sub cluster IA consisting of four isolates in that, two isolates (SP 5 and SP 6) were collected from DRR and another two isolates were collected from Nawagam (SP 27 and SP 28) and all the isolates of this sub cluster shares 32 % similarity. Whereas, sub cluster IB was comprised of 16 isolates, among which 7 isolates were collected from DRR and rest were collected from different blast endemic areas of India, all the isolates of this sub cluster have the combined similarity of 35 %. Whereas, the second major cluster consists of 5 isolates, among which two isolates were collected from Palampur and 3 isolates were collected from DRR, all the isolates in this cluster showed only 15 % similarity.

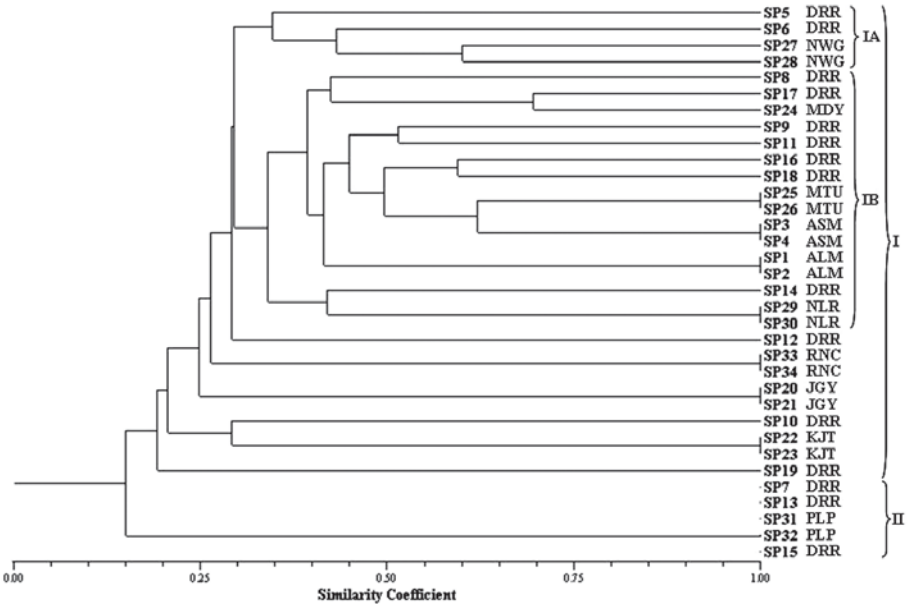

Fig. 2. Dendogram constructed from *MGM* and Pot2 primers based fingerprint data from 34 rice infecting of *Magnaporthe grisea* isolates from India. (ALM- Almora, ASM- Assam, DRR- Directorate of Rice Research, JGY- Jagityala, KJT- Karjat, MDY- Mandya, MTU- Mareturu, NLR- Nellore, NWG- Nawagam, PLP - Palampur, RNC - Ranchi).

Table 1. List of *Magnaporthe grisea* isolates collected from different parts of India

S. No	Blast isolate	Host (Rice genotype)	Place of collection	Remarks
1	SP 1	VL-Dhan 61	Almora	Susceptible to blast
2	SP 2	Swarnadhan	Almora	Susceptible to blast
3	SP 3	DR-92	Assam	Susceptible to blast
4	SP 4	Mahsuri	Assam	Susceptible to blast
5	SP 5	C101LAC	DRR	Monogenic line of Co 39 background containing *Pi-1* gene
6	SP 6	C101A51	DRR	Monogenic line of Co 39 background containing *Pi-2* gene
7	SP 7	C101PKT	DRR	Monogenic line of Co 39 background containing *Pi-4a* gene
8	SP 8	RIL 29	DRR	*Pi-7*
9	SP 9	BL-245	DRR	*Pi-2, Pi-4*
10	SP 10	A57	DRR	*Pi-1,Pi-2* and *Pi-4*
11	SP 11	Raminad str 3	DRR	International Blast differential
12	SP 12	NP 125	DRR	International Blast differential
13	SP 13	Usen	DRR	International Blast differential, known to contain *Pi-a* gene
14	SP 14	Kanto 51	DRR	International Blast differential, known to contain *Pi-k* gene
15	SP 15	Calaro	DRR	International Blast differential, known to contain *Pi-k^s* gene
16	SP 16	C 102 PKT	DRR	Monogenic line of Co 39 background containing *Pi-3* gene
17	SP 17	IR 50	DRR	Susceptible to blast
18	SP 18	Rasi	DRR	Tolerant to blast
19	SP 19	HR 12	DRR	Local susceptible Check
20	SP 20	Tella Hamsa	Jagityal	Susceptible to blast
21	SP 21	Swarna	Jagityal	Susceptible to blast
22	SP 22	RP 2421	Karjat	Susceptible to blast
23	SP 23	TN1	Karjat	Susceptible to blast
24	SP 24	TN1	Mandya	Susceptible to blast
25	SP 25	Swarna	Mareturu	Susceptible to blast
26	SP 26	MTU 1010	Mareturu	Susceptible to blast
27	SP 27	IET 10750	Nawagam	Neck blast
28	SP 28	Rasi	Nawagam	Susceptible to blast
29	SP 29	NLR 145	Nellore	Tolerant to blast
30	SP 30	BPT 5204	Nellore	Susceptible to blast
31	SP 31	HP 2216	Palampur	Susceptible to blast
32	SP 32	F 23	Palampur	Susceptible to blast
33	SP 33	S 1113	Ranchi	Susceptible to blast
34	SP 34	Improved BPT 5204	Ranchi	Susceptible to blast

Although, we collected the isolates from two different hosts from each endemic area, we observed not much variation among the isolates collected from all the endemic areas of the present study, which indicated the particular race may be more prevalent and virulent in that area.

Table 2. Sequences of the primers used in the present study

S.No.	Primer	Sequence		Annealing temperature (T_a)	No. of observed alleles (N_a)	Allele size range (bp)	Observed heterozygosity (*Ho*)	Expected heterozygosity (H_E)	Polymorphism Information Content (PIC)
		Forward	Reverse						
1	MGM - 1	tttcgtacaatcccgatg	gcgacaatgtctttttttt	58	8	200-400	0.04	0.35	0.50
2	MGM – 2	gatggggagatattccat	actcacctatcaacacttca	57	7	200-300	0.27	0.49	0.35
3	MGM -3	gtgacattagaggaaataaggt	aatcccaaaactcaaaacc	56	7	200-500	0.18	0.48	0.40
4	MGM - 4	tctagaactcaaaactcaaa	atcaccattcctgctg	55	7	200-400	0.17	0.43	0.55
5	MGM - 5	tctccctatatttctccc	aaatgatatgttgctgc	57	7	200-400	0.32	0.47	0.35
6	MGM - 6	aggcaggaagacatatgc	acagctcattaccatgcc	56	6	100-600	0.18	0.92	0.50
7	MGM - 9	gactcaaggtggagatgg	gcctccactatctctcgt	58	8	100-800	0.18	0.97	0.25
8	MGM - 10	acagccgacaggtcaaga	gccagacctcaaggaca	57	7	100-800	0.16	0.96	0.46
9	MGM - 21	gcaggtgagcaaacagcaaga	atatctcgtgcaggccggt	57	7	100-800	0.11	0.97	0.60
10	MGM - 24	gtcttgagtccaccctctttg	ccgtccctgtttcatcc	55	6	100-600	0.43	0.80	0.24
11	Pot2	cggaagccctaaagctgttt	ccctcattcgtcacacgttc	55	17	1400	0.20	0.94	0.26

Genetic frequency for heterozygsity in the population was analyzed using Hardy-Weinberg equilibrium it was revealed that few heterozygote's was observed indicating inbreeding pattern in the population this may be due to the most of the isolates collected from same region while MGM - 2, MGM - 5 and MGM – 24 primers showed significant observed heterozygosity (Table 2) indicating that MGM primers are the right choice to analyze the *Magnaporthe grisea* genetic diversity compared to other conventional primers, Similarly using Hardy–Weinberg equilibrium Li *et al.,* (29) was observed no deviations between any pair of loci, suggesting that null alleles at all the loci are rare in *M. grisea*. However, we observed great extent of variation among the isolates collected from different endemic areas. For instance, the isolates collected from Mareturu (SP 25 and SP 26), Assam (SP 3 and SP 4), Almora (SP 1 and SP 2) and Nellore grouped in the same cluster but they share only 35 % similarity. Another 9 isolates two each from Ranchi (SP 33 and SP 34), Jagityal (SP 20 and SP 21), Karjat (SP 22 and SP 23) and 3 isolates from DRR (SP 12, SP 10 and SP 19) also grouped in the first major cluster they were out grouped with two sub clusters by showing 75 % dissimilarity to the isolates in the first major cluster. We also observed some exceptions like isolates collected from coastal Andhra Pradesh (Mareturu and Nellore) shares the high similarity of 64 % with Assam isolates (SP 3 and SP 4). High similarities between isolates collected from various endemic areas of India like Uttaranchal, Himachal Pradesh those of Madhya Pradesh and Karnataka was reported earlier by Chadha *et al.,* (30) with RAPD markers contributing to the possibility of seed-borne transmission of the pathogen. Therefore it is expected that, the migration of the pathogen to a newer location operates. Due to extensive gene flow among the isolates genetic variability may arise that was evident from the presence of high variation among the isolates collected from different endemic areas. Kumar *et al.,* (23) also concluded the migration of the pathogen that results in to the wide distribution of lineages in the Indo-Gangetic Plains of India. Nguyen *et al.,* (31) also observed the significant gene flow between *P. oryzae* isolates of *indica* populations collected from North Vietnam.

The clustering analysis revealed the grouping of the isolates collected over differentials with the isolates collected from the susceptible cultivars of blast endemic areas. Based on these clustering and similarity values, a prediction of *AVR*/*avr* genes was done. The isolate (SP 14) which was virulent on differential variety *i.e.,* Kanto51 (which has *Pi-k* gene) shares 43 % similarity with the isolates (SP 29 and SP 30) collected from Nellore. Hence, we presumed that in Nellore region the fungal population may have *avr Pi-k* gene, hence deployment of varieties having *Pi-k* gene may not offer resistance to this prevalent race in that region. Interestingly, many present day ruling varieties (NLR 145) in this region have developed using Tetep (which has *Pi-K*^{h}) as a resistance source (32, 33). It is also predicted that there will be many alleles exist in *avr Pi-k* cluster in *Magnaporthe grisea* as like *Pi-k* cluster in rice It has also been reported that the *Pi-k* locus is actually a cluster of genes including *Pik-p*, *Pik-m*, *Pik-s* and *Pik-h* present on rice chromosome 11 (34). Similarly, the isolate (SP 24) collected from endemic area of South India (Mandya- Karnataka) showed 70 % similarity with the isolate (SP 17) which is collected on IR50 variety as well as 40 % with the isolate (SP 9) which is collected on BL-245 variety which is known to contain a combination of *Pi-2* and *Pi-4* genes. Hence the fungal population of Mandya region might be having both or either of *avr Pi-2* and *avr Pi-4* genes. Assam fungal population might be having *avr Pi-k*$_{S}$ gene since these isolates collected from these regions showed 64 % of similarity to the isolate collected from a differential variety (Calaro) which contains *Pi-k*$_{S}$ gene. On the other hand, tightly linked blast resistance genes at the *Pik* locus were presumed to have evolved from

the same ancestral gene. Future work regarding the function and evolution of the avirulence genes that correspond to the resistance genes at the *Pik* locus will help reveal part of the history of rice cultivar–blast fungus arms race. Although the number of isolates analyzed in this research is too small and their geographic origin is too limited, our results demonstrate the potential of the Vegetative Compatibility Group (VCG) analysis to differentiate even closed related isolates of *P. grisea*. Population structure analyses of *M. grisea* using molecular markers have contributed to the knowledge on evolutionary dynamics. Studies on genetic structure of *M. grisea* population using MGM markers SSR based fingerprinting showed that even though each isolate had a unique fingerprint, they could be grouped into distinct lineages Thus; *P. grisea* may represent a reticulate species in which there may be periodic vertical and horizontal gene transfer over time (35). Addressing such hypotheses about the true nature of genetic variability and population structure with *P. grisea* in the future will likely benefit from the use of marked field strains (36) combined with following the dynamics of specific pieces of DNA such as avirulence genes (37, 38) within and between field populations. A better understanding of these factors and the mechanisms that control population dynamics may reveal how we can most effectively manage this important plant pathogen.

Conclusion

The present study with genome specific microsatellite markers helped to understand the population diversity precisely. The concomitant grouping of the isolates collected on the differential varieties with the predominant isolates collected from the diverse agro climatic regions of India, helped in precise prediction of virulence genes spectra in the hotspot regions of India. The recent progress in genomics and sequence availability of rice and *M. grisea* can be extended further to determine the spectra of AVR genes of major blast isolates which can in turn offer a major clue in deployment of blast resistance genes in rice improvement programmes. It is anticipated that this study will lead to a better understanding of the diversity and distribution of blast pathogens both from rice and non-rice hosts and to its potential application in rice breeding programs aiming at development of durable blast-resistant rice cultivars.

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High Biomass Sorghum as a Potential Raw Material for Biohydrogen Production: A Preliminary Evaluation

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Abstract

Six high biomass sorghum lines (IS 22868, IS 27206, IS 15957, IS 16529, ICSV 93046 and CSH 22SS were evaluated for their potential as a substrate material for biohydrogen production by anaerobic fermentation using methanogens deactivated cow dung based mixed microbial consortia. The data revealed that all selected high biomass sorghum lines differed significantly for candidate biomass traits as well as for biomass composition. The high biomass lines, IS 27206 and IS 22868 recorded higher stalk and stover yield compared to others. Least biomass yield (stalk and stover) was noticed with ICSV 93046. The lignin content is low in IS 27206 and IS 15957. Highest biohydrogen yield was observed in IS 27206 followed by IS 22868 and ICSV 93046. The lignin content is negatively correlated with biohydrogen production.

Key words: Anaerobic fermentation, Biohydrogen production, High biomass sorghum, Lignin.

Introduction

Industrial development and urbanization is always associated with increased utilization of energy. This is in addition to dwindling petroleum reserves and increasing green house gas (GHG) emissions which are the major concerns of future energy needs which stress upon development of alternative and environment-friendly energy resources/technologies. International Energy Out look 2011, projects 85% growth in energy consumption by non OECD nations while 18% accounts for OECD economies by 2035, taking 2008 levels as base (1). In most of the developed and developing countries energy consumption is expected to rise by 30% within next two decades (2). The biomass research and development technical advisory committee estimated that one billion dry tonnes of biomass would be required only to replace the petroleum consumption. The United States Department of Agriculture (USDA) states that the United States could potentially produce more than 1.3 billion dry tons annually, exceeding the amount needed to replace 30% of the country's oil consumption (2).

Among different biofuel compounds, hydrogen gas is gaining edge over other such as bioethanol or biobutanol or methane mainly due to it's a high energy (122 KJ/g) content and as clean fuel (3). Hydrogen can be produced by steam reforming of hydrocarbons and coal gasification. However, hydrogen production by anaerobic fermentation from renewable resources is a more promising technology over several other alternatives. In accordance with sustainable development and waste minimization

issues, biological hydrogen production, known as "green technology", has received considerable attention in recent years.

Use of renewable and widely distributed cellulosic feed stock is considered as the effective for energy production as agricultural land alone could produce one billion tons of dry biomass annually without disrupting food and feed demands (4). Though several agro industrial materials and energy crops have been the subject of research, efforts have been on development of short duration, widely distributed and uncompetitive lignocellulosic biomass feed stock with altered structural composition. Of late, sorghum bicolor (L.) moench has emerged as the best option over switch-grass (*Panicum virgatum* L.), *Miscanthus* and other feed stock materials because of its wide adaptation in semi-arid and arid environments and its utility for food, feed, fodder and fuel (5). However, so far, sorghum has gained little importance as an energy crop compared to corn and switchgrass. Yet this has a greater yield potential as biofuel feedstock and it is estimated that corn stover, switch grass and sorghum can produce 3.14, 15.6 and 29.12 harvestable dry tons ha^{-1}, respectively (6) and subjectable for further increase to produce 33.6 to 44.8 harvestable dry tons ha^{-1} through genetic improvement.

The International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Hyderabad, India has recently identified several high biomass sorghum varieties under the ongoing biomass sorghum development program funded by Ministry of New and Renewable Energy (MNRE), Government of India. The objective of the present investigation is to understand the chemical nature of improved high biomass sorghum materials and their potential for their use as a raw material for fermentative biohydrogen production using anaerobic mixed consortia.

Materials and Methods

High biomass sorghum lines: The ICRISAT gene bank has the largest number of sorghum germplasm accessions of over 39600. A total of 412 lines (368 germplasm accessions and 44 high biomass lines of breeding program) were screened for biomass traits in 2010-11 at ICRISAT and 38 promising lines were identified for high biomass. Among them, the top six high biomass lines (IS 22868, IS 27206, CSH 22SS, IS 15957, ICSV 93046 and IS 16529) were evaluated for agronomic traits following randomized complete block design with 4 rows each of 4 m length with 75 cm × 15 cm crop geometry during rainy season of 2011. Recommended crop management practices were followed. Data from internal two rows were considered for plot yield calculation. The data on plant height (m), inter-node diameter (mm), number of internodes and stalk yield were recorded at physiological maturity while stover yield were recorded at 15% moisture level. Plot yield data were converted to kg ha^{-1} using the plot size as factor. The variety ICSV 93046 was proven commercialized sweet sorghum variety developed at ICRISAT while CSH 22SS was the first sweet sorghum hybrid from Directorate of Sorghum Research released in India for commercial cultivation and used as a national check (7) while other lies were germplasm accessions identified at ICRISAT for high biomass.

Statistical Analysis: General linear model (GLM) was used for analysis of variance (ANOVA) and to calculate significant differences among improved varieties using SAS software (SAS Institute Inc., 1991). GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA) software version 2.0 (8) was used for simple linear regression analysis between traits. The statistical significance of the differences between the means was estimated by the least significant difference and all significant results were reported at the $P \leq 0.05$ levels.

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Inoculum development for biohydrogen production: Inoculum for H_2-production was developed according to Prakasham *et al.,* (9). Briefly, hydrogen-producing mixed consortia that originated from buffalo dung compost was collected in Hyderabad city, Andhra Pradesh, India and the hydrogenotrophic methanogens were deactivated by heat treatment for 30 min at 100° C. The developed inoculum was stored under anaerobic environment for further use.

Anaerobic fermentation: Fermentation experiments were performed according to Prakasham *et al*., (9) in 250 ml serum vials as batch reactors consisting of methanogens deactivated inoculum (15%) – 15 ml, 5 g *bmr* sorghum stover material, 15 ml of nutrient stock solution (prepared using the following composition (in g/L) NH_4Cl – 0.5, KH_2PO4 – 0.25, K_2HPO_4 – 0.25, $MgCl_2.6H_2O$ – 0.3, $FeCl_3$ – 0.025, $NiSO_4$ – 0.016, $CoCl_2$ – 0.025, $ZnCl_2$ – 0.0115, $CuCl_2$ – 0.0105, $CaCl_2$ – 0.005 and $MnCl_2$ – 0.015). The final working volume of 150 ml was made up with distilled water. These flasks were deoxygenated with nitrogen gas for the development of an anaerobic environment. These flasks were incubated at 37 ± 1 °C in an orbital shaker with a rotation speed of 100 rpm to provide better mixing of the substrates. The volume of biogas produced was determined using glass syringes of 5–50 ml. All the experiments were performed in triplicates and the average values were reported.

Chemical Analysis: The analysis of sorghum samples for cellulose, hemicellulose and lignin content was done by Tappi (10) and permanganate oxidation method (11) in duplicates. The hydrogen gas measured as a percentage of the total volume was determined using a 100% hydrogen standard with gas chromatograph (GC, Agilent 4890D) equipped with a thermal conductivity detector (TCD) and 6 feet stainless column packed with Porapak Q (80/100 mesh). The operational temperatures of the injection port, the oven and the detector were 100 ^{0}C, 80 ^{0}C and 150 ^{0}C, respectively. Nitrogen gas at a flow rate of 20 ml/min was used as the carrier.

Results and Discussion

In order to have suitable sorghum varieties with improved traits for biomass production, six different high biomass varieties of sorghum (IS 22868, IS 27206, CSH 22SS, IS 155957, ICSV 930461 and IS 16529) were either developed or identified by ICRISAT, Patancheru, Hyderabad. These lines were evaluated for their agronomic traits as well as screened for production of biohydrogen by anaerobic fermentative mixed bacterial consortium.

The six high biomass sorghum lines were characterized in terms of candiate agronomic traits. The ANOVA revealed significant differences for the agronomic traits studied *i.e.* plant height (m), internode diameter (mm), number of internodes, fresh stalk yield (t ha^{-1}) and stover yield (t ha^{-1}) among the entries in the

Fig. 1. Comparative view of high biomass sorghum line (IS 27206) and grain sorghum line (PVK 801)

study. The entry IS 27206 (6.2 m) was the tallest (Fig 1) followed by IS 16529 and IS 15957 (Table - 1). The same entry, IS 27206 recorded highest average internode diameter (22.38 mm), fresh stalk yield (90.5 t ha^{-1}) and stover yield (54.5 t ha^{-1}). The next best lines for biomass yield were IS 16529 (52.1 t ha^{-1}) and IS 15957 (44.3 t ha^{-1}). The association analysis revealed that plant height (0.93), internode diameter (0.98) and fresh stalk yield (0.98) were positively correlated with stover yield or dried biomass. Therefore breeding efforts need to be intensified to improve plant height and internode diameter to genetically enhance biomass yield.

To characterize further, all high biomass sorghum lines have been analyzed for their structural composition. It is evident from the Table - 2 that all developed high biomass sorghum lines have shown cellulose content in the range of 27-52% while the hemicellulose percentage ranging from 17-23% followed by lignin composition in the range of 6.2-8.1%. This observed variations in major components of biomass i.e., cellulose, hemicelluloses and lignin do suggest that all selected high biomass sorghum lines are genetically different and show significant genetic variability in agronomic traits (Table - 1) and structural component composition (Table - 2). Lignin is considered as one of important structural chemical component which is responsible for providing the strength to plant in addition to protection from the microbial attack (9, 12-14). Less lignin content was observed in the lines, IS 27206 and IS 15957. Critical analysis

Table 1. Mean performance of high biomass sorghum lines for agronomic characteristics

Genotype	Plant height (m)	Inte-rnode diameter (mm)	Number of Internodes	Fresh stalk yield (t ha^{-1})	Stover yield (t ha^{-1})
ICSV 93046	3.9	16.75	14.5	53.6	29.9
IS 15957	5.9	21.01	14.6	71.6	44.3
IS 16529	6.1	21.94	15.1	82.7	52.1
IS 22868	5.8	20.35	13.2	74.4	43.8
IS 27206	6.2	22.38	13.8	90.5	54.5
CSH 22 SS	4.2	18.86	14.4	60.7	35.7
LSD (P<0.05)	0.8	1.90	0.8	9.3	6.4

Table 2. Chemical composition of developed high biomass sorghum varieties

High biomass sorghum line	Chemical composition (%)		
High biomass sorghum line	Cellulose	Hemicellulose	Lignin
IS 22868	45±0.353	20±0.367	6.6±0.226
IS 27206	52±0.077	17±0.042	6.2±0.226
CSH 22SS	27±0.410	21±0.268	6.6±0.403
IS 15957	33±0.494	18±0.487	6.2±0.197
ICSV 93046	49±0.113	19±0.042	7.1±0.091
IS 16529	37±0.282	23±0.254	8.1±0.247

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further indicated that holocellulose content differed from 48 to 69% depending on the line further suggesting the high biomass sorghum lines differ in their genetic constituents and biomass production during their life cycle.

Biochemical conversion of lignocellulosic biomass to biofuel require a process that involves in removal of lignin component, popularly termed as pretreatment of biomass, which is subsequently followed by a breakdown of the complex structural carbohydrates chains to fermentable sugars either by enzymatic or by chemical methods (15). The resulting sugars can then be converted to desired product by fermentation using specialized microbial flora and conditions. One of the major challenges in this biomass to biofuel conversion technology is associated with complexity of converting biomass though the efficiency of this conversion process is dependent on a host of factors which can convert the sugar chains more or less accessible to microbial strains for further metabolism associated synthesis of biofuels. Understanding these factors and manipulating them to enhance the accessibility of convertible sugars is an active area of research (16, 17). In fact, it is generally understood that higher content and easier accessibility of these complex chains of sugars are important, since these establish the optimized biofuel yield. In this context, sorghum compares well with corn stover and other herbaceous and energy biomass crops. Another major challenge in biohydrogen production by dark anaerobic fermentation is to improve the rate and the yield of hydrogen production for an economic process. Hence, biological and engineering studies must be concentrated on these issues. Since, raw material cost is another concern in biohydrogen fermentations and the above high biomass sorghum lines showed high fresh stock and stover yield, these lines were evaluated for biohydrogen production by anaerobic fermentation process using enriched hydrogen producing microbial consortia.

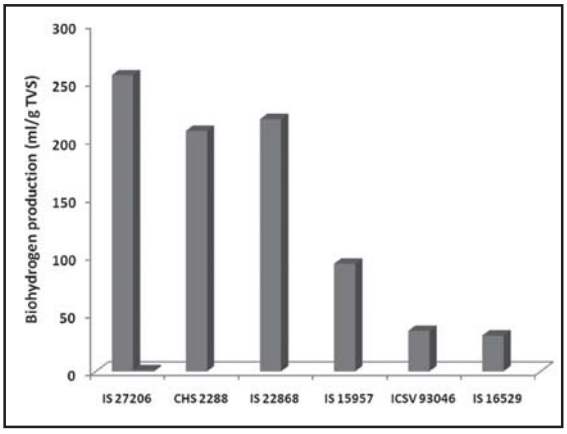

Fig. 2. Anaerobic biohydrogen production using high biomass sorghum lines as substrate material

Biohydrogen production differed with each of the high biomass sorghum lines (Fig 2). Highest biohydrogen yield was noticed with IS 27206 sorghum line followed by IS 16529 and IS 22868. The least production was observed with IS 16529 line. Such biohydrogen variation may be attributed to difference in chemical composition of the lines especially with respect to cellulose and lignin content as noticed in table - 1 and table - 2. This is because, cellulose is the major component of biomass material which upon enzymatic hydrolysis yields fermentable sugars then could be used as raw material for biohydrogen production by enriched biohydrogen producing microbial consortia developed from cow-dung as reported earlier by (16). Comparative evaluation of biohydrogen yield with respect lignin content do suggested that there is little variation in lignin content with the lines of IS 27206 and IS 15957 (Table - 2) however, a large variation in biohydrogen production values were noticed. A maximum of 240 ml of hydrogen/g TVS was observed with high biomass sorghum line IS 27206 whereas IS 15967 supported only > 100 ml of hydrogen/g TVS (Fig 2). In addition, the lines ICSV 93046 and IS 16529 consist of 7.1 and 8.1% of lignin, respective also showed

lower production of biohydrogen (>75 ml of hydrogen/ g TVS) (Fig 2). This data suggest that only cellulose and hemicelluloses are mainly responsible for biohydrogen production and whose digestion play significant role in overall product yield by anaerobic mixed biohydrogen consortia. Similar nature of linkage has been observed by Prakasham *et al.,* (9) and reported negative association of lignin content with biohydrogen production by anaerobic fermentation with mixed anaerobic consortia as inoculum when brown-mid rib sorghum was used as substrate material. The above results inidicate exploitable variation for hydrogen production in high biomass sorghum lines in terms of composition of biomass and fermentation conditions particularly pH.

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Optimization of Xylanase Secretion from *Paenibacillus macquariensis*

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Abstract

A sporulating bacteria having potential to produce xylanase has been isolated from the soil mixed with petroleum products. The isolated bacteria retained violet color upon gram straining revealing its Gram positive nature. This strain was identified as *Paenibacillus macquariensis* based on biochemical characterization and deposited in MTCC with an accession number RC1819. The bacteria showed growth at wide variability of pH ranging from pH 5.2 to 10.0 and the temperature ranging from 25 to 37°C. The optimum pH for xylanase production has been found to be in the range of pH 8.2 to 8.6 and the optimum temperature for xylanase production is found to be 37°C. It secreted xylanase in the culture medium (extra-cellular xylanase) in the presence of 2% xylan. It secreted nearly 4.50 units of xylanase per ml of culture medium. The data revealed that *Paenibacillus macquariensis* has high potential for xylanase secretion and can be used for industrial applications.

Introduction

Plant cell wall is mainly composed of cellulose and hemicelluloses and provides a protective barrier against the enzymatic attack of cellulases and xylanases (1). Xylan is a heterogeneous polymer composed of 1,4 linked β-D xylosyl residues. It is much abundant in nature being the major component of hemicelluloses of monocotyledonous cell walls. The complete degradation of system requires a multi-step process involving xylanases and various xylan debranching enzymes viz. β-xylosidase, acetylxylan-esterase, α-glucuronidase and α-arabino-furanosidase (2,3).

Xylanases in conjunction with other enzymes viz. cellulases, glucanases and proteases, are widely used in animal feed, brewing, food processing and waste treatment as well as in paper and pulp industries (4-6). Combined application of xylanase, β-1,3 and 1,4-glucanases can reduce the intestinal viscosity of feed for higher nutrition availability and improve the filtration rate and extraction yield in the brewing industry (7,8).

Xylanase (EC 3.2.1.8) acts on β-1,4 xylan and splits β-1,4 glycosidic linkage randomly (4,9). The products are xylose, xylobiose and xylo-oligosaccharides and these products are used as feedstock for food and fine chemicals (10). It is an industrially important enzyme having applications in paper industry for brightening the pulp, clarification of fruit juices (9,11-12). Use of xylanase in paper industry is eco-friendly since chemicals used in paper industry cause pollution (13-15). Here, we reported isolation of a bacteria which has been identified as *Paenibacillus macquariensis* and has been given accession number RC-1219 by the MTCC, Chandigarh. We also optimized the conditions for xylanase production by this bacteria.

Materials and Methods

***Collection of samples*:** Soil samples were collected from different places of Indore (Madhya

Pradesh) including one sample from petrol pump where soil was mixed with petroleum products.

***Screening of bacterial isolates*:** All soil samples were made semi-dried in the laboratory using autoclaved water. The 10^2 to 10^7 times dilutions were made from the suspension of different soil samples. Serially diluted soil samples were plated on Luria Broth (LB) nutrient agar plates and incubated at 37°C for 24 hours. The LB medium was comprised of bacto-tryptone, 10gm; yeast extract, 5gm; sodium chloride, 10gm; agar, 15gm per litre and adjusted to pH 10 using 0.1N sodium hydroxide. Few distinct colonies with distinct morphology were isolated and transferred to Petri-plates having Emersion medium (yeast extract, 5 gm; peptone, 5 gm; K_2HPO_4, 1 gm; $MgSO_4$. $7H_2O$, 0.2 gm per litre and pH adjusted to 9 with 1N NaOH) and incubated at 37°C for 24 hours. The Emersion medium was supplemented with 1% xylan in order to isolate xylan utilizing bacteria.

***Screening for xylanase activity*:** The colonies obtained were screened for xylanase activity using congo red dye method (16). A set of replica plates was prepared and incubated at 37°C for 24 hours. Thereafter, one of the replica plate was flooded with 0.5% congo red dye and subsequently with 1 mM solution of sodium chloride. A clear zone around the colony was considered as indication of xylanase activity. The corresponding colonies from other replica plate were inoculated individually in Emersion medium broth, allowed to grow overnight at 37°C at 200 rpm in an orbital shaker. The xylanase activity was analyzed in the medium.

***Morphological, biochemical and physiological studies of the bacteria*:** Gram staining was done using Kit from Hi-media. After staining, slides were observed in a phase contrast microscope. For biochemical and physiological studies and identification of the bacteria, the culture was sent to Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh.

Production of xylanase: The isolated bacteria was maintained on Emerson medium slants by routine sub-culturing every month and used for the production of xylanase. The growth from 24 hours old Emerson slant was scrapped into sterile Emerson medium contained in a 250 ml capacity Erlenmeyer flask and allowed to grow at 37°C on an orbital shaker with a speed of 200 rpm. A 2 ml aliquot of this suspension was used as an inoculum for 100 ml medium. The flasks were incubated at 37°C for 48 hours with a speed of 200 rpm. Thereafter, the bacterial cells were harvested by centrifugation of the broth at 10,000 x *g* for 30 minutes at 0 to 4°C in a cooling centrifuge. The supernatant contained most of the xylanase activity.

***Growth time optimization for xylanase production*:** Samples from growing broth were drawn at 4 hours intervals, centrifuged at 10,000 x *g* for 15 minutes in the cold condition (0 to 4°C). The resulting supernatants were analyzed for xylanase activity.

***Enzyme assay*:** Xylanase enzyme was assayed by measuring the release of the reducing sugars from birch wood xylan following the dinitrosalicylic acid (DNS) method (17). The D- xylose was used as standard during the colorimetric estimation. One unit of xylanase activity was taken as the amount of the enzyme required to release one micromole of the reducing sugar equivalent to one micromole of xylose per minute at 50°C under the conditions of the enzyme assay.

Optimization of culture conditions for xylanase production: For optimization of culture conditions, pHs ranging from pH 8.0 to 11.0 with a difference of 0.1 unit were maintained in the culture medium. The xylanase activity and bacterial growth was measured. The bacterial growth was measured using absorbance at 600 nm. The time of growth was also optimized in the range of 12 to 96 hours by withdrawing the aliquots aseptically at 12 hours intervals and analyzed for the xylanase activity. For

temperature optimization, growth as well as enzyme production was estimated at various temperatures viz. 30°C, 35°C, 37°C, 40°C, 42°C, 45°C and 50°C. For checking induction effect of xylan on xylanase production, two 500 ml capacity Erlenmeyer flasks were deployed marked as control and experimental. In each flask, 200 ml of Emerson medium was put and autoclaved. In experimental flask, 1% xylan was also added. Each flask was inoculated using 2% (4 ml) inoculum. Both the flasks were incubated in an orbital shaker at 37°C with a speed of 200 rpm. From each flask, 20 ml suspension was withdrawn aseptically at intervals of 12 hours. Each withdrawn aliquot was centrifuged at 10,000 x *g* for 15 minutes at 0 to 4°C in a cooling centrifuge and the supernatant was analyzed for xylanase activity. The effect of different concentrations of xylan on enzyme production was also observed using different concentrations of xylan viz. 0.5%, 1%, 2%, 3%, 4% and 5%. One control was also run with 0 % xylan. All the flasks were incubated in an orbital shaker at 37°C with a speed of 200 rpm. From each flask, 20 ml suspension was withdrawn aseptically at intervals of 12 hours and up to 84 hours. Each withdrawn aliquot was centrifuged at 10,000 x *g* for 15 minutes at 0 to 4°C in a cooling centrifuge and the supernatant was analyzed for xylanase activity.

Results and Discussion

Screening of the bacteria: A xylanase producing bacteria was isolated from the soil mixed with petroleum products. The xylanase production by the bacteria was screened by congo red dye staining method (16). Xylanase activity was taken as positive in the bacteria by the presence of clear zone surrounding the colony (Fig. 1).These zones were enhanced on treatment of the plate with 1 mM sodium chloride.

Morphological, biochemical and physiological properties of the bacteria: The Gram staining showed it to be Gram positive bacteria. Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh identified it as *Paenibacillus macquariensis* and gave it Accession number RC1819. The biochemical and physiological properties of the bacteria are shown in Table 1. The *Paenibacillus macquariensis* was grown at various temperatures ranging from 10°C to 42°C (Table 1). It showed optimum growth at 37°C. The growth was much poor at 42°C.

Effect of pH on the growth of the bacteria was observed in the pH range of 5.2 to 10.0. It was found to have good growth at all the pHs tested in the range of pH 5.2 to pH 10.0 (Table 1). These results showed that this bacteria has much tolerance of pH change and has growth in acidic to highly alkaline range.

The effect of sodium chloride on the growth of the bacteria was also checked in the range of 2 to 10% sodium chloride and was observed to exhibit same amount of growth in the presence of various concentrations of sodium chloride (Table 1). These results showed that the bacteria also has tolerance against salinity.

The bacteria is capable of hydrolyzing starch and casein. It also liquefied gelatin. However, the bacteria could not utilize citrate

Fig. 1. Screening of xylanase activity by using 0.5% Congo red dye method.

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Table 1. Biochemical and physiological characteristics of the bacteria

Tests	RC1819
Gram Staining	+
Spore staining	+
Motility	+
Growth at 10°C	-
Growth at 15°C	-
Growth at 25°C	+
Growth at 37°C	+
Growth at 42°C	-
Growth at pH 5.2	+
Growth at pH 8.0	+
Growth at pH 9.0	+
Growth at pH 10.0	+
Growth at NaCl 2%	+
Growth at NaCl 5%	+
Growth at NaCl 7%	+
Growth at NaCl 10%	+
Starch hydrolysis	+
Casein hydrolysis	+
Citrate utilization	-
Gelatin liquefaction	+
H_2S Production	-
MR	-
VP	+
Nitrate reduction	+
Indole	-
Catalase	+
Oxidase	+
Urea	-
Acid production from	
Arabinose	+
Galactose	-
Mannitol	+
Raffinose	-
Salicin	+
Xylose	-
Sucrose	-
Rhamnose	-
Meso-inositol	+
Fructose	+

added in the growth medium. The bacteria could not produce hydrogen sulfide gas and also could not reduce nitrate. The bacteria showed catalase and oxidase activities. The bacteria showed negative tests for indole and urea hydrolysis (Table 1).

The bacteria produced acid from glucose, arabinose, mannitol, salicin, fructose and *meso*-inositol. However, it could not produce acid from galactose, raffinose, xylose, sucrose and rhamnose indicating that these are not metabolized the same way (Table 1).

Earlier, authors isolated a bacteria from dung, *Bacillus halodurans*, MTCC 9512 which showed optimum growth at 55°C and at pH 10 (9).

***Production of xylanase*:** Based on the growth of the bacteria and production of xylanase, Emerson medium was selected for the growth of the bacteria.

***Time optimization for xylanase production*:** The bacterial growth and xylanase production were observed up to 96 hours. It was found that the bacteria grew logarithmically up to 84 hours as observed by measuring absorbance at 600 nm. Xylanase production was observed to increase up to 48 hours and thereafter, there was decrease in production as observed by measuring enzyme activity. The culture medium showed nearly 4.5 units/ ml of xylanase activity at 48 hours in the presence of 2 % xylan.

***Effect of xylan on xylanase production*:** The effect of xylan on the production of xylanase by the bacteria was observed by adding 1 % xylan in the growth medium. A control was also prepared simultaneously under identical conditions having no xylan in the growth medium. The xylanase production was observed at 12 hours intervals. There was little xylanase production in the control flask whereas bacteria grown in the growth medium having 1% xylan showed significant production of xylanase. It was

. **Table 2.** Effect of xylan concentration on xylanase activity at different time intervals

Time (hours)	0% xylan (U/ml)	0.5% xylan (U/ml)	1% xylan (U/ml)	2% xylan (U/ml)	3% xylan (U/ml)	4% xylan (U/ml)	5% xylan (U/ml)
12	0.18± 0.01	0.89 ± 0.08	1.34 ±0.13	1.60 ± 0.15	2.05 ± 0.20	3.74 ± 0.35	2.40 ± 0.20
24	0.20 ±0.01	1.16 ± 0.10	2.49 ± 0.20	3.74 ± 0.30	4.45 ± 0.40	4.72 ± 0.40	4.36 ± 0.40
36	0.27± 0.01	1.25 ± 0.12	2.58 ± 0.25	4.18 ± 0.40	5.52 ± 0.50	6.41 ± 0.55	5.25 ± 0.50
48	0.30± 0.01	1.34 ± 0.13	2.76 ± 0.25	4.36 ± 0.40	6.41 ± 0.60	7.21 ± 0.70	6.85 ± 0.60
60	0.10± 0.01	1.42 ± 0.14	2.49 ± 0.24	4.27 ± 0.40	7.03 ± 0.70	8.01 ± 0.75	8.46 ± 0.75
72	0.30± 0.01	1.07 ± 0.10	2.23 ± 0.20	4.18 ±0.40	5.52 ± 0.50	8.54 ± 0.75	8.99 ± 0.80
84	0.10± 0.01	0.89 ± 0.08	0.98 ± 0.08	3.74 ± 0.35	4.81 ± 0.45	7.12 ±0.70	9.08 ± 0.85

observed that there was initially up to 48 hours increase in xylanase production and thereafter decrease in xylanase production was observed (Table 2). When 1% more xylan was added after 48 hours of growth, there was increase in xylanase production up to 96 hours. The data indicated that due to extra-cellular xylanase, xylan present in the medium exhausted and upon further addition of xylan increased xylanase production.

The effect of various different concentrations of xylan viz. 0.5, 1, 2 , 3, 4 and 5 % xylan was also checked on xylan production. The data are given in Table 2. These data showed that there was more production of xylanase with increase in xylan concentration up to 4% in the growth medium. At lower concentration of xylan, increase in xylanase production was observed up to 48 hours only whereas at higher concentration of xylan, increase in xylanase production was observed for a longer time (Table 2). The present results showed that although xylanase isolated from *Paenibacillus macquariensis* is not thermophilic but it can be exploited as good source of xylanase production for industrial applications.

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Simple, Rapid and Sensitive Method for Determination of Nitrofurantoin in Human Plasma by using Liquid Chromatography / Tandem Mass Spectrometry

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Abstract

A simple, rapid and sensitive method using an isocratic Liquid chromatography coupled with Tandem mass spectrometry was developed and validated for the assay of nitrofurantoin in the Human Plasma. The Mass transition of nitrofurantoin and losartan (Internal standard) were M/z 237.000/151.800 and M/Z 421.300/ 179.274 in ESI Negative ionization. Linearity was observed between the nitrofurantoin concentration and the peak area ratio from 10.107 to 999.900ng/mL with r^2 value of 0.99. Plasma samples containing nitrofurantoin were extracted with ethyl acetate. The observed recovery of nitrofurantoin was 80.2 %. The intra-day and inter-day accuracy ranged from 83.61 to 107.16% and from 93.13to 103.02% respectively, at Low, middle and high level concentrations. The method will be used in the determination of the pharmacokinetic parameters of nitrofurantoin after oral administration of nitrofurantoin formulation in human Plasma.

Keywords: Nitrofurantoin, LCMS/MS, Bioanalytical, Validation, Human Plasma, 5-nitrofurans

Introduction

Nitrofurantoin is an antibiotic used for the treatment and prophylaxis for urinary tract infections. Nitrofurantoin is more soluble in urine due to the presence of urea and creatinine. Considerable amount of nitrofurantoin and its metabolites are excreted in urine. This may be the reason; possibly, nitrofurantoin is contraindicated in patients with a creatine clearance of 60mL/min or less, though it is not contraindicated in rest of the population. (6, 21) Therefore, regular monitoring is required in patients receiving nitrofurantoin. Several methods are reported for routine drug monitoring by using different analytical methods such as LC-MS/MS and other conventional methods (1-20). The authors suggest that performing analysis by LC-MS/MS (3) includes derivatisation techniques and is no longer beneficial with the recent advances in the techniques available. There is a need for an established method for analysis which is not tedious and cost consuming, as derivatisation requires more reaction time as well as more amounts of solvents and chemical consumption. More over it is also required to see that the new methods being developed does not require more retention time so that analysis is simple and faster. Therefore, authors proposed a simple, sensitive, accurate and precise method for determination of nitrofurantoin by LC-MS/MS method for measuring nitrofurantoin plasma concentration.

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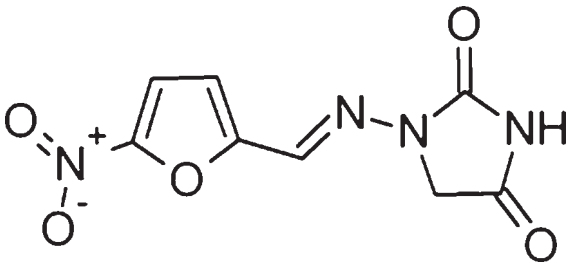
Stricture of Nitrofurantoin



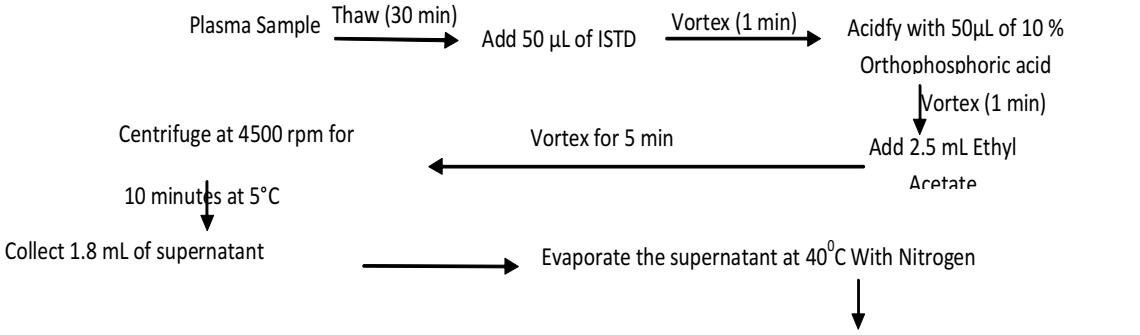
Materials and Methods

Materials: Nitrofurantoin and Losartan analytical standards were obtained from Biocon India Pvt Ltd, HPLC grade Acetonitrile and methanol were obtained J.T. Baker, USA, Ammonium acetate (GR Grade Merck), Dimethyl sulphoxide (AR Grade Merck), Orthophosphoric acid (GR Grade Merck),Glacial acetic acid(GR Grade Merck),Water was de-ionized and triple distilled.

Preparation of Nitrofurantoin standard solutions: Standard solutions of Nitrofurantoin (1.0 mg/ml) was prepared by dissolving an accurately weighed amount of 10.00 mg of Nitrofurantoin in 5 ml of Acetonitrile and add 50 µL of Dimethyl sulphoxide, sonicated for 5 minutes and the volume was adjusted to 10 ml with Acetonitrile. The standard solution was stored subsequently at 2-8 ^{0}C. The appropriate concentrations of standard solution were prepared by diluting the stock solution with 80: 20 Acetonitrile: water.

Preparation of Losartan Internal standard solutions: Standard solution of Losartan (1.0 mg/ml) was prepared by dissolving an accurately weighed amount of 10.00 mg of Losartan in 5 ml of methanol, sonicated for 5 minutes and the volume was adjusted to 10 ml with methanol. The standard solution was stored subsequently at 2-8 ^{0}C. 10.00µg/mL Losartan was prepared by diluting the stock solution with 80: 20 methanol: water.

Sample preparation



Instrumentation and conditions : The Thermo Scientific HPLC (Model: Surveyor) and LC-MS/MS system interfaced to LC Quan Software in a windows platform The Reverse phase C8 analytical column (Kromosil C 8, Length: 4.6×50 mm, Particle size: 5 µ) was used for the separation of Nitrofurantoin and internal standard, the mobile phase consists a mixture of Acetonitrile and 5mM Ammonium Acetate at a ratio of 60:40 respectively. The mobile phase was degassed by passing through a 0.22 µm membrane filter (Millipore, Bedford, MA, USA) prior to use and operated through a single pump (Isocratic) at a flow rate of 0.5 ml/min. The injector was filled with an injector loop of 10 µl. Tandem mass spectrometric detection and quantification

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was performed using multiple reaction monitoring (MRM).

Results and Discussion

Development conditions for rapid extraction of Nitrofurantoin from Human Plasma: Reported extraction techniques for determination of nitrofurantoin from human plasma [3-6, 8-11, 14-19] is more laborious and involves more tedious process like derivatisation. More over, they involve cost efforts and in addition to this, they are time consuming. While developing a method, one needs to look at the chromatographic conditions and extraction process for minimizing the disadvantages of older techniques or methods. In the process, mobile phase selection and optimization, column selection is critical to minimize run time, solvent consumption and injection volumes. It was observed that, a novel method can be developed with advantages that will eliminate derivatisation, more solvent consumption, high run time and rising costs. The new method should offer the benefits of using small columns, reducing plasma and solvent consumption to arrive at an extraction procedure which will have more extraction recoveries. Conditions for simple and rapid HPLC separation with MS/MS Electro-spray Negative ionization mode were developed using an isocratic elution with a mobile phase composed of Acetonitrile and 5mM Ammonium acetate at a ratio of 60:40% v/v. Thus the ions formed for drug and Internal standard in ESI Negative mode due to the addition of Hydroxyl ion (OH^-) in carbonyl function (C=O) present in the drug (Fig.1). These conditions gave a well defined, sharp peak of Nitrofurantoin and Losartan (ISTD) with a retention time of approximately 1.21 and 1.51minutes. Under these conditions an amount of Nitrofurantoin as low as 1ng/mL could be detected. With these retention times, analysis could be completed in about 3.0 minutes.

Method validation

Linearity: The quantification of the chromatogram was performed using the peak area ratio of Nitrofurantoin and Losartan (ISTD). Nine standard solutions were prepared.

(10.248, 25.621, 51.242, 102.484, 256.209, 457.515, 667.906, 861.814 ng/mL and 1013.899 ng/mL) and subjected analyses by HPLC-MS/MS. Three precision and accuracy (P&A) batches were injected. The peak area ratio was determined and plotted versus the concentration of Nitrofurantoin. Statistical analysis using least square regression analysis indicated excellent linearity for Nitrofurantoin with the concentration range studied as shown in Table 1. In constructing the standard curve, samples of Nitrofurantoin in Human Plasma identical to those in the standard solutions were prepared and the Nitrofurantoin response ratios were plotted against the concentrations of Nitrofurantoin in ng/mL as shown in Fig. 2. The linearity of the concentration and response relation was established over the range of 10.248 – 1013.899 ng/mL ($R^2 = 0.9898$). Fig. 3 shows the LC-MS/MS chromatograms of pure drug. (Nitrofurantoin), Fig. 4 shows the LC-MS/MS chromatograms of drug-free Human plasma and Fig. 5 shows the LC-MS/MS chromatograms of standard Plasma sample containing the drug at a concentration of 10.248ng/mL.

Accuracy and precision: The intra-day accuracy and precision of the assay was evaluated by analyzing six replicates of the Plasma containing Nitrofurantoin at three different concentrations. The intra-day precisions of the analyzed samples are determined by Relative Standard Deviation (RSD) range from 2.11% to 11.01%, while the intra-day accuracy ranged from 83.61% to 107.16%. The inter-day precision of the assay was measured by analyzing six replicates of Nitrofurantoin Plasma samples for three consecutive days. The inter-day precision of the analyzed samples as determined by Relative Standard Deviation (RSD) range from 6.48% to 12.37%, while the inter-day accuracy ranged from 93.13to 103.02%.

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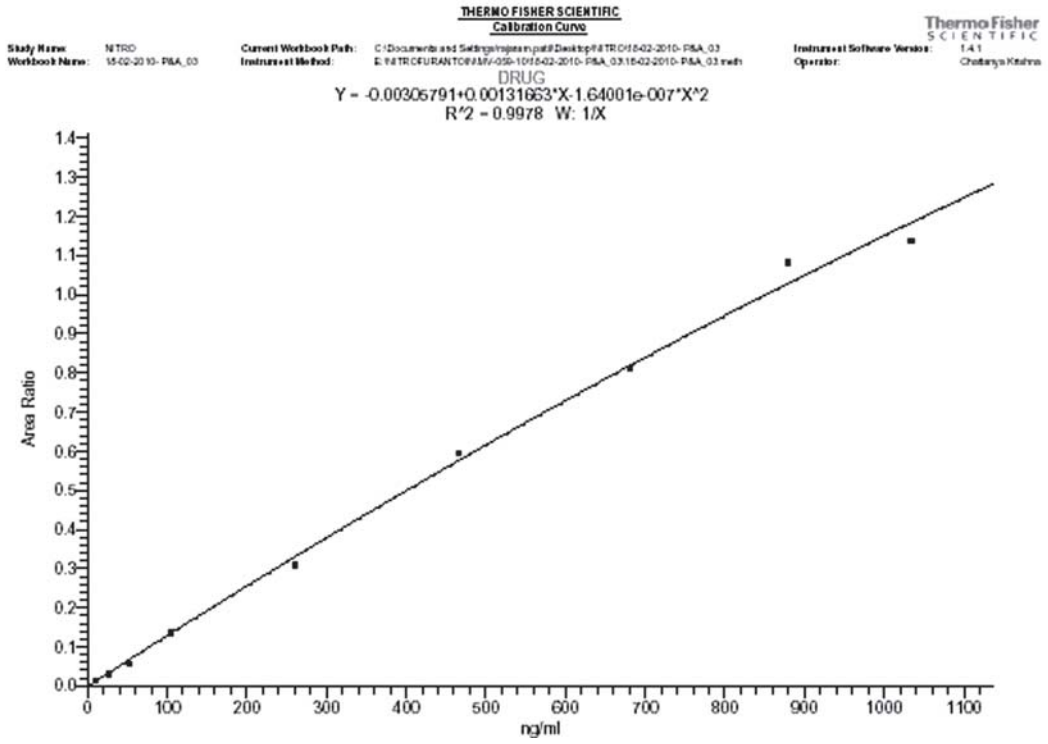

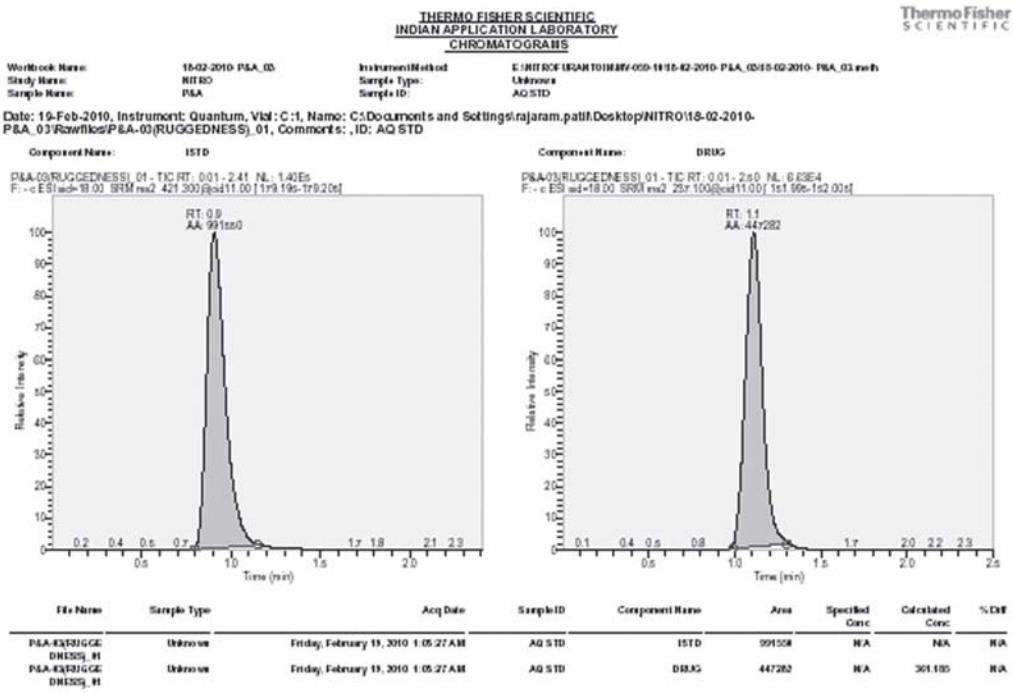

THERMO FISHER SCIENTIFIC
INDIAN APPLICATION LABORATORY
CHROMATOGRAMS

ThermoFisher SCIENTIFIC

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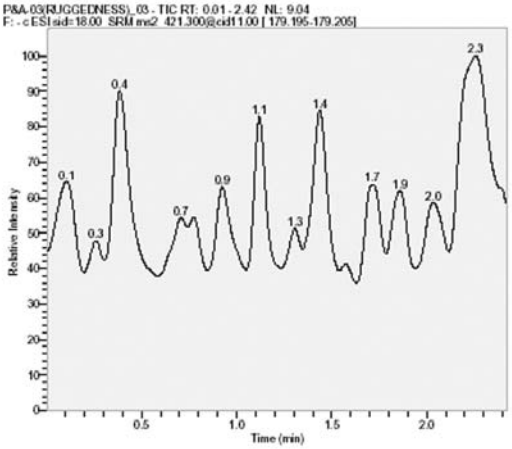
Study Name:	NITRO	Sample Type:	Unknown
Sample Name:	P&A	Sample ID:	AQ STD

Date: 19-Feb-2010, Instrument: Quantum, Vial: C:3, Name: C:\Documents and Settings\rajaram.patil\Desktop\NITRO\18-02-2010-P&A_03\Rawfiles\P&A-03(RUGGEDNESS)_03, Comments: , ID: BLK

Component Name: ISTD

P&A-03(RUGGEDNESS)_03 - TIC RT: 0.01 - 2.42 NL: 9.04
F: - c ESI sid=18.00 SRM ms2 421.300@cid11.00 [179.195-179.205]



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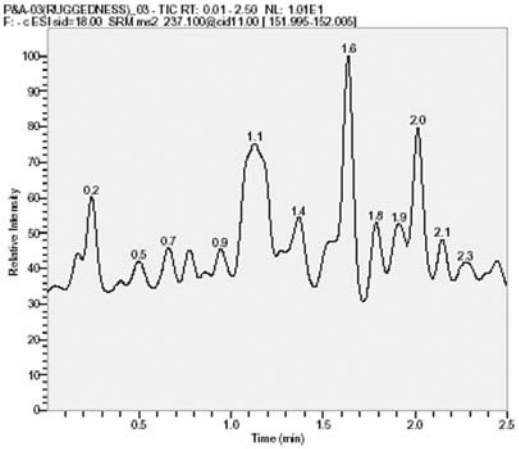

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P&A-03(RUGGEDNESS)_03	Blank	Friday, February 19, 2010 1:12:24 AM	BLK	DRUG	N/A	N/A	NC	N/A

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ThermoFisher SCIENTIFIC

Workbook Name:	18-02-2010- P&A_03	Instrument Method:	E:\NITROFURANTOINMV-059-10\18-02-2010- P&A_03\18-02-2010- P&A_03.meth
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Component Name: ISTD

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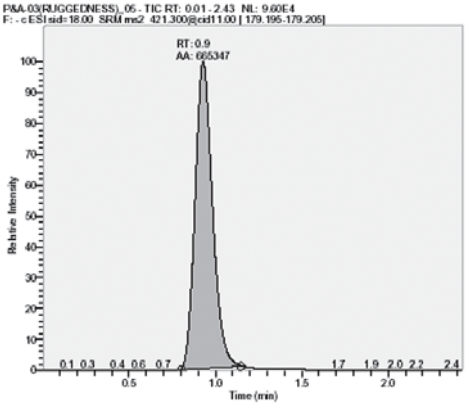

Component Name: DRUG

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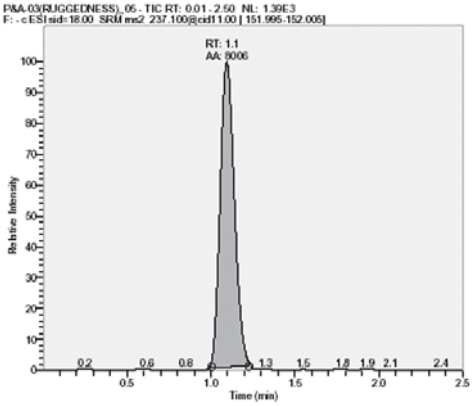

File Name	Sample Type	Acq Date	Sample ID	Component Name	Area	Specified Conc	Calculated Conc	% Diff
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P&A-03(RUGGEDNESS)_05	Standard	Friday, February 19, 2010 1:19:19 AM	A	DRUG	8006	10.451	11.479	9.84

Table 1. Curve parameter summary and back-calculated calibration curve concentration for Nitrofurantoin in human Plasma

STANDARD (CC ID)	A	B	C	D	E	F	G	H	I
ACTUAL (NG/ML)	10.2483	25.6209	51.2417	102.4835	256.2086	457.5154	667.9057	861.8138	1013.8986
CC1(P &A 1)	9.005733337	26.9988858	52.4 4977771	105.372637	270.932378	437 .078142	703.6463876	762.3971 521	1078.044345
CC2(P &A 2)	10.50949	324.6 9850	649.650613	105.233318	269.1729 69	435.934921	682.8 839761	837. 077386	1032. 090417
CC3(P & A 3)	11.1994415	123. 60441	646. 42190002	109 .828 016	265.539938	440. 858154	67 9.4623121	842.5 262955	1027.5 47215
MEAN	10.2382	25.100	649.5074	106. 8113	268.5484	4 37.9571	688.6642	814.0003	10 45.894
SD	1.12173	1.73259	3.01649	2.6134 6	2.74993	2.57661	13.08724	44.93553	27.93553
%CV	10.96	6.9	6.09	2.45	1.02	0.59	1.9	5.5	2.67
%NOMINAL	99 .9	97.97	96.62	104.22	104. 82	95.73	103.11	94.45	103.16

Acceptance Criteria: 1) Precision for all CC std 1-9 should be within 15% and for LOQ should be within 20%.
2) Accuracy for all CC std 1-9 should be within 85-115% and for LOQ should be within 80-120%.

Note:- a - Standard was not considered for calculation.
NA- Not Applicable

Recovery: The absolute recovery was calculated by comparing the peak areas of Nitrofurantoin and Losartan standards to those assessed by extraction of Nitrofurantoin and Losartan at the three different concentrations. Results of absolute recovery of Nitrofurantoin and Losartan were 103.160% and 94.889% respectively as shown in Table 2.

Conclusion

This method was validated in human Plasma for its specificity, sensitivity, linearity, accuracy, precision (repeatability & reproducibility), % recovery, stability of samples (Freeze thaw, Bench top and Auto sampler stability, short-term and long term stability of stock solution and Internal Standard), dilution integrity and for ruggedness. Method is applicable to quantify the Nitrofurantoin in clinical samples for further evaluation of pharmacokinetics.

Acknowledgement

The author would like to thank Dr.S.S.Makone and Dr.Vinay P. Shedbalkar for their continuous encouragement and remarks.

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Table 2. Within and between batch precision and accuracy

Acceptance criteria

1) The within and between batch mean Precision for LQC, MQC and HQC samples should be within 15% and 20% for the LOQ QC
2) The within and between batch mean Accuracy for LQC, MQC and HQC samples should be within 85-115% and 80-120% for the LOQ QC

QC ID	LOQQC	LQC	MQC	HQC
Actual (ng/ml)	10.3129	28.2546	357.6527	861.8138
QC-PA1	8.03635398	27.065301	356.704618	893.1308
	8.41787092	30.770766	342.647412	864.6573
	8.35451263	36.393436	361.322031	866.0097
	8.94058529	28.173236	357.13257	879.201
	9.02181804	29.208245	364.6488	833.9273
	8.96339905	29.028796	357.699024	811.64
QC-PA2	9.7851	31.2179	322.2158	806.1628
	10.5656	33.7762	322.0735	707.4497
	9.8086	29.6063	325.5442	790.0107
	9.1712	29.3972	319.8673	727.5572
	9.4565	29.6731	314.3319	780.1988
	8.3166	23.9637	285.4347	665.0917
QC-PA3	11.0094524	26.863036	345.577665	781.7984
	11.4023056	25.54112	365.126322	783.5845
	11.6242023	24.274589	362.855776	842.7417
	10.8603541	30.151343	338.160634	806.9359
	11.7378058	28.922102	352.064364	827.8195
	9.6742967	29.920648	354.000862	778.6509
Mean	9.7304	29.1082	341.5226	802.5871
SD	1.20344	3.04063	22.13759	59.85233
%CV	12.37	10.45	6.48	7.46
%Nominal	94.35	103.02	95.49	93.13

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First Evidence for Aluminum-maltol driven B to Z-DNA Conformational Transition in Poly d(GC). d(GC): Relevance to Alzheimer's Disease

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Abstract

Higher concentration of Aluminum (Al) has been observed in Alzheimer's, Parkinson's, and Amyotrophic lateral sclerosis and there was an apparent localization of the Al in chromatin in the brain cells. Studies of Al-DNA interaction showed that cross-linking occurs in DNAs of all base ratios, including poly d(AT).d(AT) and poly d(GC).d(GC). In the present investigation, interaction of Al with poly d(GC).d(GC) and poly d(AT).d(AT) was studied using Al-maltol, a hydrolytically stable Al-compound, at neutral pH. We found that Al at micromolar level caused conformational transition from B-DNA to Z-DNA in poly d(GC). d(GC). Chelation by EDTA could reverse the Z-DNA caused by Al-maltol back to B- DNA. We propose that $Al(mal)_2^+$ and $Al(mal)^{2+}$, major cationic species of Al-maltol at neutral pH are involved in the above transition in poly d(GC).d(GC). The biological relevance of DNA transition in Alzheimer's disease is discussed.

Key words : Aluminum maltol, DNA transition, EDTA, Z DNA

Introduction

The interest in the biological role of Aluminum has been stimulated by its debatable involvement in Alzheimer's disease (1-3), dialysis encephalopathy (4) and ALS- Parkinson dementia in Gaum and Kii peninsula (5-6), Aluminum has been found in the nuclear region of brain neuronal cells that contain neurofibriallry tangles, in Alzheimer's disease and ALS-Parkinson dementia (7-8). Aluminum is reported to be associated with cellular toxicity through its interaction with DNA (9). More detailed studies located the aluminum in the chromatin of brain neuronal nuclei both in Alzheimer's victims and in animals subjected to CNS injection of aluminum (9-12). Alzheimer's disease is complex neurodegenerative disorder in which etiological factors are not clearly established although unproven hypothesis have included trauma, aluminum toxicity and infectious agents (13 -15). We reported that the genomic DNA in the hippocampus of the Alzheimer's brain undergoes helical transition from B-DNA to Z-DNA. We hypothesized that aluminum, abnormal proteins like Ab and tau, oxidative stress, and metal homeostasis might play role in the conformational transition (16). In the present investigation Aluminum maltol, an Al compound was tested for its interaction with Poly d(GC).d(GC) and Poly d(AT).d(AT). Karlik and Eichhorn (17) reported that interaction of Al with Poly d(GC). d(GC) using aluminum chloride results in cross linking of DNA by aluminum without any conformational change. Previous studies from our lab reported that Al-maltol could induce Z-DNA in $(CCG)_{12}$ repeats at neutral pH (18). Since the effects of aluminum depend

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upon the species present under a given set of conditions, this prompted us to carry out to study the Al-DNA interaction using Al maltol at neutral pH.

Materials and Methods

Chemicals: Poly d(GC).d(GC) (Sodium salt), poly d(AT).d(AT) (Sodium salt), Aluminum chloride hexahydrate, Maltol, HEPES, EDTA and EtBr were procured from Sigma chemicals. Poly d(GC).d(GC) and poly d(AT). d(AT) was used without further purification. Al-maltol has been synthesized according to the method of Finneagen *et.al.* (19). Purity of Al-maltol has been checked by UV and IR spectroscopy.

Circular dichroism (CD) Studies*:* The Circular dichroism spectra (190-300 nm) were recorded for poly d(GC).d(GC) and poly d(AT).d(AT) in the absence and presence of Al-maltol (50-200μM) in 1μM HEPES at pH 7.0 on a JASCO-J-715 Spectropolarimeter at 20°C by using 1 mm cell. Each spectra is the average of four recordings.

Results

CD spectra: Poly d(GC).d(GC) was titrated with Al-maltol (50-200μm) and CD spectra were recorded for each titration at pH.7.0 in 1mM HEPES buffer. Normal B-DNA conservative spectrum is observed for poly d(GC).d(GC) alone (Fig 1.a). At 50μm level of Al maltol there is disappearance of large positive peak at 190nm, a strong B-DNA peak (Fig 1.b). The group of spectra (Fig 1.c,d&e) which possesses features of left-handed Z –DNA helix were treated with 100, 150 and 200 μM of Al-maltol respectively. The spectra have two negative peaks at 290nm and at 198 nm. There is also shift in the 200nm cross-over of the B-DNA to lower wavelength (<190nm), which is a trademark of Z-DNA (20). The DNA and A-maltol complex displays the helix stabilization characteristics. There is an increase in the magnitude of the 198nm negative peak as the concentration of Al-maltol is increased. Al-maltol treated poly d(GC).d(GC) spectra have isobestic point at 276nm. The spectra also revealed a small change in the magnitude of the 270nm positive peak and the disappearance of negative peak of the B form at 250nm as the concentration of A-maltol is increased. This reveals strong hydrophilic interactions of the Al-maltol. EDTA (100 μM) could reverse the Z-DNA caused by Al-maltol to B-DNA (Fig. 1f).

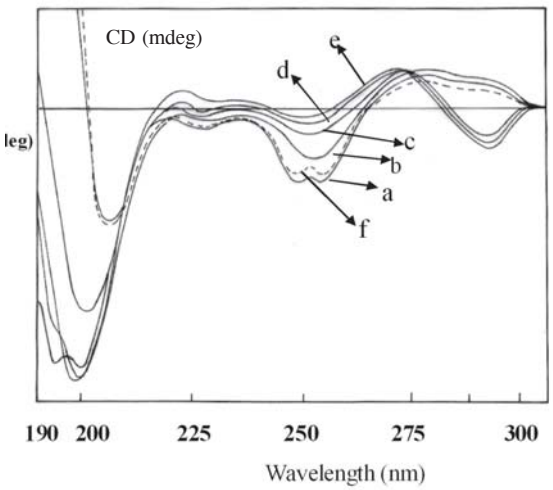

Fig. 1. Effect of Al-maltol on the CD spectra of Polyd d(GC).d(GC) in 1μM HEPES buffer (pH 7.0). a : Poly d (GC).(GC) b, c, d and e represent the molar ratio of Al-maltol/DNA [P] 0.25, 0.33, 0.41 and 0.5. f : Al (Al-maltol/ DNA [P] of 0.5) chelation by EDTA (30μM)

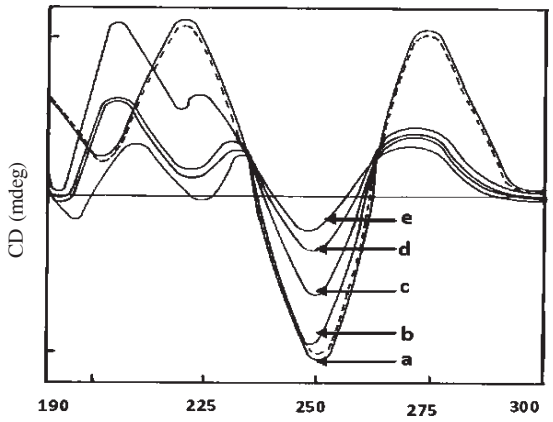

Fig. 2. Effect of Al-maltol on the CD spectra of Polyd d(AT).d(AT) in 1μM HEPES buffer (pH 7.0). a : Poly d (AT).(AT) b, c, d and e represent the molar ratio of Al-maltol/DNA [P] 0.25, 0.33, 0.41 and 0.5. f : Al (Al-maltol/ DNA [P] of 0.5) chelation by EDTA (30μM).

For the comparative study interaction of Al-maltol with Poly (AT).d(AT) was also studied. As the concentration of Al- maltol is increased there is a decrease in the magnitude of both positive and negative peaks at 260nm and 250nm respectively. The 225nm positive peak of the Poly(AT).d(AT) has been shifted to lower wavelength as the concentration of Al-maltol is increased. But Al-maltol did not cause any helical transition (Fig. 2).

Discussion

Poly d(GC). d(GC) undergoes Z-DNA conformation from B-DNA by various factors like high salt concentration (20), methylation of deoxycytidine (21), high pressure (22), several cations like Mg, Cd, Hg, Ni, and Co (23), binding of peptides (24), polyamines like spermine (24) and negative supercoiling in in vivo system (25). The Z-DNA is first reported by optical studies demonstrating that a polymer of alternating deoxyguanosine and deoxycytidine residues ($d(CG)_n$) produced a nearly inverted circular dichroism spectrum in a high salt solution (20). The Z-DNA helix is built from a dinucleotide repeat with the deoxycytidines in the anti conformation and deoxy guanosines are in the unusual syn conformation (26, 27). Karlik (17) reported that Aluminum stabilizes a portion of calf thymus DNA at neutral pH and Al $(OH)^{2+}$ has been attributed for the stabilization. At acidic pH Al destabilized the DNA by intrastrand cross links. Studies on interaction of Al with Poly d(GC). d(GC) showed interstrand cross link at low pH (< 6) (17). The primary binding of Al is the phosphate group and through electrostatic interaction. There exists controversy regarding its binding with nitrogen bases. Ahmed *et al* (28) using FTIR spectroscopy reported that Al cation bind mainly through the backbone PO_2 group and guanine bases. The cross linking of DNA strands by Al reported by Karlik *et al* (17) also evidenced Al binding to bases since the ability of metals to produce cross links appear to result from affinity for the nucleotide bases. Al binds weakly to basic phosphate group of DNA, while basic and chelating phosphate groups of nucleotides di and triphosphates do bind Al strongly. Al- maltol is a water soluble ($6x\ 10^{-2}M$) and hydrophilic (28). Al- maltol don't under go hydrolysis chemistry from pH 2 to 12. Though conductivity studies indicated that Al-maltol remains uncharged in aqueous solutions, the presence of charged species can not be ruled out. According to Corain *et al* the speciation of Al- maltol reveals that the thermodynamically predicted species which dominate at pH 7.0 for Al-maltol are $Al(mal)_2^+$ and $Al(mal)_2^{2+}$ (29). In the present study, under given experimental conditions Al acts as monovalent and divalent rather than the trivalent Al (Al^{3+}) ion and movovalent being predominant and moreover the Al-maltol complex species, $Al(mal)_2^{2+}$ and $Al(mal)_2^+$ bind to DNA. It has been shown that Al $(OH)^{2+}$ do not cross link the DNA strands (29). Binding of $Al(mal)_2^+$ and $Al(mal)^{2+}$ to the GC polynucleotide might play role in the B-DNA to Z-DNA conformational transition. There exists no definite mechanism to explain the role of metals in causing structural transitions. The possible ways through which Al-maltol can cause structural transition in GC polynuclelotide are given below. Through phosphate binding, which alleviates electrostatic repulsion thereby stabilizing base pairing and base stacking. Al species could have an influence on the water shell of DNA, which would make the helix more flexible for conformational change. It has been suggested that reduction of DNA phosphate repulsion (charge screening) and polymer hydration state are important determinants of conformation and structural transitions (30) . Another mechanism would be base-back binding of phosphate coordinated metal ion to N-7 of guanosine there by stabilizing the syn conformation of the guanosine. The H(N1) sites of guanosine base are not available for binding with metals at neutral pH and this leaves N-3 and N-7 as targets. Among theses N-7 is the most likely target for cation interaction while binding to N-3 is exceptionally found (31-33). We propose that Al in its $Al(mal)_2^{1+}$ and $Al(mal)^{2+}$ forms stabilizes syn conformation by base-back binding of the

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phosphate coordinated metal ion to N-7 of guanosine. It is evident from the Ahmad *et.al.*, (28) report that the chelation via N-7 of the guanine bases and the nearest oxygen of the $(PO_2)^-$ groups prevail in Al-calf thymus DNA complexes. It has been reported that cytosine has no major binding site for Al. In case of Cytosine, N-1-C-6 bond of projects onto or near to the ribose ring, and N-3 is directed away from the phosphate moiety there by simultaneous binding of the metal ion to phosphate and the N-3 is not feasible and will be in anti conformation (31). Evidence of base binding of Al in the present investigation is also supported based on the alterations in the CD spectra of Poly d (GC).d(GC) and Al complex, since alterations in CD spectra for DNA and polynucleotides have been observed for base – binding metals (34). The involvement of the ionic species in the interaction of DNA is also evident from its dissociation with native DNA regeneration upon treatment with EDTA. Investigations carried out on chromatin from intact nuclei of AD –affected brains offered evidence that Al (III) markedly increased the affinity of histone H1for DNA, thus suggesting its potential ability to inhibit the correct gene expression in vivo (35). This observation is interpreted as being due to the cross-linking action of Al (III) between histones, proteins, and DNA. The relevant bonds might involve two carboxylate ligands pending from a histone segment and a phosphate oxygen belonging to a DNA nucleotide.

The ability of Al-maltol to induce Z-DNA in poly d(GC).d(GC) might be important in chromatin condensation and in altering the gene expressional pattern. It is of considerable interest as biological function is often correlated with the structure at the molecular level. Moreover potentially Z-DNA forming sequences are highly dispersed through the human genome (36). It has been speculated, based on immunohistochemical data, that Z-DNA is about one-seventh as abundant as B-DNA (37). In particular interest with AD, it is observed from the Human Genome Sequence that the Z-DNA conforming GC rich sequences are observed in 5´ regions of AD specific genes like PS1, PS2, and APOE (38). It is interesting to mention that some of these genes have been over expressed in AD and have significant role in AD pathogenesis. Z-DNA formation excludes nucleosome formation and could affect the placement of nucleosome formation, as well as organization of chromosomes (39) and is considered to be involved in both transcriptional activation and inactivation (Herbert and Rich, 1996). It is theoretically postulated that the guanosine base present in DNA would be more susceptible to hydroxy radical induced DNA damage if the conformation of the DNA is Z rather than B or A because of the greater exposure of the bases (40). Restriction endonucleases and methylases are incapable of cleaving their respective recognition sites in Z-DNA conformation (41).

Conclusion

In conclusion the GC rich regions possessing Z-DNA conformation has great relevance to gene expression and G* oxidation. These events like to throw light in understanding metal induced neuronal cell death in neurodegenerative disorder.

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Vital medicine *Asparagus racemosus* willd

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Abstract

Use of plants as a source of medicine has been inherited and is an important component of the health care system in India. Among these plants *Asparagus racemosus* is an important medicinal plant which has been used worldwide. A lot of medicinally importance attributes have been assigned to this herb. It has been used by tribes located in distinct area of India from primeval time. Key component of this herb is saponins. Recent developments in transgenic research have opened up the possibility of the metabolic engineering of biosynthetic pathways to produce these high-value secondary metabolites. The present review is a pragmatic approach to accrue the findings on this very important herb.

Key words: *Asparagus racemosus*, Saponins, Ethnopharmacology, Disease.

Introduction

India's diversity is unmatched due to the presence of 16 different agro-climatic zones, 10 vegetation zones, 25 biotic provinces and 426 biomes. Of these, about 15000-20000 plants have good medicinal value. However, only 7000-7500 species are used for their medicinal values by traditional communities. In the Indian system of medicine, most practitioners formulate and dispense their own recipes (1). The age-old tribal knowledge of plants is an important aspect of ethno botanical research. The tribal tracts are the storehouse of information and knowledge on the multiple uses of plants (2) . Potential plants for Ayurvedic medicines have been reported by Kumar (3).

These plants are not only used for common diseases but also for fetal diseases. The Asparagus genus (Asparagaceae) has over 300 species, which are widely distributed in temperate and tropical regions. Its medicinal properties are reported in traditional systems of medicine such as Ayurveda, Siddha and Unani (4). It is used in the treatment of diarrhea, rheumatism, diabetes and brain complaints (5). During previous investigations influence of fertilizers on growth (6) and biochemical composition (7) and *in vitro* propagation of *Asparagus racemosus* (3) was studied.

Pharmacological applications of Asparagus racemosus

Aphrodisiac : An aphrodisiac is a substance that is used in the belief that it increases sexual desire.

The herbs have been traditionally used as Vajikaran Rasayana herbs because of their putative positive influence on sexual performance in humans. Aphrodisiac property of this herb was investigated by Pandey *et al.,*(8). Detectable level of Phytoecdysteroids in *Asparagus racemosus Willd.* seeds were revealed by Dinan *et al.,* (9)

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who did his research on 16 *Asparagus* species. Herbal *preparation* of *Asparagus racemosus Willd.* {lyophilized aqueous extracts of *Asparagus racemosus* Willd, *Chlorophytum borivilianum* Sant. F., *Curculigo orchioides* Gaertn, *Dactylorhiza hatagirea* (D. Don) Soo and *Orchis latifolia* Linn. (200 mg/Kg body weight) is formulated by Thakur *et al.,*(10) treat heat induced testicular damage in mice. Administration of this recipe results in significant amelioration of sexual behavior and the mount, intromission and ejaculatory latencies were significantly reduced (11).

Cognitive disorders : *Asparagus racemosus* Willd. is a well-known nervine tonic in the Ayurvedic system of medicine. 'Mentat', an herbal psychotropic preparation containing *Asparagus racemosus Willd* has been found to be effective in the treatment of alcohol abstinence induced withdrawal symptoms such as tremors, convulsions, hallucinations and anxiety in ethanol administered rats (12) due to its anticonvulsant and anxiogenic action. Parihar and hamnani (13) investigated neuroprotective properties of extracts of *Asparagus racemosus* Willd, *Convolvulus pleuricauas* and *Withania somnifera* against free radicals induced damage in different brain regions in experimental animals. Strategies to rescue or protect injured neurons usually involve promoting neuronal growth and functions or interfering with neurotoxic processes.

Galactogague : *Asparagus racemosus* Willd. root are one of the chief source of galactagogue. It has been shown to promote growth as well as increase in weight of mammary lobulo-alycolar tissue and milk yield in weaning rats by systemic administration of the alcoholic extract (14). The extract increased the weight of mammary glands in post partum and estrogen-primed rats. The alcoholic extract of *Asparagus racemosus* has been shown to increase the prolactin levels in female rats (14). Randomized controlled trial of *Asparagus racemosus* (Shatavari) as a lactogogue in lactational inadequacy was also studied by Sharma *et al.*(15). *A. racemosus* along with some other herbal substances in the form of a commercial preparation is reported to enhance milk output in women complaining of scanty breast milk, on 5th day after delivery (16). Patel and Kaniker (17) have also shown galactogogue effect of roots of *Asparagus racemosus* in buffaloes.

Immunoadjuvant and immunomodulater activity : The immunoadjuvant potential of *Asparagus racemosus* Willd. aqueous root extract was evaluated by Gautam *et al.,* (18) in experimental animals immunized with diphtheria, tetanus, pertussis (DTP) vaccine. Gautam *et al.*,(19) have studied possible immunoregulatory effects of *Asparagus racemosus* Willd. In Administration of *Asparagus racemosus, Sida cordifolia* in combination with Levamisole was the more effective in producing immunomodulatory effect in immunosuppressed (by Cyclophosphamide) birds (19).

Anti-tussive activiy : The plants have been used as antitussives agents due to their anti-inflammatory, antibiotic, antiviral, demulcents, expectorant and mucolytic properties, related with their ability to elaborate active principles such as aldehydes, alcohols, alkaloids, essential oils, glycosides, flavonoids, gums, ketones, lactones, mucilages, oleoresins, pectin, phenols, tannins and terpenoids. Asteraceae (Compositae), Lamiaceae (Labiatae), Boraginaceae, Rosaceae and Brassicaceae (Cruciferae), was the principal families reported, perhaps their secondary metabolites as i.e. sesquiterpenes and essential oils (20). The flowers, leaves, and aerial parts are most frequently used. The mainly common form of preparation is as decoction or infusion (tea) and the administration type is usually oral. The methanol extract of *Asparagus racemosus* root showed significant antitussive activity on sulfur dioxide induced cough in mice (21).

Adaptogenic activity : Adaptogenic drugs are those which are useful to counteract stressful factors by promoting non-specific resistance of the body (22). Antiulcerogenic action of an ayurvedic herbo-mineral formulation 'Satavari mandur' (SM) was investigated for its efficacy in the treatment of coldrestraint stress-induced gastric ulcer in rats (23). Rege *et al*., (24) administered orally the aqueous, standardized extract of *Asparagus racemosus* to experimental animals, following which they were exposed to a variety of biological, physical and chemical stresses. Bhattacharya *et al.,* (25) undertook a study to investigate the adaptogenic activity of 'Siotone' (a herbal formulation consisting of Withania somnifera, Ocimum sanctum, *Asparagus racemosus* Willd*., Tríbulus terristris* and shilajit) against chronic unpredictable, but mild, foot shock stress induced perturbations in behaviour (depression), glucose metabolism, suppressed male sexual behaviour, immunosuppression and cognitive dysfunction in albino rats

Anti-diarrhea activity : Diarrhea is increased fluidity, frequency or volume of bowel movements. It may be acute or chronic. Since the *Asparagus racemosus Willd.* root extract is composed of saponins, alkaloids, flavonoids, sterols and terpenes its root has been used traditionally in Ayurveda for the treatment of diarrhoea and dysentery. The plant extracts showed significant inhibitor activity against castor oil induced diarrhoea and PGE_2 induced enteropooling in rats. Both extracts also showed significant reduction in gastrointestinal motility in charcoal meal test in rats (26). It has been reported that asparagus decreases gastric emptying time (27). Nanal et al., (28) found Satavari to be extremely effective in the treatment of *Atisar* (diarrhoea), *Pravahika* (dysentery) and *Pittaj shool* (gastritis) as described in Ayurvedic texts such as *Sushruta Samhita* and *Sharangdhar Samhita*. Ethanol and aqueous extracts of *Asparagus racemosus Willd.* roots exhibited significant anti-diarrhoeal activity against castor oil induced diarrhoea in rats demonstrating an activity similar to loperamide(26). Other studies have shown that the methanolic extracts of asparagus root reduced intestinal propulsive movement, castor oil-induced diarrhoea and intestinal fluid accumulation (29).

Anti ulceric activity : In Ayurveda, *Asparagus racemosus* Willd*.* has also been mentioned for the treatment of ulcerative disorders of stomach and Parinama Sula, a clinical entity akin to the duodenal ulcer diseases (30). Nanal *et al.,* (28) studied the effect of *Asparagus racemosus* Willd*.* on *Amlapitta* (hyperacidity), *Grahani* (ulcerative colitis), *Parinam shool* (septic ulcer) and *Vataj shool* (spastic colon) and observed an amelioration of symptoms. Singh *et al*.,(31) showed that Shatavari promptly and persistently relieve the pain and burning sensation as well as other dyspeptic symptoms due to duodenal ulcer. The juice of fresh root of *Asparagus racemosus* Willd. has been shown to have definite curative effect in patients of duodenal ulcers Mangal *et al.,* (32) had done his study on human and found that *Asparagus racemosus* Willd*.* treatment increase lifespan of gastric mucosal epithelium cells as well as secretion and viscosity of gastric mucus. Antiulcerogenic action of an ayurvedic herbo-mineral formulation 'Satavari mandur' (SM) was investigated for its efficacy in the treatment of coldrestraint stress-induced gastric ulcer in rats (23). *Asparagus racemosus* Willd. along with *Terminalia chebula* reported to protect gastric mucosa against pentagastrin and carbachol induced ulcers, by significantly reducing both severity of ulceration and ulcer index (33). In another study by Sairam *et al.,*(34), the methanolic extract of fresh roots of *Asparagus racemosus* showed significant protection against acute gastric ulcers induced by cold restraint stress, acetic acid, pylorus ligation, aspirin plus pylorus ligation and cysteamine induced duodenal ulcers. Bhatnagar

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et al., (35) evaluated the anti-ulcer effect of *Asparagus racemosus* Willd. on indomethacin induced ulcers in rats. *Asparagus racemosus* has been found to be effective in dyspepsia, being associated with anti-ulcerogenic activity (36).

Anti depressant: Depression is a common chronic recurrent syndrome, often refractive to drug treatment affecting quality of life and overall productivity (37).

EuMil, is a herbal formulation comprising the standardised extracts of *Withania somnifera* (L) Dunal, *Ocimum sanctum* L, *Asparagus racemosus* Willd and *Emblica officinalis* Gaertn., the results indicate that EuMil has significant adaptogenic and anti-stress, activity, against a variety of behavioral, biochemical and physiological perturbations, induced by unpredictable stress, which has been proposed to be a better indicator of clinical stress than acute stress (38). Singh *et al*.,(37) administered orally the methanol, standardized extract of *Asparagus racemosus* roots to rats, following which they were exposed to a variety of biological, physical and chemical stresses. The results show that methanolic extract of *Asparagus racemosus* decreases immobility in forced swimming test and increases avoidance response in learned helpless indicating antidepressant activity. Same result previously received by Rege *et al*., (24).

Anticancer : *Asparagus racemosus* is well known for its immunomodulater activity (18), phytoestrogenic properties and use as a hormone modulator (39). Treatment with *Asparagus racemosus* Willd. *Tinospora cordifolia*, *Withania somnifera*, and *Picrorhiza kurrooa* significantly inhibited ochratoxin A-induced suppression of chemotactic activity and production of inflammatory cytokines interleukin (IL)-1 and tumor necrosis factor (TNF)-alpha by macrophages (40). Moreover *Asparagus racemosus* Willd. induced excess production of TNF-α when compared with controls. The crude saponins obtained from asparagus shoots were found to have antitumor activity. It inhibited the growth of human leukemia HL-60 cells in culture and macromolecular synthesis in a dose and time-dependent manner (41). Total extract, polar and non-polar extracts, and their formulations, prepared from medicinal plants mentioned in Ayurveda, namely, *Withania somnifera* (Linn Dunal) (Solanaceae), *Tinospora cordifolia* (Miers) (Menispermaceae), and *Asparagus racemosus* (Willd.) (Liliaceae) exhibited various immunopharmacological activities in cyclophosphamide (CP)-treated mouse ascitic sarcoma (42). Rao (43), studied inhibitory action of DMBA induced mammary carcinogenesis. Agrawal *et al.,* (44) proved that the aqueous extract of the roots of *Asparagus racemosus* has the potential to act as an effective formulation to prevent hepatocarcinogenesis induced by treatment with diethylnitrosamine. Anti-cancer activity of asparagus extract was also proved by Seena *et al*., (45). There are several studies that indicate a lower rate of breast cancer in populations with a high exposure to phytoestrogens (46) which is found predominantly in asparagus However; contradictory studies also exist regarding this evaluation. Studies found no association between phytoestrogens and breast cancer (47).

Antilithiatic effect : Antilithiatic effect of *Asparagus racemosus* Willd on ethylene glycol-induced lithiasis in male albino Wister rats was studied by Christina *et al.,* (48). Oral administration of *Asparagus racemosus* ethanolic extract reduce oxalate, calcium and phosphate ions in urin which are the main cause of renal stone formation (49). An *in vitro* assay technique was set up to determine the phagocytic and microbicidal activity of a monocyte-macrophage cell line using Candida species as test organisms. The utility of candidicidal assay in experimental and clinical studies was discussed by Rege and Dahanukar(50).

Antiparasitic effect : Antiplasmodium activity is identified by kigondu *et al*.,(Kigondu, Rukunga

et al. 2009). Sairam *et al*., (51) have reported antiulcerogenic activity of methanolic extract of fresh roots of AR in the cold restraint stress (CRS), pyloric ligation, aspirin plus pyloric ligation induced gastric ulcer models and cysteamine induced duodenal ulcer model. AR was found to be effective in the CRU, AL, and cysteamine induced ulcer models, but was ineffective in PL and ASP models. Effectiveness of *Asparagus racemosus* ethanol extract compare to the methanol and distill water extract of the same plant was reveled by S.Alok *et al*.,(52). It was found that the ethanolic extract of *Asparagus racemosus* Willd. had an inhibitory potential on lithiasis induced by oral administration of 0.75% ethylene glycolated water to adult male albino Wistar rats for 28 days. The ethanolic extract, significantly reduced the elevated level of calculogenic ions in urine and it elevated the urinary concentration of magnesium,which is considered as one of the inhibitors of crystallization (52).

Antiparasitic activity *:* The alcoholic extract of the root was found to possess *in vitro* antibacterial activity against *Staphylcoccus aureus* and *Escherichia coli*. However, the aqueous extract was found to be inactive (53). The hexane, aqueous and alcoholic extracts of the root at concentration of 200 mg /ml were devoid of any *in vitro* antibacterial activity against *Bacillus subtilis, Escherichia coli, Proteus vulgaris, Salmonella typhimurium, Pseudomonas aeruginosa* and *Staphylococcus aureus* using the agar well diffusion test (54). The juice of the root showed fungitoxicity against three plant fungi viz., *Helminthosporium sativum* (60.7%) *Colletotrichum falcatum* (58.2) and *Fusarium oxysporum* (60.7%) (55). The root bark showed marked antibacterial, against eight bacteria viz., *Micrococcus pyogenes* var. *aureus, Bacillus subtilis, Diplococcus pneumoniae, Streptococcus pyogenes, Escherichia coli, Salmonella typhosa, Vibrio comma* and *Shigella dysenteriae*; antitubercular against two mycobacteria *Mycobacterium phlei* and *Mycobacterium* 607 and antifungal actions against four fungi viz., *Microsporum gypseum, Trichophyton mentagrophytes, Candida albicans* and *Helminthosporium sativum* (56). The methanol fraction of the leaves using the disc diffusion test at a concentration of 4000 and 5000 ppm was found to inhibit *Proteus vulgaris* while it was devoid of any activity against *Escherichia coli, Klebsiella aerogenes* and *Pseudomonas aerogenes* (57) fresh juice of the plant showed antibacterial activity against *Staphylococcus* (56). The extract of the plant showed moderate toxicity against *Rhizoctonia solanil* (58).

Anticandidal activity of *Asparagus racemosus Willd.* against six species of candida (*Candida albicans, Candida tropicalis, Candida krusei, Candida guillermondii, Candida parapsilosis* and *Candida stellatoida)* had evaluated by Uma *et al.,* (59). *Asparagus racemosus* extract showed high degree of inhibition against *candida* in compare to any other antibiotics. Antibacterial activity of *Asparagus racemosus* was studied against *Escherichia coli, Shigella dysenteriae, Shigella sonnei, Shigella flexneri, Vibrio cholerae, Salmonella typhi, Salmonella typhimurium, Pseudomonas putida, Bacillus subtilis and Staphylococcus aureus* by Mandal *et al*., (60). *Asparagus racemosus* extract activity against leishmania and plasmodium has also been demonstrated (61).

Antidiebetic activity : More than 100 medicinal plants are mentioned in the Indian system of medicines including folk medicines for the management of diabetes, which are effective either separately or in combinations (62). *Asparagus racemosus* is consistently used by the tribal communities for the treatment of diabetes (63,64) as well as in modern medicine. As describe above metformin drug for diabetes increase Ca++ level in mitochondria, same mechanism was evaluated by Hannan *et al*., (65)

Table 1: Ethnopharmacological Importance of *Asparagus recemosus* (Willd.)

S.No.	Therapeutic use of *Asparagus*	Proposed mechanism *racemosus*of action	References
1.	Aphrodisiac	Plant compound structurally and/or functionally similar to ovarian and placental oestrogens.	10,8,9
2.	Alzheimer's disease	Antioxidative mechanism· Regulate neurotransmitters · Neuritis regeneration	13
3.	Galactogague	Activate adenohypophysis (anterior pituitary gland) to produce prolactin	13,68,17
4.	Immunoadjuvant and immunomodulater activity	Modify the antigenicity of immunization components.· Activate T cells· Up-regulation of Th1 (IL-2, IFN-g) and Th2 (IL-4) cytokines.	18,19,24,70
5.	Antitussive activiy	Anti-inflammatory property	20,21
6.	Adaptogenic activity	Modulate stress mediators (corticosteroids, catecholamines, and nitric oxide)	69,25
7.	Anti-diarrhea activity	Balancing the way fluid moves through intestines· Anti bacterial and anti viral activity· Reduce inflammation	79
8.	Antiulceric activity	Inhibit lipid peroxidation and protein oxidation.Antioxidant activity	2,21,35,71,23,34
9.	Anti depressant activity	Serotonin reuptake inhibitors· Increase GABA level in the brain	Antioxident 24,38,69
10.	Anticancer activity	Immunomodulater activity· Enhance production of inflammatory cytokines interleukin (IL)-1 and tumor necrosis factor (TNF)-alpha by macrophages· Inhibited the growth of human leukemia HL-60 cells	69,72,41,46,44,73
11.	Antilithiatic effect	*Asparagus racemosus* ethanolic extract reduce oxalate, calcium and phosphate ions in urin which are the main cause of renal stone formation.	34,48,49,50
12.	Antiparasitic activity	Inhibition of parasite through antiparasitic agents which break parasite resistance system. Biochemical of this plant act by two ways:· Destruction of metabolic pathways of parasite. Disturb physical resistance system of parasite.	21,35,50,57,61
13.	Antidiebetic activity	Hypoglycaemic activityIncreased intracellular Ca (2+)	65,74,75,76

revealed that constituents of *A. racemosus* root extracts have wide-ranging stimulatory effects on physiological insulinotropic pathways. The dried ethanolic extract 250 mg per kg body weight and the inorganic parts 90 mg pure ash/ kg body weight of the root revealed hypoglycaemic activity in a single dose effect on the oral glucose tolerance test GTT in fasting albino rats (66).

Anti anemic : Ayurvedic treatment of aplastic anemia is basically directed at treating the immune dysfunction and improving normal bone marrow production. Immuno-modulatory herbal medicines like Ashwagandha (*Withania somnifera*), Shatavari (*Asparagus racemosus*), Bala (*Sida cordifolia*), Nagbala (*Sida humilis*), Yashtimadhuk (*Glycerrhiza glabra*), Guduchi (*Tinospora cordifolia*) and Punarnava (*Boerhaavia diffusa*) are used. Asparagus is high in folic acid, which is essential for the production of new red blood cells and may therefore be helpful in preventing anemia. It is a rich source of folate and vitamin K. folate helps to get rid of the problem of anemia. Vitamin K is found to play a role in regulating the process of blood coagulation. *Shatavari churna* with milk or *shatavarisidhdha ghrut* (medicated ghee) is recommended for women suffering from anemia especially due to the loss of blood through periods. Hence, *asparagus* is a vital drug to cure anemia.

Antioxytocic : The alcoholic extract of the root exhibited antioxytocic activity. The saponin-glycoside A4, mp 191-95° C in doses of 20-50 µg/ml produced a specific and competitive block of the pitocin syntocinon -induced contraction of rat, guinea pig and rabbit uteri *in vitro* as well as in situ. The saponin also blocked the spontaneous uterine motility. It was also found that the hypotensive action of syntocinon in cat was unaffected by previous administration of saponin A4 (67).

Conclusion *:*

From the above description (concluded in Table 1), it may be concluded that *Asparagus racemosus Willd.* could be a useful natural herb which posses no side effects compare to allopathic drugs and can be used to cure many fatal dieses like cancer, gonorrhea, piles, diabetes etc. There are many unraveled applications of this herb remain uninvestigated in relatively newer areas of its function. Hence, phytochemicals and minerals of these plants will enable to exploit its therapeutic use. The drug is without having any serious toxicity or side effects known till date and thus can be safely used in humans for acute and chronic treatment regime.

In order to have a excellent medicine it is very necessary to coordinate the quality of raw materials, in process materials and the final products, it has become essential to develop reliable, specific and sensitive quality control methods using a combination of classical and modern instrumental method of analysis. *In vitro* induction of stress response is in progress to increase secondary metabolites in this plant using various abiotic and biotic elicitors. This would help in conservation of this species and provide pharmaceutical component in less time and cheap cost.

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Cloning, Expression and Purification of Haemagglutinin and Neuraminidase gene of highly Pathogenic Avian Influenza H5N1 in *Escherichia coli*

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Abstract

The looming influenza virus pandemic requires simple and quicker vaccination strategies to prevent higher mortality and morbidity both in chickens and in humans. The process of current influenza vaccines manufacturing using embryonated eggs cannot help in controlling a future pandemic as it is too slow and the yield obtained by these methods are quit lower. In this study, we have developed a bacterial expressed rHA and rNA for using as a subunit vaccine against avian influenza which could also be used as a diagnostic tools against avian influenza. The HA and NA genes were cloned individually into *Escherichia coli* expression vector pRSETA. The rHA and rNA were expressed in *E.coli* and were purified using Ni-NTA chromatography. The haemagglutinin activity of the *E.coli* expressed rHA was analyzed against various RBCs. The rHA and rNA expressed *E.coli* can be used as a quicker and cheaper subunit vaccine candidate against avian influenza and also as a tools for diagnosis.

Key words: Highly pathogenic avian influenza, haemagglutinin, neuraminidase, vaccine, diagnosis.

Introduction

Avian influenza is a highly contagious disease caused by type A influenza virus which belongs to the family *Orthomyxoviridae* (1). The highly pathogenic avian influenza (H5N1) virus is a significant threat not only to the poultry industry but for human too. The 1997 H5N1 outbreak in Hong Kong clearly indicated that these viruses can transmit efficiently from poultry to humans and has the potential to cause high mortality in both hosts (2). It repeatedly proven its ability to spread in humans as multiple episodes of human transmission associated with high mortality have been reported.

The effective control of the disease can be achieved either by vaccination or by antiviral drugs. On the other hand, rapidly evolving drug resistance among various highly pathogenic isolates showing resistance to antiviral drugs amantadine and rimantadine (3,4) necessitates the development of potential broad spectrum prophylactic vaccines for newer strains. Currently licensed seasonal influenza vaccines is an egg based vaccine which are only partially protective, particularly in populations at highest risk of severe disease, the very young and the elderly. In addition, there is a need for novel approaches for enhancing immune responses to emerging influenza isolates. However, more research is needed in the discovery of such vaccines, adjuvants, and dosing regimens to be able to supply the world with a safe and effective vaccine against avian influenza viruses. Majority of these

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vaccine development targets the two major structural antigens namely hemagglutinin (HA) and neuraminidase (NA). Antibodies to HA seem to play a major role in controlling the disease while NA is reported to assist in controlling virus replication but, it does not protect an animal from being infected (5,6). On the other hand, there are 16 HA subtypes and 9 NA subtypes have already been reported. The newer avian influenza virus can posses one among many probabilities of HA and NA (7).

There are several alternative strategies have been developed to produce pandemic and seasonal influenza vaccines which involves mammalian cell culture and baculovirus expression systems. Several studies have demonstrated the use of HA and NA either in the form of whole virus or expressed in various platform such as baculovirus and DNA vaccine expressing HA and NA aid partial protection in animals. None the less, the development of large quantities of such vaccines in mammalian cell culture and baculovirus may not fulfill the necessity of a pandemic as millions and millions of vaccine doses would be required. Few reports demonstrated the use of yeast as an expression system for producing avian influenza antigens (8,9,10,11) which can be used as potential pandemic vaccine candidates. However, expression in yeast based system consumes considerable time for producing bulk vaccines. Hence, development of such vaccine in a bacterial expression system may be an attractive alternative to produce faster, cheaper and bulk vaccines. Development of avian influenza HA in *E.coli* expression system has already been described (12) yet, not many reports describe the development of such cheaper and quick vaccine against avian influenza. Furthermore, the recombinant vaccines produced in bacteria will not only avoid the use of live virus but also expected to help in reducing complications associated with the whole virus vaccines (13).

In an attempt to produce a faster and safer vaccine against avian influenza, the aim of this study was to examine the feasibility of *E.coli* expressed avian influenza HA and NA as a subunit vaccine against potential influenza pandemic. The HA and NA gene of H5N1 strain (A/Hatay/2004/H5N1) was cloned into *E.coli* expression vector, the protein was expressed and purified from *E.coli*. The possibility of using the rHA and rNA as a vaccine candidates discussed.

Material and methods

Strains, Plasmids and culture conditions : The cDNA of HA and NA gene of Avian Influenza virus H5N1 strain (A/Hatay/2004/H5N1) was obtained from the International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India. *Escherichia coli* TOP10 (F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Δ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Ä(*ara leu*) 7697 *gal*U *gal*K *rps*L (StrR) *end*A1 *nup*G) cells were used for all the cloning and plasmid propagation work and BL21(DE3) pLysS (F– *omp*T *hsd*SB(rB–, mB–) *gal dcm* (DE3) pLysS (CamR) was used for the expression of rHA and rNA genes and the bacterial cells were grown and maintained in LB broth and/or LB agar containing appropriate antibiotics.

Cloning of HA and NA into pRSET A vector: The HA and NA gene of avian influenza H5N1 was PCR amplified from the cDNA using specific primers employing standard PCR protocols. The gene encoding NA was amplified using forward primer (5'- ATCGGCTAGCATGA ATCC AAATCAG AAGATAATAA-3') and a reverse primer (5'- ATCGAAGCTTCT ACTTGTCAA TGGTGAATG-3') consisting *Nhe*I and *Hind*III restriction endonuclease sites respectively. The gene encoding HA was amplified using forward primer (5'- ATCGGCTAGCAT GGAGAAAATA GTGCTTCT-3') and a reverse primer (5'- ATCGAAGCTTTTAAATGCAAATTCTGCATTG-3') consisting *Nhe*I and *Hind*III restriction endonuclease sites (underlined) respectively. A PCR reaction mixture containing 50 ng of cDNA, 1xPCR reaction buffer, 200µM of each dNTPs, 25 picomoles of each forward and reverse primers specific for HA and NA, 0.5 units of Phusion DNA polymerase enzyme was prepared

and the reaction volume made to 50µl using nuclease free water. A PCR reaction with an initial denaturation at 98°C for 2 min; denaturation at 98°C for 20 sec, annealing at 55°C for 30 seconds, extension at 72°C for 60 sec (25 cycles) and the final extension for 72°C for 10 min was performed to amplify the HA and NA gene. The PCR products were analyzed on 1% agarose gel electrophoresis and the PCR products were purified using GenElute Extraction Kit (Sigma, USA). The purified HA and NA amplicon were cloned into pRSET 'A' (Invitrogen, USA) in-frame with the N-terminus his-tag after digestion with *Nhel* and *Hind III* (New England biolabs, USA) and the transformed *E.coli* cells were plated on LB agar containing 100µg/ml of ampicillin. The resulted colonies were screened by restriction digestion with *Nhel* and *HindIII*. The clones releasing HA and NA genes were analyzed by DNA sequencing to confirm the clone and the sequence.

Expression of HA and NA gene in E.coli: The positive clones of HA and NA were transformed into BL21 (DE3) pLysS cells and grown to an optical density 0.6 at 600nm. The cells were induced with 0.5mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) for four hours. The cells were harvested four hours post induction and the expression was analyzed in SDS-PAGE and western blot using HisProbe-HRPO.

Purification of rHA and rNA using Ni-NTA chromatography: The clones expressing the HA and NA were scaled up to 2 liter and the cells were induced as described above. After four hours, the cells were harvested by centrifuging at 5000rpm for 30 minutes. The supernatant was discarded and the pellet was resuspended in 50mM sodium phosphate buffer (pH-8) containing 300mM NaCl. The cells were lysed by sonication (40% amplitude, 9 sec pulse on and 5 sec pulse off). The lysate was filtered through a 0.22µM filter and the rHA and rNA was purified from the filtered lysate by Ni-NTA chromatography under native conditions. The lysate was passed through a pre-equilibrated Ni-NTA Superflow resin (Qiagen, USA) at a speed of 1ml / min. The column was washed with 50mM sodium phosphate buffer containing 300mM NaCl, 30mM imidazole (Sigma, USA) to remove the unbound protein and the bound protein was eluted with 300mM imidazole in 50mM sodium phosphate buffer containing 300mM NaCl. All the fractions were analyzed on SDS-PAGE and the fractions containing target protein were pooled. The pooled fractions were dialyzed against 50mM sodium phosphate buffer containing 200mM NaCl and the protein concentration was determined using BCA protein assay reagent (Pierce, Rockford, IL). The protein was concentrated through a vivaspin20 (10,000 MWCO) and analyzed on SDS-PAGE and the final protein concentration was estimated using BCA reagent.

***Haemagglutination assay*:** Different erythrocyte species such as 1% horse, 0.5% chicken and 0.75% guinea pig were used to analyze the haemagglutination activity of the rHA as described previously (14). Briefly, the erythrocyte species were suspended in phosphate buffered saline (PBS), pH 7.4. The rHA was adjusted to a protein concentration of 1mg/ml. In a 'U' bottomed 96 well plate, 50µl of rHA antigen was serially (two fold) diluted from 1/2 and proceeded till 1/1024 and 50µl of different erythrocyte species were added. The reaction mixture was incubated at room temperature for 45minutes. The HA titer was defined as the highest antigen dilution that yielded complete haemagglutination of the test erythrocytes.

Results and Discussion

In the present study the haemagglutinin and neuraminidase gene of highly pathogenic avian influenza was cloned into *E.coli* expression vector and expressed in *E.coli*. The *E.coli* expression platform is an easy, cheaper yet efficient expression system for the expression of heterologous protein. Many such proteins expressed in *E.coli* are reported to be useful for wide range of applications such as diagnostic assays and vaccines. In an effort to develop a quicker and cheaper subunit vaccine and diagnostic tool for avian influenza, the HA and

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NA of highly pathogenic influenza virus was cloned, expressed and purified from *E.coli*.

Cloning of rHA and rNA gene in E.coli: The HA and NA genes were amplified from the cDNA of H5N1 avian influenza was analyzed in 1% agarose gel (Fig.1A). The agarose gel showed a 1.7kb HA and 1.3kb NA band confirming the gene specific amplification of HA and NA gene. Both the amplicons were further confirmed by DNA sequencing. The sequencing results were matched with the HA and NA respectively and was found to be 100% homologous. The purified HA and NA PCR products were digested with *NheI* and *Hind III* and cloned into similarly digested pRSET-A vector. The clones were confirmed by restriction digestion with *NheI* and *HindIII* which showed a 1719 bp HA and 2.9 kb pRSET A vector for HA and 1362 bp NA and 2.9 kb pRSET A vector for NA (Fig.1B) confirming the presence of cloned HA and NA genes. The pRSET-A-HA and The pRSET-A-NA was confirmed by DNA sequencing for the presence of respective genes and any errors in the reading frame of HA and NA genes which showed that both HA and NA genes were in-frame with the N-terminus His tag and had no errors within the reading frame of the gene.

Expression of rHA and rNA gene in E.coli : The positive transformants were analyzed in a 2ml culture for the expression of of rHA and rNA in SDS-PAGE (Fig. 2A) which showed a 66kDa rHA and a 50kDa rNA protein. The western blot using His-probe-HRPO also revealed a 66 kDa rHA and a 50kDa rNA confirming the expression of rHA and rNA respectively (Fig. 2B). The positive cultures expressing rHA and rNA were scaled up and the expression of the gene was analyzed by SDS-PAGE and western blot. The expression levels of rHA and rNA in the 2ml mini culture was lower as evidenced by the SDS-PAGE. However, the large scale cultures showed better expression. The HA gene used in this experiment is native and any kind of codon optimization has not been performed in the gene

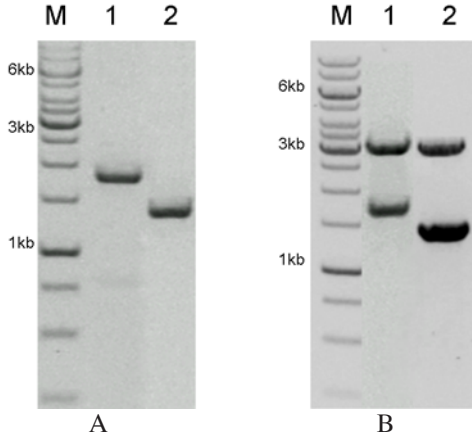

Fig. 1. Cloning of HA and NA into pRSET-A. (A). The HA and NA gene was amplified using gene specific primers. The amplification of HA resulted in 1.7kb product and the amplification of NA gave a 1.36kb PCR products. M-marker; 1-HA, 2-NA. (B). The HA and NA were digested with NheI and HindIII and were cloned into similarly digested pRSET-A vector. The clones were confirmed by restriction digestion with NheI and HindIII which released a 1.7kb HA and 1.36kb NA gene. The band running at 3kb size is the pRSET-A vector. M-marker; 1-HA, 2-NA.

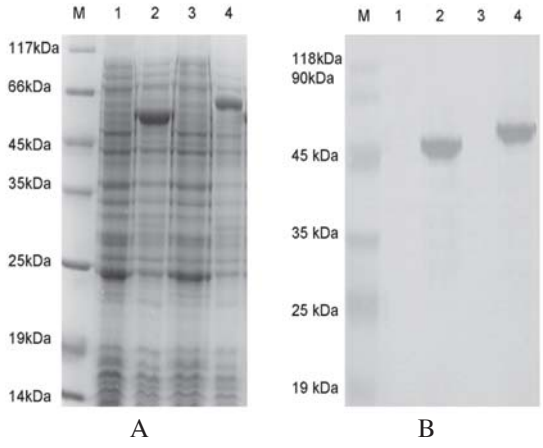

Fig.2. Expression of rHA and rNA in *E.coli*. The positive transformants were induced with 0.5mM IPTG and the cells were harvested 4 hours post induction and analyzed in a 12% SDS-PAGE (A) and western blot (B) which showed a 50kDa NA and 66kDa HA protein. M-Marker; 1-uninduced NA cell lysate; 2-induced NA cell lysate; 3-uninduced HA cell lysate; 4-induced HA cell lysate.

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coding sequences which could also be the reason for these lower expression levels.

Purification of rHA and rNA gene in E.coli: The rHA and rNA proteins were cloned in such a way that the expressed gene will have a N-terminus His-tag so as to enable the purification simpler and quicker. The cells expressing rHA and rNA were scaled up in a 2 liter flasks and the rHA and the rNA proteins were purified through Ni-NTA chromatography. The elution fractions were analyzed on SDS-PAGE and confirmed by western blot which showed a 66 kDa rHA and 50kDa rNA protein (Fig. 3). The fractions containing purified proteins were pooled. The purified HA and NA proteins were dialyzed against 50mM sodium phosphate buffer to remove the imidazole and were adjusted to a final concentration of 1mg/ml before storing at -80ºC for further analysis.

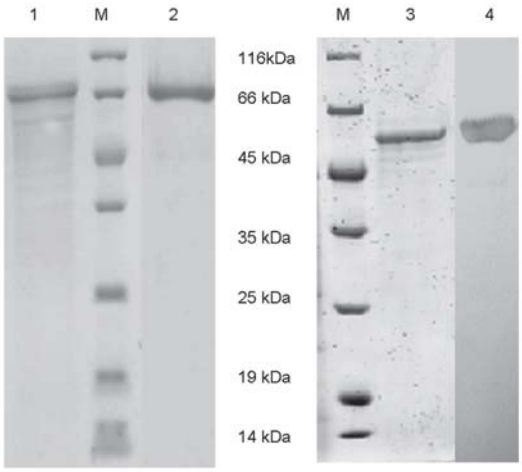

Fig.3. Purification of rHA and rNA from *E.coli*. The clones expressing rHA and rNA were induced with 0.5mM IPTG and the cells were harvested 4 hours post induction. The rHA and rNA were purified through Ni-NTA column chromatography. The purified fractions were analyzed in a 12% SDS-PAGE and western blot. M-Marker; 1-SDS-PAGE showing purified rHA; 2-western blot showing purified rHA; 3-SDS-PAGE showing purified rNA; 4-western blot showing purified rNA

Haemagglutination assay *:* The purified rHA protein was adjusted to a final concentration of 1mg/ml and the haemagglutination activity was analyzed using various RBCs. The guinea pig RBC showed the maximum haemagglutination titer followed by chicken and horse. The rHA protein showed a HA titer of 32 with chicken RBC (Fig. 4), 64 with guinea pigs RBC, 4 with horse RBC and no agglutination with sheep RBC. This clearly demonstrates the use of bacterial expressed rHA in diagnostic assays and as a candidate for subunit vaccine.

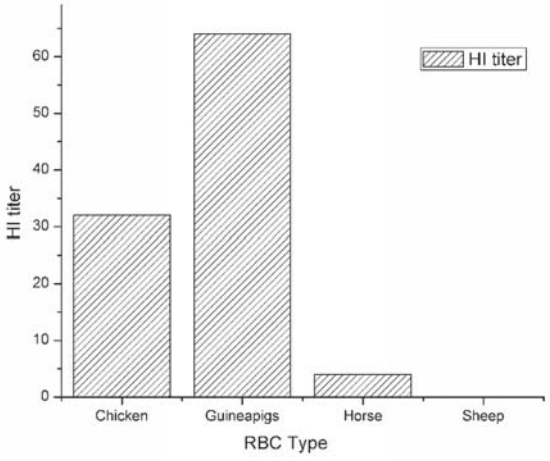

Fig. 4. Haemagglutination activity of purified rHA. The purified rHA protein was adjusted to a final concentration of 1mg/ml and the haemagglutination activity was analyzed using various RBCs. The guinea pig RBC showed the maximum haemagglutination titer followed by chicken and horse. The rHA protein showed a HA titer of 32 with chicken RBC, 64 with guinea pigs RBC, 4 with horse RBC and no agglutination with sheep RBC.

Conclusion

A variety of reports support the role of rHA and rNA as potential vaccine and diagnostic tools for avian influenza. Although, both HA and NA antigens are known to elicit neutralizing antibody response in animals (15, 16, 17), antibodies to HA is known to play a major role in virus control while antibodies to NA helps in partial not complete protection. Even though HA alone can provide maximum protection in animals from

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the disease (15, 16, 18, 19), few studies have already reported that addition of NA with HA improving the vaccine efficiency (17, 20). Hence, a detailed study on the combination of these two antigens as a vaccine candidate is a necessity. These rHA and rNA can also be used in the diagnosis of avian influenza as there are 16 HA subtypes and 9 NA subtypes have already been reported and for each type the HA and NA antigens can be readily expressed and purified in *E.coli* with in a very short duration. In addition to the possibility of using as subunit vaccines these antigens expressed in *E.coli* also pave a way for the development of easier and quicker diagnostic tools for avian influenza.

Acknowledgement

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Chemical Modification of Recombinant Human Interferon Beta-1a Using Linear and Branched mPEGs

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Abstract

Interferon-beta-1a (IFN-β-1a) is used clinically in the treatment of multiple sclerosis. Similar to other biological molecules, IFN-β-1a has a relatively short serum half-life and is rapidly detected by the host's immune system. PEGylation is a common approach to increase the blood circulation time of therapeutic proteins. In the present study, IFN-β-1a was PEGylated using linear methoxy polyethylene glycols (mPEGs) with molecular weights of 5 and 20 kDa and also 40 kDa branched mPEG-SPA. Prior to PEGylation, the mPEGs were activated by succinimidyl propionic acid (SPA). PEGylation was evaluated by size-exclusion HPLC (SE-HPLC) and Ninhydrin method. In the designed experiments, the factors of mPEG molecular weight, pH, and the molecular ratio of protein to mPEG fractions were studied. The results were analyzed using Design-Expert statistical software and the significant factors were determined. Then, in order to find the optimum conditions, Taguchi method (L_9 array) was used by considering the significant factors. Consequently, the optimum conditions for PEGylation, using 20 kDa linear mPEG-SPA was found to be at pH 8 and the protein to mPEG molar ratio of 1/40. The extents of protein modification were obtained 45.5% and 46.8% by HPLC and Ninhydrin methods, respectively. Optimum PEGylation with 40 kDa branched mPEG-SPA was obtained at pH 8 and protein/mPEG of 1/40. In this case, the extents of protein modification were obtained 46.5% and 47.7% by HPLC and Ninhydrin methods, respectively. The biological activity test showed that the PEGylated protein retained about 80% of its activity, were compared to that of the unmodified protein.

Key words: Beta interferon, Biological activity, mPEG-SPA, PEGylation, Taguchi method.

Introduction

Interferons (IFNs) are a family of cytokines that mediate antiviral, antiproliferative and immunomodulatory effects in response to biological and chemical stimuli. Two types of IFNs are recognized based on their physical and biological properties; type I which contains IFNs-α, -β, -κ, -τ, and -ω, and type II the only member of which is IFN-γ (1-5). Recombinant forms of IFN-β-1a (Avonex, Biogen Idec, Rebif and Serono) and IFN-β-1b (Betaferon, Schering AG) have been approved for the treatment of multiple sclerosis (MS), while non-recombinant forms of IFN-β (e.g., Feron and Toray) have been approved in Japan for the treatment of chronic viral hepatitis C (HCV). Therapeutic proteins have poor pharmacokinetic profiles because of their rapid clearance from blood circulation. Polyethylene glycol (PEG) attachment to the proteins offers a solution to these problems. The antiviral and immunomodulator drug, IFN-β-1a, is a common example (6).

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PEG conjugation (PEGylation) is an established technology that donates many beneficial effects to the proteins, including increased circulation half-life, reduced immunogenicity and antigenicity and decreased toxicity (7). Since the initial demonstration of PEGylated proteins as therapeutic agents, several proteins have been PEGylated and shown useful properties in clinical applications (8).

In the PEGylation technology, the size and structure of the PEG moiety, play important roles in the pharmacokinetic and pharmacodynamic properties of the resulting protein conjugates. Increasing the molecular size by PEGylation in particular, slows the renal ultrafiltration and prolongs the residence time of the drugs in blood circulation (9). Therefore, the current PEGylation technology needs high-molecular-weight PEG reagents to obtain favorable pharmacokinetic profiles (9-11). In fact, branch-structured PEGs allow for a higher molecular weight of up to 60 kDa as compared to linear PEGs with less than 30 kDa molecular weights. In addition, the branched PEGs act as if they are much larger than linear PEGs of the same molecular weight and show more effectiveness in protecting the proteins from proteolytic degradation and in reducing immunogenicity (7, 9).

A linear 5-kDa PEG was conjugated to interferon alpha (IFN-α), in an early attempt (12); however, this conjugate did not make any significant improvements in increasing the circulation half-life, compared to the unmodified IFN (13). To improve the pharmacokinetic properties, a linear 12-kDa PEG was conjugated to IFN and the resulting conjugate (PEG-Intron, Schering–Plough), showed a significant increasing in the circulation half-life, when compared to the unmodified IFN, with measurable serum concentrations detected after single weekly administrations (14).

The next generation of PEGylated IFNs was obtained by conjugating a branched 40-kDa PEG structure to IFN via an amide linkage. This product, Pegasys; Roche, showed superior efficacy over the unmodified IFN, with a significant increasing in the circulating half-life and reduced renal clearance, resulting in a strong antiviral response throughout a once-weekly dosing schedule (15-17). Similar success was recently achieved using a trimer-structured 43-kDa PEG, which is the slightly modified form of the branched PEG prepared by attachment a 3-kDa PEG to the branched 40-kDa PEG (18). This mono-PEG43K-IFN was absorbed slowly and had markedly reduced clearance in rats, thereby increased the half-life, approximately 40-fold compared to the native IFN (19).

Initial progress on PEG-IFN-β-1a has already been reported (9, 20, 21), but a general strategy for creating tailored PEGylated IFN-β-1a has not been developed since 1990 (22). Similar success was recently achieved by conjugating a branched PEG structure to IFN-ß via an amide linkage, as mentioned above. Its product showed superior efficacy over unmodified IFN-β, with a significant increasing in the circulating half-life and reduced renal clearance, resulting in a strong antiviral response throughout a once-weekly dosing schedule (1, 23-26).

For protein PEGylation, PEG must be initially activated in order to be able to react with the functional groups on the protein surface, mostly ε-amino group of lysine. In previous studies, methoxy-PEG (mPEG) has been activated by succinimidyl propionic acid (SPA) as an useful activator for antibodies and pancreatic islet PEGylation (19, 25, 28).

In the present study, we prepared and characterized IFN-β-1a modified with 5 and 20 kDa linear mPEG-SPAs and also, 40 kDa branched mPEG-SPA. We also reported the optimum conditions for PEGylation and biological activity of the PEGylated protein relative to the unmodified one.

Materials and Methods

Materials: Recombinant IFN-β-1a was obtained from National Institute of Genetic Engineering

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and Biotechnology (NIGEB, Tehran, Iran). mPEG (20 kDa) and SPA derivatives of mPEG (5 and 40 kDa) were purchased from SUNBIO (Anyang City, South Korea).

mPEG activation by SPA: mPEG-SPA was prepared and characterized according to Perry and Kwang (27). Briefly, dried mPEG (1 mmol) was dissolved in 20 mL of dry toluene. Then, 0.365 g of potassium tert butoxide was added to the reaction mixture, which was then stirred for 6 h at 80 °C under nitrogen atmosphere. Then, 5 mL of methyl 3- bromo propionate was added slowly to the reaction mixture, and was stirred for another 20 h at room temperature under nitrogen atmosphere. Then, the reaction mixture was filtered and precipitated by adding dry diethyl ether. The dried precipitated product was dissolved in 25 mL of 1 N NaOH solution and stirred for 2 h at room temperature under nitrogen environment. HCl (6 N) was then slowly added to the solution, until obtaining pH 3. Chloroform was further added to the solution in order to extract the reacted mPEG. Finally, the reacted mPEG was reprecipitated by cold ethyl ether. Ten grams of dried precipitated product and 0.52 g of N-hydroxy succinimide were dissolved in 50 mL of methylene chloride under nitrogen atmosphere. Then, 0.74 g of N, N-dicyclohexylcarbodiimide was added to the solution which was stirred in ice bath for 20 h. After removing the precipitated dicyclohexyl urea, the product was precipitated by adding dry diethyl ether and dried in vacuum, overnight. The final product (mPEG-SPA) was characterized by Fourier Transform Infrared (FT-IR) Spectrometer and stored in vacuum at -20°C until using (28, 29).

IFN PEGylation: IFN was conjugated to mPEG-SPA derivatives of different molecular weights (5, 20 and 40 kDa) at the defined pH and protein/mPEG, according to the designed experiment, introducing in the following sections. The reaction conditions were optimized to obtain high conjugation yields in a reproducible manner. Briefly, IFN solution (0.3 mg/mL) in phosphate buffered saline (PBS, 20 mM, pH 7.2) was filtered through a 0.45 µm pore size syringe filter. mPEG–SPA was dissolved in 100 µL of dimethylsulfoxide and added dropwise to the cold IFN solution (1.5 mL). Conjugation was allowed to proceed at 4°C with an end-to-end rotation and quenched with the addition of glycine (final concentration of 15 mM). The IFN–mPEG conjugate solution was then stored at 4°C (19, 30).

Size-exclusion-HPLC (SE-HPLC): The composition of IFN–mPEG conjugates was analyzed using an HPLC system (Younglin SDV30 PLUS, South Korea) consisting of a Waters SP930P pump and M730D UV detector equipped with an Autochro data module. The samples were loaded on to a 8 mm × 300 mm Shodex protein column KW-802.5 (Showa Denko KK, Japan) through a guard column and eluted with PBS (20 mM, pH 7.4) as a mobile phase. The flow rate was adjusted to 0.8 mL/min and the elutes were monitored by UV detection at 280 nm. The unmodified protein fraction as HPLC response was calculated from the difference between the unmodified protein peak area before and after the reaction (30).

Ninhydrin test: Ninhydrin method was used to determine the degree of modification by measuring the number of free amino groups on the surface of the modified and unmodified proteins. Fifty microliters of the protein solution was mixed with 100 µL of distilled water, 50 µL of 4 M acetate buffer (pH 5.1) and 200 µL of Ninhydrin reagent. The mixture was heated at 100 °C for 15 min, cooled at 10 °C for 10 min, and diluted with 1 mL of 50% ethanol. After centrifuging at 14000 rpm, the optical absorbance was measured at 570 nm. The degree of modification was calculated from the difference between the free amino groups before and after the reaction (31).

Design of experiments, full factorial design at two levels: The first step (using Yates table (32)) identified the most effective variables on the PEGylation reaction using 5 and 20 kDa linear mPEG-SPAs. The selection of these factors was according to the literature on IFN PEGylation (25,

30, 33). According to our experiments, an appropriate time for IFN PEGylation was 2 h (data not shown). The evaluated variables and their levels are showed in Table 1. Yates table design is presented in Table 2. All of the experiments were performed in duplicate, unless stated otherwise.

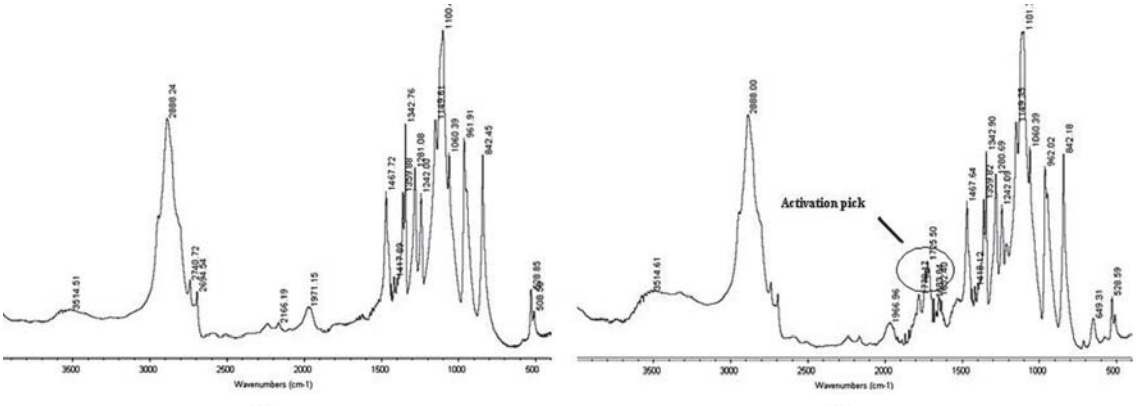
Design of experiments, Taguchi statistical design: Taguchi statistical design was selected to determine the optimum conditions for IFN PEGylation, using 5 and 20 kDa linear mPEG-SPAs (34). An L_9 Taguchi array (34) was designed based on three variables (determined from the previous design) at three levels (Table 3) (30). The design (L_9 array) is shown in Table 4. All of the experiments were performed in duplicate unless stated otherwise.

Design of experiments, one factor at a time design: One factor at a time design was selected to study the impact of one variable (the ratio of protein to mPEG fractions) on single response (HPLC or Ninhydrin test) for PEGylated IFN using 40 kDa branched mPEG-SPA (32). To obtain the optimum conditions for PEGylation of IFN with 40 kDa branched mPEG-SPA, the effect of the ratio of protein to mPEG fractions (at values of 1:10, 1:20, 1:30 and 1:40) at two different pHs of 7.4 and 8.0 was investigated.

Determination of antiviral activity: The specific antiviral activity of IFN-ß- 1a was determined using a cytopathic effect (CPE) bioassay that measures the ability of proteins to protect HeLa cells (grown at 37 °C/5% CO_2) challenged with the Vesicular Stomatitis Virus (VSV). HeLa cells suspended in 10 mL of Dulbecco´s modified eagles medium (DMEM) containing 5% fetal bovine serum, 9.53 g/L DMEM medium, 0.1 g/L of sodium pyruvate, 0.3 g/L of L-glutamine, 2.5-3.5 g/L of sodium bicarbonate and 50 µg/mL of kanamycin-neomycin, were added to a 96-well microtiter plate and incubated for 24 h at 37° C. Serial dilutions of duplicate IFN-β-1a standards and test samples were then added, and the cells were incubated for further 24 h. The medium was removed, and VSV virus was added. The cells were incubated for 48 h, then 50 µL of 5 mg/mL of 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl-2*H*-tetrazolium bromide (MTT) in PBS was added. After 1 h of incubation, the medium was removed and the wells were washed with 100 µL of PBS. The cells and dye were then solubilized with 100 µL of 1.2 N HCl in 2-propanol, and the optical absorbance of medium was then measured at 570 nm (1).

Results and discussion

Activation of mPEG: The synthesized mPEG-SPA was analyzed by FT-IR spectroscopy (FT-IR system: NICOLT, Lexus 670, USA). The FTIR spectrum of mPEG-SPA showed a peak at 1,725 cm^{-1}, indicating the presence of a carbonyl bond. These spectra indicated that the mPEG-SPA was successfully synthesized (Figure 1).



(a) (b)

Fig. 1. FTIR spectrum of (a) 20 kDa mPEG and (b) 20 kDa mPEG-SPA.

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Table 1. The selected variables and their corresponding levels for full factorial design.

Variable	Low level	High level
	(1)	(2)
A: Polymer molecular weight (KDa)	5	20
B: pH	7.4	8.0
C: The ratio of protein to mPEG fractions	1:10	1:40

Table 2. Yates table design (full factorial design) for screening the factors.

Treatment Combination[a]	Polymer MW (kDa) (A)	pH (B)	Protein/mPEG fractions (C)	HPLC Response	Ninhydrin Response
1	5	7.4	1:10	0.879	0.128
a	20	7.4	1:10	0.868	0.135
b	5	8.0	1:10	0.860	0.153
ab	20	8.0	1:10	0.809	0.208
c	5	7.4	1:40	0.655	0.352
ac	20	7.4	1:40	0.588	0.415
bc	5	8.0	1:40	0.612	0.405
abc	20	8.0	1:40	0.545	0.468

[a] The low level of any variable is denoted by I, and the high level of any variable is denoted by its lower-case letter.

Table 3. The selected variables and their corresponding levels for Taguchi design.

Variable	First level (1)	Second level (2)	Third level (3)
A:Polymer molecular weight (KDa)	5.0	12.5	20.0
B: pH	7.4	7.7	8.0
C: The ratio of protein to mPEG fractions	1:10	1:25	1:40

Protein PEGylation: The degree of polymer attachment to IFN-β was assessed by HPLC for PEGylated proteins using linear polymers with molecular weights of 5 and 20 kDa, and also the branched polymer of 40 kDa. The samples were loaded on a SE-HPLC column to separate the PEGylated and non-PEGylated proteins on the basis of their molecular size, as evaluated by the Autochro 3000 software. For each test, the ratio of the non-PEGylated protein peak to the total peaks represented the response. Therefore, in this procedure, a lowers ratio leads to higher levels of polymer attachment to the protein. Furthermore, Ninhydrin test was also done to obtain the total amount of polymer attached to the protein. In this test, the reaction of Ninhydrin with free amino groups on the protein surface showed that the PEGylated protein reacts with lower levels of Ninhydrin in comparison to the non-PEGylated protein. Consequently, in this test,

Table 4. An L_9 Taguchi array for IFNs PEGylation.

Trial Number	Polymer MW (kDa)	pH	Protein/mPEG fractions	HPLC Response	Ninhydrin Response
1	5.0	7.4	1:10	0.879	0.128
2	12.5	7.7	1:10	0.838	0.179
3	20.0	8.0	1:10	0.805	0.218
4	5.0	7.7	1:25	0.774	0.256
5	12.5	8.0	1:25	0.712	0.271
6	20.0	7.4	1:25	0.621	0.280
7	5.0	8.0	1:40	0.605	0.412
8	12.5	7.4	1:40	0.611	0.390
9	20.0	7.7	1:40	0.579	0.431

the result is represented as the ratio of the absorption difference between the non-PEGylated and PEGylated to the absorption of non-PEGylated protein. Therefore, in Ninhydrin test, a higher ratio shows higher levels of polymer attachment to the protein.

Full factoriel design: By considering the obtained results by HPLC and Ninhydrin tests, full factorial design was employed to screen the main effective factors on PEGylation, using linear polymers with molecular weights of 5 and 20 kDa. Table 2 shows data regarding the full factorial design using linear PEGs of 5 and 20 kDa. According to Table 2, by increasing the level of pH, polymer molecular weight, and the ratio of protein to mPEG fractions, HPLC responses have decreased and Ninhydrin responses increased, indicating that the degree of PEG attachment to the protein has increased. Diwan and Park reported that by increasing the polymer molecular weight, pH, and the ratio of protein to mPEG fractions during PEGylation of IFN-β with mPEG-SPA, the degree of PEGylation increased (28). By considering the analysis of variance (ANOVA) and p-values (Table 5) less than 0.01, the most important factors were determined. These results show that PEG molecular weight (A), pH (B), and the ratio of protein to mPEG fractions (C) are important but the effect of their interactions are not significant.

Taguchi statistical design: By considering the important factors, Taguchi method (L_9 array) was used to obtain the optimum conditions using the linear PEGs. Table 4, shows the obtained results by Taguchi design using linear PEGs of 5 and 20 kDa. According to Table 4, by increasing the levels of pH, polymer molecular weight and the ratio of protein to mPEG fractions, HPLC responses have decreased and Ninhydrin responses increased, indicating an increase in attachment of PEG to the protein. This shows that the amount of protein PEGylation has been improved by increasing the level of the factors. Hence the optimum conditions for PEGylation that were obtained by software are as follows: pH 8; the ratio of protein to mPEG fractions 1:40; PEG molecular weight 20 kDa.

One factor at a time design: Figure 2 shows the obtained results for one factor at a time design using 40 kDa branched PEG. PEG attachment to the protein increased following increases in pH and the ratio of mPEG to protein, as HPLC responses decreased and Ninhydrin responses increased. It shows the optimum condition for PEGylation reaction using the branched 40 kDa PEG, as pH 8 and the ratio of protein to mPEG fractions 1:40. At this point, the non PEGylated protein component is at its minimum and the numbers of the reacted amino groups are at their maximum. Table 6 shows the optimum conditions

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and the predicted and experimental responses obtained by HPLC and Ninhydrin tests from Taguchi and one factor at a time designs. It is observed that under similar conditions, the branched PEG could produce more PEGylated protein.

Determination of antiviral activity: In this study, results of the biological activity of PEGylated IFN-β are reported as qualitative and quantitative responses. The qualitative results are presented in Figure 3, obtained by an optical microscope from the infected HeLa cells. These images show the cells' health conditions and biological activity, indicating that cells with distinct shapes, sizes and cell walls were still viable.

Also, the optical absorption by the HeLa cells' growth medium was measured and compared. A change in the color of HeLa cells' growth medium indicates the viability of cells and the amount of cell density. Changing the yellow tetrazole to the purple formazan occurs just when the reductase enzymes are active, because tetrazol reacts with the reductase enzymes (MTT Test). Therefore, such changes in the color of the medium are used to measure the quantity of viable cells. The levels of optical absorption by the purple formazan solution was measured and compared with the two states: the solution containing the relevant cells and the control cells. Figure 3a shows the cell growth medium used as control.

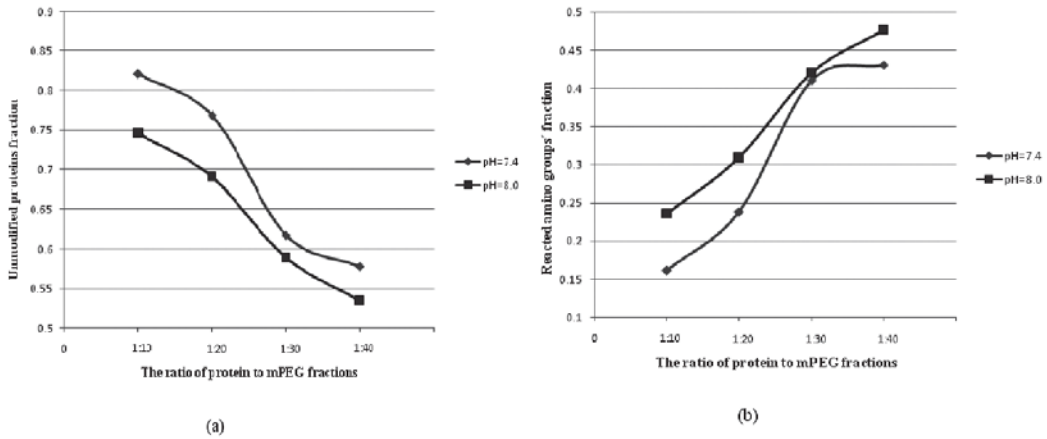

Fig. 2. The effect of different ratios of protein to mPEG at two different pHs on the extent of PEGylation progress; (a) HPLC results and (b) Ninhydrin results obtained from one factor at a time design for 40 kDa mPEG.

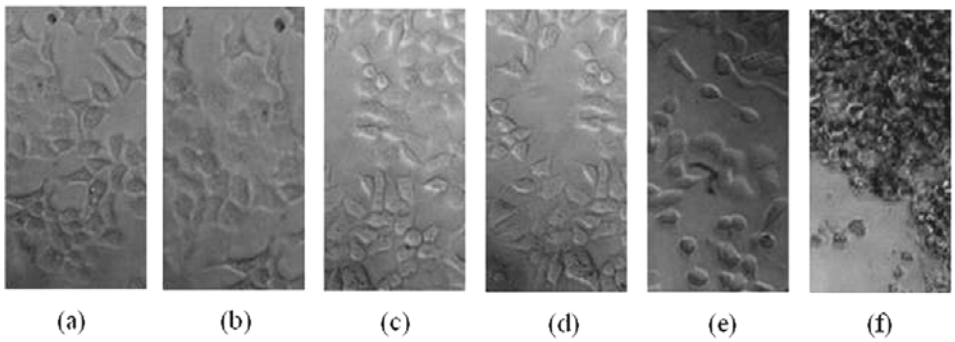

Fig. 3. Biological activities in the hela cells. (a) Control cell (b) Control cells+interferon before the PEGylation reaction, (c) control cells+interferon before PEGylation reaction+virus, (d) control cells+PEGylated interferon, (e) control cells+PEGylated interferon+ virus, (f) control cells+virus.

Table 5. ANOVA table for 2^3 full factorial design.

Source of variation[a]	p-value for HPLC results	p-value for Ninhydrin test results	
Model	<0.0001	<0.0001	Highly significant
A	0.0007	0.0012	Highly significant
B	0.0026	0.0014	Highly significant
C	<0.0001	<0.0001	Highly significant
AxB	0.0686	0.0483	Not significant
AxC	0.4573	0.5972	Not significant
BxC	0.9865	0.6604	Not significant
AxBxC	0.5600	0.6390	Not significant

[a]A: PEG MW, B: pH, and C: the ratio of protein to mPEG fractions

Table 6. Optimum conditions obtained from the Taguchi design for linear 20 kDa PEG and one factor at a time design for the branched 40 kDa mPEG.

Design type	Polymer MW (kDa)	pH	Ratio of protein/mPEG fractions	HPLC test		Ninhydrin test	
				Response 1[a]	Response 2[b]	Response 1[a]	Response 2[b]
Taguchi design (for linear PEGs)	20	8	1:40	0.569	0.545	0.438	0.468
One factor at a time design (for branched PEG)	40	8	1:40	-	0.535	-	0.477

[a] Predicted response by software
[b] Experimental response

Table 7. Quantitative assay of cell viability by MTT.

The related photo in Figure 3	The growth medium characteristic	Optical absorbance (at 570 nm) (the amount of cell viability)
a	Control cell	-
b	Control cell+interferon before PEGylation reaction	0.469
c	Control cell+interferon before PEGylation reaction+virus	0.334
d	Control cell +PEGylated interferon	0.270
e	Control cell +PEGylated interferon+ virus	0.260
f	Control cell+virus	0.050

IFN-β concentration is kept constant in all the samples.

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Figure 3 (b-e) show intact HeLa cells which have normal shapes and sizes. Figure 3f shows the biological activity of HeLa cells in the presence of virus and absence of IFN-β. In the absence of IFN-β the cells were detected at the bottom of the microplate (indicating the cytopathic effect of virus). In Table 7, by considering image b as representing 100% protein biological activity and addition of the virus to the growth medium, IFN-β biological activity decreases by about 70% (image c).

By PEGylating the protein, its biological activity, as compared to image b, has decreased by about 40% (image d). Then, by adding virus to the growth medium it is obvious that the IFN-β biological activity in image e, compare to image c, has calculated about 80%. The studies that have been published regarding PEGylated IFN-α-2b (PEG-Intron) and PEGylated IFN-α-2a (Pegasys) support our observation that non-targeted PEGylation has a deleterious effect on the activity of interferons. In the case of PEG-Intron, reaction involving succinimidyl carbonate is used to attach the linear 12 kDa PEG (35), while for Pegasys an N-hydroxysuccinimide ester derivative of the branched 40 kDa PEG was used to modify the protein [13]. The use of such chemical reactors has resulted in the modification of numerous individual sites as well as a significant reduction in the *in vitro* specific antiviral activity.

With regard to the PEG-Intron, lysine, tyrosine, histidine, serine, and cysteine residues are modified, resulting in 14 different monoPEGylated positional isomers. The antiviral activity of the mixture is 28% of that of the unmodified protein and ranges from 6 to 37% for the individual species (36). In the case of Pegasys, only lysine residues are modified, resulting in 6 different monoPEGylated positional isomers, with the mixture having an antiviral activity of only 7% of the unmodified protein (13). For rat IFN-β, the PEGylated protein, which retains essentially full *in vitro* antiviral activity, had improved pharmacokinetic parameters as compared to the unmodified protein (23). Baker *et al.* have shown that PEGylated IFN-β-1a retains approximately 50% of its activity when compared to the unmodified protein (1).

Relative to native IFN-β-1b, the monoPEGylated compounds possess excellent activity. The retention of about 20-70% of antiviral activity in these derivatives compares favorably to the marketed PEGylated IFN-R drugs, PEG-INTRON and Pegasys, where *in vitro* antiviral activities are about 28% or 7% of the unmodified protein, respectively (13,14). It is apparent that the conjugates with the highest molecular weight, such as the 40 kDa PEG polymers have lower antiviral activity *in vitro* (25).

Conclusions

mPEG-SPAs of 5, 20 and 40 kDa were covalently attached to IFN-β-1a and the optimum conditions for this reaction were determined by different methods of experimental design. For 5 and 20 kDa mPEGs, the optimum molecular weight, pH and the ratio of protein to mPEG were found to be 20 kDa, 8 and 1:40, respectively. Under optimum conditions, the percentages of protein modifications were obtained 45.5% and 46.8% by HPLC and Ninhydrin methods, respectively. For 40 kDa mPEG reactions, the optimum molecular weight, pH and protein to mPEG molar ratio were found to be 40 kDa, 8 and 1:40, respectively. Under optimum conditions, the percentage protein modifications were obtained 46.5% and 47.7% HPLC and Ninhydrin methods, respectively. Further increases in polymer concentration, may increase the extent of protein coating. Based on the results of the present and previous studies (1,24,30), it can be concluded that the optimum conditions for PEGylation of IFNs vary with changing molecular weight of mPEG or using different activating agents. The biological activity test showed that the PEGylated protein retained about 80% of its activity, were compared to that of the unmodified protein, While, in other studies the maximum biological activity were obtained about 50%.

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Enhanced Production of Glutathione from *Saccharomyces Cerevisiae* using Metabolic Precursor and Purification with New Approach

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Abstract

This paper reports on the enhanced fermentative production of glutathione by *Saccharomyces cerevisiae* NCIM 3454 using a statistical approach and its successive purification by alternative technique. In the first step, one factor at-a-time method was used to examine the effect of carbon sources, nitrogen sources and pH on glutathione (GSH) production. Subsequently, statistical mathematical model was used to identify the optimum concentrations of the key nutrients for higher GSH production. Glutathione production increased significantly from 55.28 to 148.45 mg/L when *Saccharomyces cerevisiae* NCIM 3454 was cultivated using optimized medium, as compared to basal medium. Further, glutathione production was considerably increased to 163.12 mg/L by using cysteine amino acid as one of the metabolic precursor. In this study aqueous two phase system (ATPS) was found to be most useful technique to abolish contaminating proteins in glutathione purification. Further enhanced purification was carried out by adsorption chromatography (ion exchange) using a variety of Amberlite resins.

Keywords: Glutathione, *Saccharomyces cerevisiae*, fermentation, precursor, chromatography.

Introduction

Glutathione (α-glutamyl-L-cysteinylglycine, GSH) is the most abundant water soluble non-protein consisting of thiol group which is widely distributed in living organisms and predominantly, in eukaryotic ells (1). It functions in many cellular processes including the protection of cells against xenobiotics, carcinogens, radiation and reactive oxygen species (2,3) hence it has medicinally important value in areas like health care, functional foods, cosmetics and its commercial demand is intensifying (4). Other functions of GSH include storage and transport of cysteine, regulation of cell proliferation, synthesis of deoxyribonucleotides, and regulation of leukotriene and prostaglandin metabolism (5). It also works as a neurotransmitter, neuromodulator and regulator in cell proliferation and apoptosis (6). An imbalance of GSH is observed in a wide range of pathologies including, cancer, neurodegenerative disorders, cystic fibrosis, HIV and aging.

Normally, most of the glutathione is present in the reduced form GSH while several additional forms of glutathione are present in (microbial) cells, tissues, and plasmas. Oxidized form of glutathione (glutathione disulfide, GSSG) upon oxidation of GSH, can in turn be reduced to GSH by glutathione reductase at the expense of NADPH (7). It is less easily oxidized than its precursors, cysteine and ã-glutamyl cysteine (8). It can be produced by using chemical synthesis (9) enzymatic methods (10) or by direct fermentative methods (11, 12). Although production of GSH by enzymatic method gives maximum concentration (up to 9 g/L) but

simultaneously use of three amino acids as precursors increases the production cost. Due to this reason GSH production by fermentation has been extensively studied, in which sugars as substrates can be used on industrial scale to nullify the production cost and to increase the GSH concentration in the medium (13). GSH production can be increased by either increasing the biomass or by changing amino acids. *Saccharomyces cerevisiae* and *Candida utilis* are currently utilised to produce glutathione on an industrial scale (13).

The conventional one factor at-a-time optimization method optimizes only one parameter at a time in which other parameters are kept constant. The statistical procedure makes available an alternative methodology to optimize a particular process by considering mutual interactions among the variables and gives an estimate of the combined effect of variables selected for study on the final result. Response surface methodology (RSM) employed in this study is based on the fundamental principles of statistics, randomization, replication and duplication, which simplifies the optimization by studying the mutual interactions among the variables over a range of values in a statistically valid manner. It is also known as full factorial central composite design (CCD). Previously, RSM was successfully employed for the production of GSH by *Saccharomyces cerevisiae* (14). Industrially, GSH is produced on a large scale by yeast fermentation (15). Although final purification step must satisfy extremely high purity for pharmaceutical purposes, the crude cell extract includes a lot of impurities which limit the efficiency of GSH recovery by crystallization (16). There are only a few studies reported on industrial separation of GSH.

In the present study, *S. cerevisiae* was screened for GSH production. Media optimization was done by one factor at-a-time and a statistical method i.e. RSM. The effect of addition of amino acids on GSH production was also studied. The present work also includes the development of an effective procedure for isolation and purification of GSH from fermentation broth. The effect of ATPS on partitioning behavior of GSH and contaminating proteins was also studied. ATPS parameters were studied with respect to PEG molecular weight, PEG concentration and salt concentration to concentrate GSH in one phase and unwanted proteins in other phase. Further the effective purification process was done successively with respect to operating parameters such as selection of an ion exchange resin and elution system.

Materials and Methods

Materials: Media components such as glucose, maltose, lactose, fructose, sucrose, galactose, starch, yeast extract, peptone, beef extract, malt extract, casein peptone and agar were purchased from Hi-Media Lab. Ltd, Mumbai, India. Magnesium sulphate, Potassium dihydrogen phosphate, Sodium chloride, Zinc chloride, Calcium chloride, Ammonium chloride, Ammonium sulphate and ethanol AR grade were purchased from S. D. Fine Chemicals Ltd, Mumbai, India. Glycine, hydrochloric acid, acetic acid, sodium acetate, Amberlite IR 120H, Amberlite XAD 16, PEG 1500, PEG 4000, PEG 6000 were purchased from S. D. Fine Chem. Ltd., Mumbai, India. Indion CAM and Indion 830 were purchased from Ion exchange (India) Ltd., Mumbai.

Maintenance of cultures and Inoculum development: The strains of *S. cerevisiae* NCIM 3454 was procured from NCIM (National Collection of Industrial Microorganisms) Pune, India and maintained on MGYP agar medium (malt extract, 0.3 %; glucose, 1.0 %; yeast extract, 0.5 %; peptone, 0.5 %). All slants were grown for 24 h aerobically at 30 °C. For inoculum, saline solution (5 mL) was added to the fully grown slant and 1 mL cell suspension was transferred to 25 mL of the seed medium (MGYP

broth) in a 250 mL Erlenmeyer flask and incubated at 30±2 °C and 180 rpm for 18 h.

Media optimization by one factor at-a-time method

Effect of initial pH: In order to investigate the effect of initial pH on GSH production, fermentation runs were carried out by adjusting initial pH (before autoclaving) of the medium in the pH range of 4 to 8. The pH was adjusted using 1 N HCl and/or 1 N NaOH. Each media was inoculated with 3 % of inoculum suspension and placed on a shaker for 24 h at 180 rpm at 30±2 ºC.

Effect of carbon sources: Glucose in the media was replaced with different carbon sources *viz* galactose, glycerol, sucrose, soluble starch, maltose, lactose, fructose at a concentration of 5.2 % to determine their effect on growth and GSH production. Each media was inoculated with 3 % of inoculum suspension and placed on a shaker for 24 h at 180 rpm at 30±2 ºC.

Effect of nitrogen sources: The effect of various organic nitrogen sources on the formation of GSH by *S. cerevisiae* NCIM 3454 was investigated. Peptone in the production media was substituted with different organic nitrogen sources such as yeast extract, malt extract, beef extract, casein peptone at 4.84 %. The 50 mL of autoclaved medium was inoculated with 3 % of mycelium suspension of and incubated for 24 h at 180 rpm at 30±2 ºC. Simultaneously, the effects different inorganic nitrogen sources such as sodium nitrate, ammonium sulphate, ammonium chloride at 4.84 % were also studied.

Media optimization by Response Surface method (RSM): A central composite rotatable design (CCRD) for three independent variables (glucose, yeast extract and magnesium sulphate) was used to obtain the combination of values that optimizes the response within the region of three dimensional observation spaces, which allows one to design a minimal no. of experiments. The experiments were designed using the software, Design Expert Version 6.0.10 trial version (StatEase, Minneapolis, MN). The medium components (independent variables) selected for the optimization were glucose, yeast extract, and magnesium sulphate. Regression analysis was performed on the data obtained from the design experiments. Coding of the variables was done according to the following Eq. 1

$$xi = (Xi - Xcp) / \Delta Xi, \quad i = 1, 2, 3, \ldots, k \qquad (1)$$

Where xi, dimensionless value of an independent variable; Xi, real value of an independent variable; Xcp, real value of an independent variable at the centre point; and ÄXi, step change of real value of the variable i corresponding to a variation of a unit for the dimensionless value of the variable i. The experiments were carried out at least in triplicate, which was necessary to estimate the variability of measurements, i.e. the repeatability of the phenomenon. Replicates at the centre of the domain in three blocks permit the checking of the absence of bias between several sets of experiments. The relationship of the independent variables and the response was calculated by the second order polynomial Eq. 2:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta i\, X_i + \sum_{i=1}^{k} \beta_{ii}\, X_i\, X_j + \sum i \sum j\, \beta_{ij}\, X_i\, X_j \qquad (2)$$

Where Y is the predicted response; β_0 a constant; β_i the linear coefficient; β_{ii} the squared coefficient; and β_{ij} the cross-product coefficient, k is no. of factors. The second order polynomial coefficients were calculated using the software package Design Expert Version 6.0.10 to estimate the responses of the dependent variable. Response surface plots were also obtained using Design Expert Version 6.0.10.

Effect of amino acids as a stimulator for GSH production: Effect of cysteine, glycine, glutamic acid, methionine, serine, tyrosine and lysine on GSH production was studied at different concentrations. To 50 mL of autoclaved medium

was inoculated with 3 % of mycelium suspension of and incubated for 24 h at 180 rpm at 30±2 ºC.

Analytical determinations

Determination of dry cell weight (DCW): Fermentation broth was centrifuged at 8000 rpm for 10 min and biomass was separated. Separated biomass was suspended in 25 mL, 40 % ethanol and kept for extraction for 2 h on a rotary shaker at 30±2 °C. The extract was again centrifuged at 8000 rpm for 15 min and GSH concentration in the supernatant was determined by alloxan method. Biomass obtained after centrifugation was dried on a preweighed filter paper at 100 °C to constant weight for determination of dry cell weight.

Estimation of GSH **:** GSH forms colored compound upon reaction with alloxan so the concentration of GSH can be determined by UV spectrophotometer, at 305 nm. One gram per liter alloxan was prepared in 0.1 M HCI solution. Glycine (0.1 M) and 0.24 M $NaHPO_4$–NaH_2PO_4 buffer (pH 7.6) were prepared in deionized water. The standard curve was prepared accordingly using standard GSH. Each of the standards was added to a cuvette containing 3.5 mL 0.24 M $NaHPO_4$–NaH_2PO_4 buffer (pH 7.6) and 0.5 mL 0.1 M glycine. The reaction was started by addition of 1 mL alloxan solutions and the durations of the reactions were 20 min. Similar reaction was carried out for extract of GSH from fermentation broth, replacing standard GSH *(17)*.

Purification of GSH

Aqueous two phase system **(ATPS):** *Effect of PEG molecular weight, PEG concentration and ammonium sulphate concentration:* Four different ATPS's systems were studied to find out effect of molecular weight of PEG (1500, 4000, 6000, and 8000), 20 %; ammonium sulphate, 10 %; cell extract, 10 % and deionized water, 60 %. Similarly, to study the effect of PEG 6000 concentration on partitioning of GSH and proteins five different systems were prepared each containing PEG 6000 at different concentration (5 %, 10 %, 15 %, 20 %, and 25 %), ammonium sulphate 10 %, cell extract 10 % and concentration of deionized water was adjusted to make final volume to 100 %. To find out the effect of ammonium sulphate concentration, four different systems were prepared each containing ammonium sulphate at different concentration (10 %, 12 %, 15 %, 20 %), PEG 6000 20 %, cell extract 10 % and concentration of deionized water was adjusted to make the final volume to 100 %.

In all above mention cases, entire system was mixed and phases dispersed by vortex mixer for 1 min at 30±2 °C. Phases were allowed to separate at 30±2 °C for 12 h. Visual estimates of the volumes of top and bottom phases were made. Samples were carefully extracted from the phases and analyzed for GSH content by alloxan method and for protein content by Bradford method. Kd was calculated for each system by the formula given below:

$$Kd = \frac{\text{Concentration of GSH in top phase}}{\text{Concentration of GSH in lower phase}} \quad (3)$$

Ion exchange chromatography

Selection of optimal binding pH for GSH using different resins: The optimum binding pH for GSH on different selected resins like Indion CAM-I, Amberlite XAD-16, Indion 830, Amberlite IR 120H was determined. Based on this, a suitable ion exchange resin was selected for further study. The resin (0.5 mL) was added to each of the tubes, and equilibrated to different pH *viz.*, 3, 4, 5, 6, 7 and 8 by washing twice with equilibration buffer (5 mL of equilibration buffer was added to each tube, and kept for 2 h on rocker shaker). Glycine-HCl buffer (100 mM) was used for pH 3, acetate buffer (100 mM) was used for pH 4 and 5, and phosphate buffer (100 mM) was used for pH 6. In each tube, 2 mL of sample

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was added and were kept for 2 h on rocker shaker for equilibration. The resin was allowed to settle and the supernatant was used to quantify for GSH content. The resin was washed twice with the equilibration buffer. Adsorbed GSH was calculated by subtracting amt obtained in supernatant from the initial sample of the same pH. Thus, bound percentage was calculated using the following formula.

$$\% \text{ BOUND} = \frac{\text{Total GSH content of bound}}{\text{Total GSH content in loaded}} \times 100 \quad (4)$$

The pH with maximum binding was binding pH, and that with least binding can be used as elution pH. The resins were regenerated with 0.5 M NaOH and reused.

Determination of static binding capacity and further purification by column chromatography: Different dilutions of the filtered sample containing different concentrations of GSH (0.5 to 3.5 mg/mL) were prepared using phosphate buffer of pH 7. These dilutions were loaded to 0.5 mL of Amberlite IR 120 H previously equilibrated with binding buffer at 25±2 °C) and kept for equilibration. After equilibration it was allowed to settle; the supernatant was removed and quantified for GSH. The isotherms obtained by plotting concentrations of GSH adsorbed (q*) as (µg/mL of resin) *vs* corresponding equilibrium concentration of GSH in the supernatant (C*) (*i.e.* unadsorbed concentration; µg/mL), this signify the nature of adsorption. The amt of GSH bound to the adsorbent q* was calculated as the difference between the total amt of GSH loaded and that present in the supernatant after 2 h of equilibration. Maximum adsorption capacity q_{max} was determined from the plot of q* *vs* C* (isotherm) and the type of isotherm was found by plotting a graph of 1/q* *vs* 1/C*.

$$\frac{1}{q^*} = \frac{k_d}{q_{max}} \times \frac{1}{C^*} + \frac{1}{q_{max}}$$

Adsorption isotherm equation:

$$q^* = \frac{q_{max}\, C^*}{(k_d + C^*)} \quad (5)$$

Where k_d = Langmuir isotherm constant.
Rearranging Eq. (5), we get,

(6)

Value of k_d can be determined from straight-line plot of 1/q* against 1/C*. The intercept of such plots on the 1/q* axis is at $1/q_{max}$ and slope is k_d/q_{max}.

A batch study was carried out on strong cation exchange resin Amberlite IR 120H packed in column of diameter 1.1 cm, with bed height 5.7 mL. The headspace of the column was filled with buffer completely to avoid any air gap and then equilibrated with acetate buffer (pH 4.0). Supernatant obtained after ammonium sulphate precipitation was mixed with buffer and loaded on the column until exhaustion point. Concentration of GSH to be loaded was calculated according to static binding capacity of the resin. The pH of the sample was kept at 4 to ensure binding of the GSH to the matrix. Volumetric flow rate of 0.5 mL /min was maintained by peristaltic pump. Washing with same equilibrating buffer to remove the unbound or weakly bound was done.

Three different elution strategies were employed to elute the adsorbed GSH from Amberlite IR 120H resin, as elution with 1.5 M NaCl, elution by changing the buffer pH and elution with 1 %. Each time volumetric flow rate was maintained at 0.5 mL/min and 3 mL fractions were collected at a time and analyzed for GSH concentration by alloxan method.

Results and Discussion

Media optimization by one factor at-a-time method

Effects of pH: At an initial pH 6.0, maximum production of GSH, 62.18 mg/L (biomass, 5.55

g/L) was observed. It was found that GSH production along with biomass concentration was lesser than all pH values other than 6.0; hence pH was found to be a significant factor for GSH production. The results are found in accordance with Santos *et al.* (18). They reported maximum production of GSH, 60.5 mg/L (after 24 h) at an initial pH of 6.0 for using *S. cerevisiae* ATCC 7754.

Effects of carbon sources: As during fermentation process, carbon source plays dual characteristic role, by acting as major constituent for building of cellular material and as an energy source (19, 20). Fig. 1 shows the effect of different carbon sources on GSH production. None of the carbon sources increased the production further. Glucose as a sole carbon

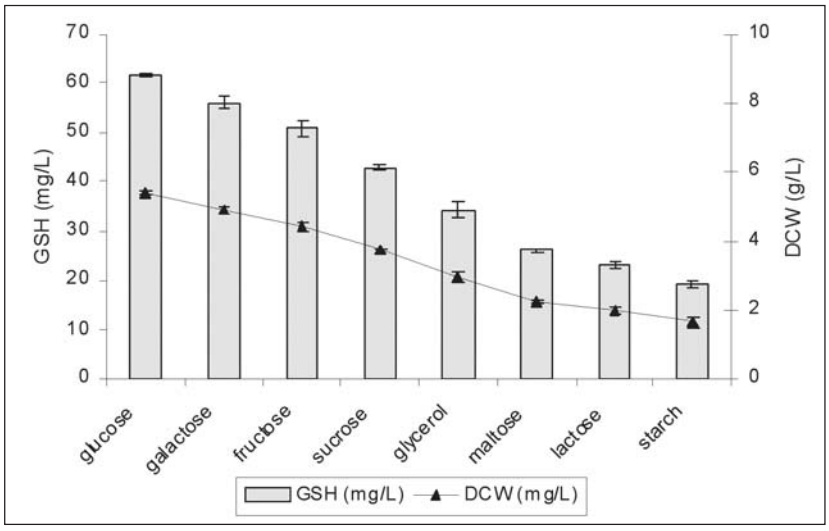

Fig. 1. Effect of carbon sources

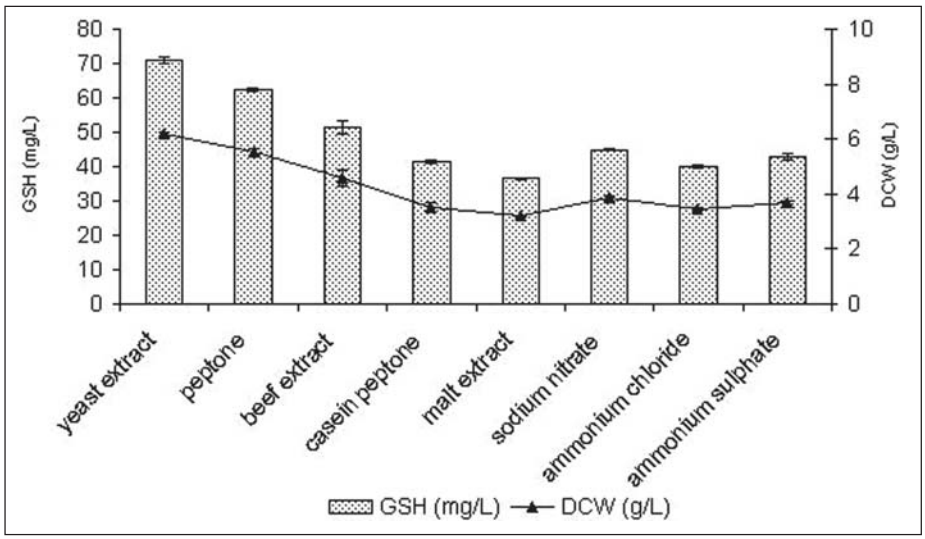

Fig. 2. Effect of nitrogen sources

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source supported the maximum GSH production 61.87 mg/L (biomass, 5.39 g/L). Santos *et al.* (18) reported glucose as a sole carbon source for maximum production of GSH (154.5 mg/L) using *S. cerevisiae* ATCC 7754. Liu *et al*. (14) and Cha *et al. (21)* screened various carbon sources for production of GSH using *S. cerevisiae* ATCC 7754 and *S. cerevisiae* FF-8 respectively, found glucose as the best carbon source for maximum GSH production as 115.28 mg/L and 204 mg/L respectively.

Effects of nitrogen sources **:** The effects of nitrogen sources on GSH production and cell growth by *S. cerevisiae* NCIM 3454 were shown in Fig. 2. Yeast extract supported maximum GSH production, 70.96 mg/L (biomass, 6.18 g/L) and followed by peptone, 62.32 mg/L (biomass, 5.54 g/L). Hence, yeast extract was chosen as a nitrogen source for further studies. Sodium nitrate was found to be the best among inorganic nitrogen sources screened with GSH production, 44.9 mg/L (biomass, 3.86 g/L). Previously, Cha *et al.* (21) studied the effect of various organic and inorganic nitrogen sources on GSH production using *S. cerevisiae* FF-8. Maximum GSH production of (204 mg/L) was found in presence of 3 % yeast extract as nitrogen source followed by tryptone (67.4 mg/L). Liu *et al.* (14) reported peptone (4.84 %) to be the best nitrogen source for GSH production (115.28 mg/L) using *S. cerevisiae* ATCC 7754. Rollini & Manzoni (22) studied the effect of different fermentation parameters on GSH volumetric productivity by *S. cerevisiae* CBS 1171 and reported ammonium sulphate to be best nitrogen source for GSH volumetric productivity.

Growth curve and production profile: The growth curve and production profile of the GSH using *S. cerevisiae* NCIM 3454 were carried out with respect to time (Fig. 3). The production of the GSH was observed from 6 h of fermentation (20.66 mg/L) and reached a maximum at 48 h (128.28 mg/L). Dry cell weight (DCW) was also found to be maximum (10.78 g/L) at the end of 48 h. GSH production and biomass concentration did not change at 54 h and 60 h indicating that the organism might have reached the death phase.

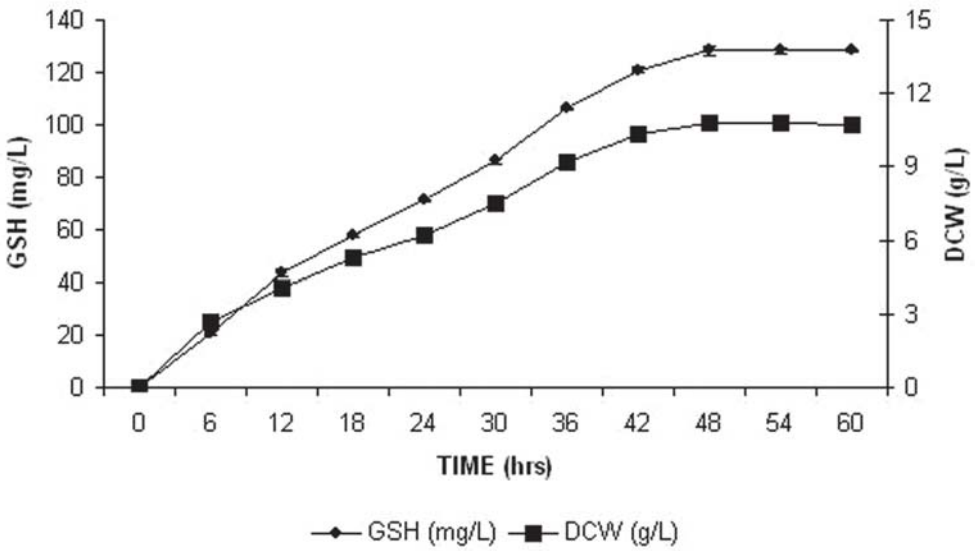


Fig. 3. Growth curve and production profile of GSH

Media optimization by Response surface methodology (RSM): The Central composite rotatable design (CCRD) gave quadratic model for the given set of experimental results. Eq. 8 represents the mathematical model relating the production of GSH with the independent process variables and the second order polynomial coefficient for each term of the equation determined through multiple regression analysis using the Design Expert. The coded values, a CCRD matrix of independent variables along with responses of each experimental trial is given in Table 1. The results were analyzed by using ANOVA i.e. analysis of variance suitable for the experimental design used, and cited in Table 2. The ANOVA of the quadratic model indicates that the model is significant. The Model F-value of 270.75 implies the model to be significant and is calculated as ratio of mean square regression and mean square residual. Model P-value (Prob > F) is very low (< 0.0500), again signifying the model to be significant.

The *P* values were used as a tool to check the significance of each of the coefficients, which, in turn are necessary to understand the pattern

Table 1. The CCRD matrix of independent variables in coded form and actual values with their corresponding response in terms of production of glutathione by *S. cerevisiae* NCIM 3454

Sr. No.	Glucose (%)	Yeast extract (%)	$MgSO_4$ (%)	GSH (mg/L)
1	-1 (3.0)	-1 (3.0)	-1 (1.0)	85.780 ± 0.95
2	1 (9.0)	-1 (3.0)	-1 (1.0)	48.220 ± 1.02
3	-1 (3.0)	1 (9.0)	-1 (1.0)	96.630 ± 1.10
4	1 (9.0)	1 (9.0)	-1 (1.0)	110.25 ± 1.21
5	-1 (3.0)	-1 (3.0)	1 (2.0)	79.350 ± 0.99
6	1 (9.0)	-1 (3.0)	1 (2.0)	69.240 ± 1.41
7	-1 (3.0)	1 (9.0)	1 (2.0)	75.320 ± 1.31
8	1 (9.0)	1 (9.0)	1 (2.0)	105.21 ± 0.82
9	0 (6.0)	0 (6.0)	0 (1.5)	137.63 ± 1.10
10	0 (6.0)	0 (6.0)	0 (1.5)	137.32 ± 1.11
11	0 (6.0)	-0 (6.0)	0 (1.5)	138.54 ± 0.71
12	0 (6.0)	0 (6.0)	0 (1.5)	138.24 ± 0.77
13	-1.68 (0.95)	0 (6.0)	0 (1.5)	74.200 ± 1.62
14	1.68 (11.05)	0 (6.0)	0 (1.5)	70.980 ± 0.88
15	0 (6.0)	-1.68 (0.95)	0 (1.5)	42.170 ± 0.94
16	0 (6.0)	1.68 (11.50)	0 (1.5)	88.510 ± 0.32
17	0 (6.0)	0 (6.0)	-1.68 (0.66)	150.91 ± 1.02
18	0 (6.0)	0 (6.0)	1.68 (2.34)	139.32 ± 0.74
19	0 (6.0)	0 (6.0)	0 (1.5)	137.21 ± 0.83
20	0 (6.0)	0 (6.0)	0 (1.5)	139.77 ± 0.34

[a] Results are mean ± SD of three determinations
Values in the parenthesis indicate the real values of variables

of the mutual interactions between the test variables. The *F* value and the corresponding *P* values, along with the coefficient estimate, are given in Table 2. The smaller the magnitude of the *P*, the more significant is the corresponding coefficient. Values of *P* less than 0.050 indicate the model terms to be significant. The coefficient estimates and the corresponding *P* values suggests that, among the test variables used in the study, B, C, A^2, B^2, AB, AC and BC (where A = glucose, B = yeast extract, C = $MgSO_4$) are significant model terms. B, A^2, B^2 and AB ($P < 0.0001$) have the largest effect on GSH production. Other intearctions were found to be insignificant.

The corresponding second-order response model for Eq. (7) that was found after analysis for the regression was:

GSH (mg/L) = 139.13 – (0.70 x glucose) + (13.38 x yeast extract) – (2.29 x magnesium sulphate) – (24.94 x glucose^2) – (27.50 x yeast extract^2) + (0.71 x magnesium sulphate^2) + (11.40 x glucose x yeast extract) + (5.47 x glucose x magnesium sulphate) – (5.12 x yeast extract x magnesium sulphate) (7)

The fit of the model was also expressed by the coefficient of regression (r^2), which was found to be 0.996, indicating that 99.6 % of the confidence level of the model to predict the response (GSH yield). The "Pred R-Squared" of 0.975 is in reasonable agreement with the "Adj R-Squared" of 0.992. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Here, the ratio of 47.095 indicates an adequate signal. The special features of the RSM tool, "contour plot generation" and "point prediction" were also studied to find optimum value of the combination of the three media constitutes for the maximum production of GSH. These predicted values were experimentally verified. Table 3 documents the predicted and experimental yields of GSH by various media combination. It was observed that medium containing (%), glucose 5.67, yeast extract, 7.13 and magnesium sulphate, 0.66 yielded maximum (148.45 mg/L) GSH.

Table 2. Analysis of variance (ANOVA) for the experimental results of the central composite design (Quadratic Model)

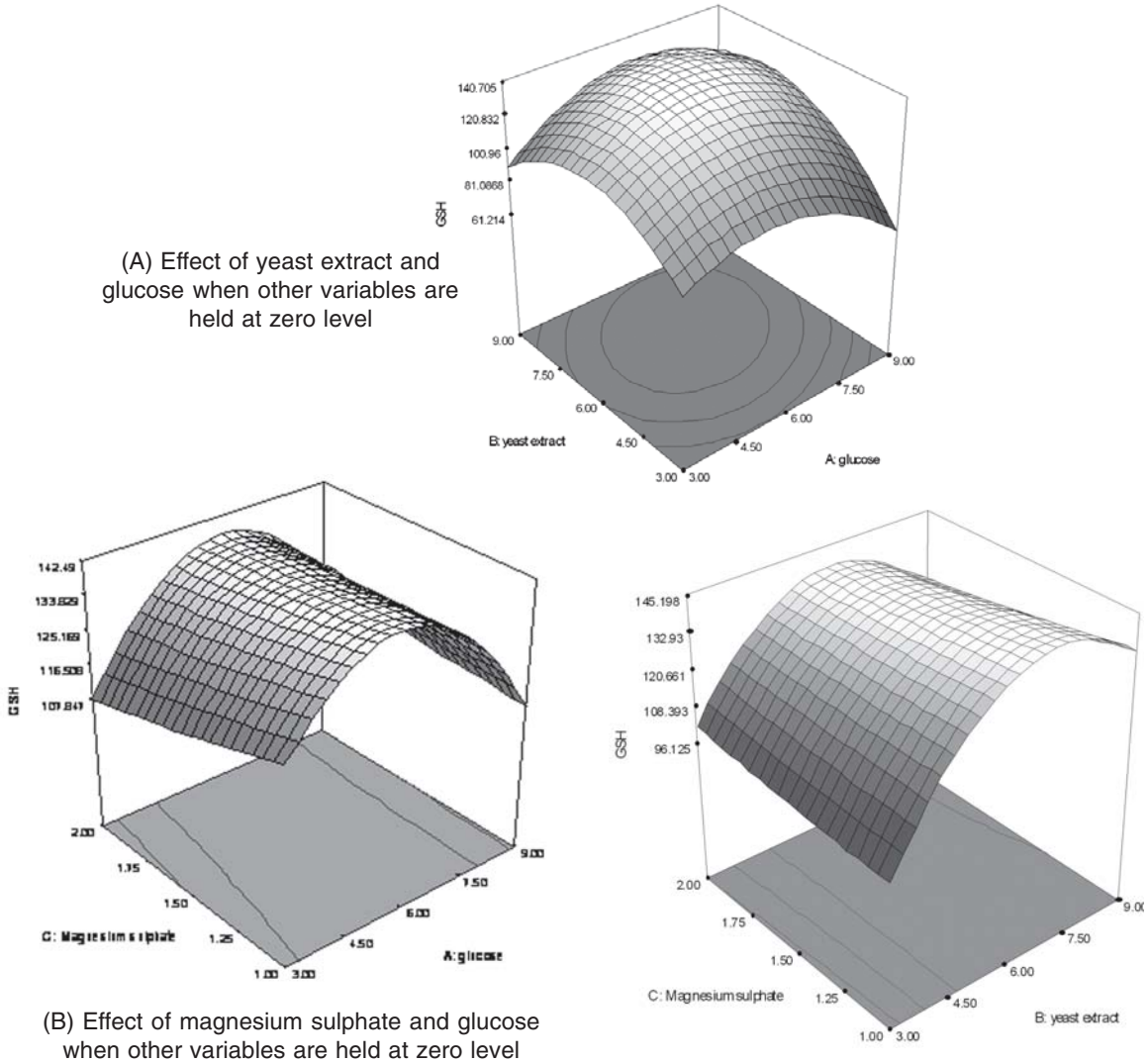
Factor[a]	Estimate Coefficient	Sum of squares	Standard Error	DF[b]	F value	p
Intercept	139.13	61.28	1.25	1	270.75	< 0.0001
A	-0.70	6.71	0.82	1	0.73	0.4159
B	13.38	2445.59	0.82	1	264.87	< 0.0001
C	-2.29	71.52	0.82	1	7.75	0.0213
A^2	-24.94	8953.57	0.80	1	969.70	< 0.0001
B^2	-27.50	10888.97	0.80	1	1179.31	< 0.0001
C^2	0.71	7.18	0.80	1	0.78	0.4009
AB	11.40	1039.22	1.07	1	112.55	< 0.0001
AC	5.47	238.93	1.07	1	25.88	0.0007
BC	-5.12	209.51	1.07	1	22.69	0.0010

[a] A = glucose, B = yeast extract, C = $MgSO_4$
[b] Degree of freedom, $p < 0.05$ (models are significant), $R^2 = 0.99$

Table 3. Predicted and experimental yields of GSH by validation

Sr. No	Optimum concentrations (%)			GSH (mg/L)	
	Glucose	Yeast extract	$MgSO_4$	Predicted	Experimentally verified[a]
1	5.67	7.13	0.66	149.65	148.45 ± 1.031
2	5.68	7.14	0.66	149.65	148.38 ± 1.113
3	5.73	7.19	0.66	149.65	147.92 ± 1.091

[a] Results are mean ± SD of at least three determinations



(A) Effect of yeast extract and glucose when other variables are held at zero level

(B) Effect of magnesium sulphate and glucose when other variables are held at zero level

(C) Effect of magnesium sulphate and yeast extract when other variables are held at zero level

Fig. 4. Contour plot and surface plot for GSH

The three-dimensional graphs were generated for the possible combination of the three factors. Graphs for interactions are revealed here to highlight the roles played by these factors (Fig. 4). From the central point of the contour plot the optimal process parameters were identified.

Effect of amino acids as a stimulator for GSH production: The effect of cysteine on GSH production was shown in Fig. 5. GSH production increased to 163.12 mg/L with the addition of 3 mM cysteine simultaneously biomass concentration was found to be decreased with cysteine addition. The inhibition could be through metal chelation of specific enzymes concerned with carbohydrate metabolism. Alfafara *et al.* (23) studied the effect of amino acids on GSH production and reported cysteine as the key amino acid for GSH production. Fig. 6 shows the effect of glycine, glutamic acid, methionine, serine, tyrosine and lysine on GSH production. Glycine, glutamic acid, and methionine increased

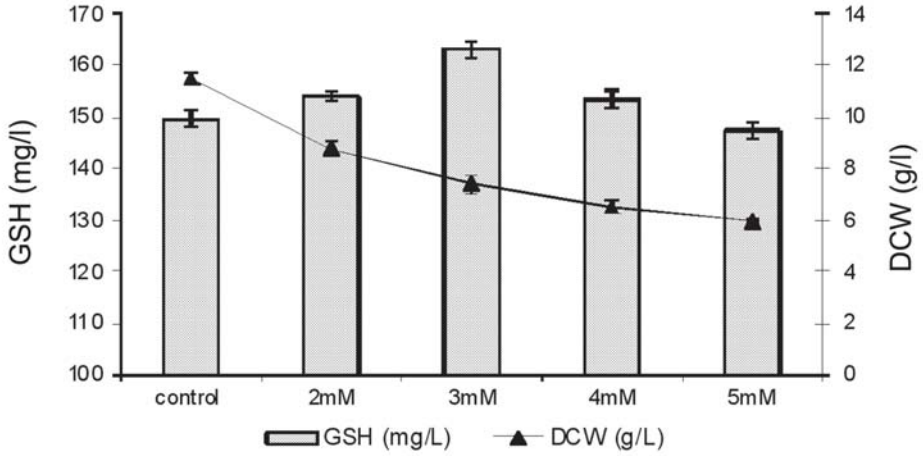


Fig. 5. Effect of cysteine on GSH production

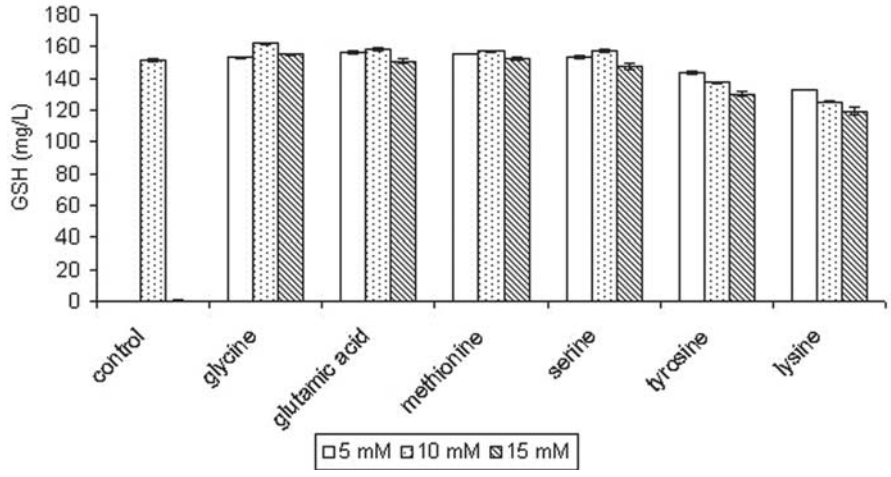


Fig. 6. Effect of amino acids on GSH production

GSH production when added at concentration of 10 mM. Methionine is a precursor for cysteine biosynthesis and hence could have a positive effect on GSH production. Tyrosine and lysine showed inhibitory effect on intracellular GSH content. Wen *et al.* (24) studied utilization of amino acids to enhance GSH production in *S. cerevisiae* T65 and reported cysteine to be the most important amino acid, which increased intracellular GSH content greatly but inhibited cell growth at the same time. Methionine, glycine, serine, and glutamic acid were also reported to have positive effect on GSH production. Cha *et al.* (21) observed similar effect amino acid addition on GSH production using *S. cerevisiae* FF-8.

Purification of GSH

Aqueous Two Phase System : *Effect of PEG molecular weight, PEG concentration and ammonium sulphate concentration:*

The effect of molecular weight of PEG on partitioning of GSH and protein was studied. The partitioning of GSH in top phase increased as molecular weight of PEG was increased. Maximum Kd (3.66) was achieved with PEG 6000 followed by with PEG 1500 (2.48) and PEG 4000 (2.85). With PEG 8000 Kd value was 2.27

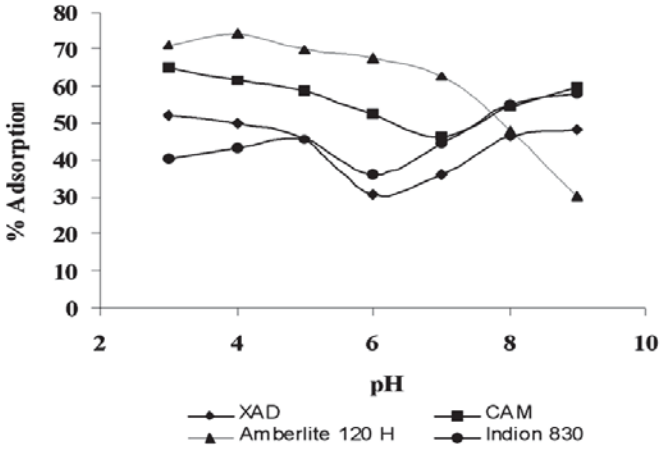


Fig. 7. Selection of optimum binding pH for GSH on different resins

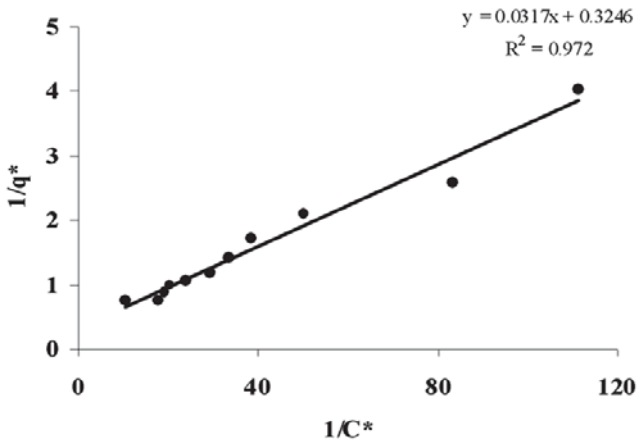


Fig. 8. Langmuir adsorption isotherm of GSH using Amberlite IR 120H

indicating that GSH was partitioned in both phases. Also percent protein in top phase was 38.23 % indicating that remaining proteins were in bottom phase with PEG 8000. Total percent protein in top phase was found to be (53.48 %) with PEG 8000 which was undesirable. Results suggested that with use of PEG 6000 around 61.87 % unwanted proteins could be separated from GSH cell extract in a single step. Hence, PEG 6000 was selected for further studies.

The effect of concentration of PEG on partitioning of GSH and protein was further taken in to consideration. As partitioning of GSH in top phase increased as concentration of PEG was increased. Maximum Kd (3.55) was achieved at PEG 6000 concentration of 20 % which decreased to 3.1 with further increase in PEG concentration. This could be due to increased viscosity of the system at high PEG concentration which in turn causes resistance to mass transfer. The percent protein in top phase was found minimum (38.36 %). The effect of ammonium sulphate concentration on partitioning of GSH and protein was also studied. At 12 % ammonium sulphate concentration maximum Kd (3.7) was achieved. The percent proteins in top phase were 38.5 % indicating that remaining were separated from GSH cell extract. At low salt concentrations percent proteins in top phase were less which may be due to solubilization of proteins at low salt concentration (salting in effect). At high salt concentration percent protein in top phase were found to be increased which may be because of the fact that proteins tend to precipitate and are retained in upper phase.

Ion exchange chromatography

Selection of optimal binding pH for GSH on different resins: –The percentages of GSH bound on various matrices at different pH are shown in Fig. 7. The adsorption of GSH was found to be maximum on Amberlite IR 120H (at pH 4.0, 74.39 %), Indion CAM (at pH 3.0, 65.34 %), Amberlite XAD-16 (at pH 3.0, 52.14 %) and Indion 830 (at pH 5.0, 45.60 %). Since maximum binding was on Amberlite IR 120H resin at pH 4.0 it was selected for further studies.

Determination of static binding capacity and further purification by column chromatography: Equilibrium adsorption isotherm was studied to find out adsorption isotherm pattern for purification of GSH using Amberlite IR 120H resin. The adsorption isotherm data was used to determine the adsorption capacity of the matrix. The GSH adsorption followed a typical Langmuir type of isotherm as shown in Fig. 8 for Amberlite IR 120H resin. The maximum capacity of the matrix (q_{max}) for GSH was found to be 3.081 mg/mL. A plot of 1/q* Vs 1/C* (Fig. 9) gave a linear correlation confirming the adsorption to be of the Langmuir type. Lower elution efficiency with 1.5 M NaCl (8.64 %) and 100 mM phosphate buffer of pH 8 (5.91 %) was observed (Table 5). Elution with 1 % H_2SO_4 showed the best elution pattern. Percent elution was very high (89.19 %) as compared to salt and pH elution.

Conclusions

Optimization of the fermentation medium could increase the GSH production by *S. cerevisiae* NCIM 3454 from 55.28 mg/L to 148.45 mg/L. L-cysteine acted as stimulator in production of glutathione (163.12 mg/L). Aqueous two phase system was found to be good alternative during purification of GSH prior to adsorption chromatography. System containing PEG 20 %, and ammonium sulphate 12 % gave maximum protein recovery of GSH in top phase and separating maximum proteins in bottom phase. Amberlite IR 120H showed maximum GSH adsorption (74.39 %) at pH 4 and maximum elution was achieved with 1 % H_2SO_4.

Acknowledgement

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NEWS ITEM

Expansion of education and research infrastructure need of the hour: President Pratibha Patil

The expansion of country's education and research infrastructure requires an immediate consideration, according to President Pratibha Patil at present there are nearly 3,500 engineering colleges in India with over 4.5 lakh students graduating every year, but the capacity was falling short of the requirement. She said that we need an expansion of our education and research base. Also Smt. Patil added that Research generates new knowledge, but its ultimate usefulness lies in how extensively it is used and the number of people it benefits. From the invention of the wheel to the WiFi mode of communication, from the construction of simple dwelling units to high-rise buildings to highways, metro-systems and satellites - the role of technology and innovation has become even more critical in the 21st century as we live in a knowledge era.

Education system in India needs revision: Kapil Sibal

Speaking at the higher education conclave organized by the Indian Chamber of Commerce, Kapil Sibal Minister of Human Resource Development, stressed upon ways to improve the existing academic patterns. He said that most of our Universities are not forward-looking and follow archaic academic systems and policies because their highest decision making bodies - the academic and executive councils - are run by people with vested interests. Sibal told that the statutes and ordinances in most Universities have outlived their utility and need revision. The academic and executive councils comprise people who are often politically motivated and do not want such changes. He criticized the dichotomy in the policies of Universities across the country, causing a severe disparity in levels of learning and elucidated on the plan of a single engineering entrance exam for the entire country, which stressed the need for revision of present education system.

Research is the key: Abdul Kalam

Former President of India APJ Abdul Kalam spoke about the need for medical educational institutes to focus on research at the 14th annual convocation of the Rajiv Gandhi University of Health Sciences (RGUHS) and he said that, "Research and teaching are the focus of medical education in India in the present century and that Institutes should work towards promoting research and teaching research." He added that universities are judged on the quality of research being conducted by the students and teachers. The former president also emphasised on the importance of medical students and practitioners to be involved in rural India and that all the MBBS and MD doctors should work in primary health centres, as it directly connects the rural population. He also mapped out several areas in medicine in need of dire attention from the graduates. "We need to look at eliminating tuberculosis and also ensure that there is no occurrence of HIV-Aids. People should be educated in preventive care when it comes to HIV-Aids and hepatitis," he also said, adding that work needed to be done to ensure that medicines in the country were affordable.

India to be as friend with China and US: Prime Minister

Prime Minister Manmohan Singh said that, Large and dynamic countries like China cannot be contained, making it clear that India aims to have cooperative ties with both China and the US. Dr. Singh said China was India's largest neighbour, sharing a long border and was also our biggest trading partner in goods. With US, he said, India's relations were transformed in 2005 which laid the framework for the India-US civil nuclear agreement. Manmohan Singh also said that three million people of Indian origin live and work in US and that the country is also India's largest business partner. Dr. Singh about his meeting with the South Korean president said that the two sides will talk about giving depth and greater meaning to the Comprehensive Economic Partnership Agreement, strengthen people-to-people contacts, boost cooperation in science and technology, and coordinate thinking in matters relating to security and global events.

SCIENTIFIC NEWS

Powerful New Stem Cells Cloned from Cheek Tissue

Cloned oral progenitor cells (PCs) isolated from cheek tissue were demonstrated to be potently immunosuppressive in a dose- and contact-independent manner. Oral mucosal lamina propria progenitor cells (OMLP-PCs) are a novel, clonally derived PC population of neural crest origin with the potential to differentiate down both mesenchymal and

neuronal cell lineages. According to the findings of School of Dentistry, Cardiff University, the OMLP-PC line was derived from cells taken from the inside lining of cheeks of patients undergoing routine dental procedures. Tests showed that even small doses of the cells could completely inhibit lymphocytes. Complete inhibition of lymphocyte proliferation was seen at doses as low as 0.001% OMLP-PCs to responder lymphocytes, while annexin V staining confirmed that this immunosuppressive effect and was not due to the induction of lymphocyte apoptosis. The findings of the study, demonstrates for the first time that OMLP-PC immunomodulation, unlike that for mesenchymal stem cells, occurs via a dose- and HLA II-independent mechanism by the release of immunosuppressive soluble factors. The results suggest that OMLP-PCs are suitable candidates for allogenic tissue engineering and may have wide-ranging potential for immune-related therapies, such as to prevent rejection of transplanted organs and to treat the form of diabetes resulting from lymphocyte attack of insulin-producing cells., These cells are extremely powerful and offer promise for combating a number of diseases where we just harvest a small biopsy from inside the mouth.

Y. Harini

Modified Protein May Reduce Heart Attack Damage

Scientists at Department of Pathology and Lab Medicine at the University of North Carolina modified a protein in the heart that greatly reduced cell damage after heart attacks, according to new research. The modified protein reduced cell damage by 50% in mice without causing damaging inflammation. These findings came during research looking at ways to prevent heart failure induced by heart attacks. The protein is called focal adhesion kinase (FAK). It organizes cell structure by triggering various processes that help the cells stay alive. FAK is important for basic processes in all cells, and it appears to be important for cell survival, growth and migration in a number of cell types, but is especially critical in the heart. In mice with the new SuperFAK gene, researchers saw a massive activation of FAK after heart attack, and not as many heart cells died compared to unmodified mice. Three days after the induced heart attack, the SuperFAK mice had approximately 50% less heart injury than the unaltered mice. This benefit was maintained for eight weeks. FAK also plays a role in the development of a number of cancers and it has been associated with tumor growth and metastases. Some chemotherapy agents hinder FAK, leaving the heart susceptible to damage. Progressive cardiac damage is a typical adverse effect of several chemotherapeutic treatments. In the future, the investigators hope to develop drugs to target FAK to protect the heart during chemotherapy or following a heart attack.

J. Aruna Kumari

The Immune System Reacts More Vigorously to Hydrophobic Nanoparticles

A recent study showed that the degree to which the immune system reacts to foreign nanoparticles is directly related to how hydrophobic the particles are. Understanding the interactions of nanomaterials with the immune system is essential for the engineering of new macromolecular drug or vaccine delivery systems. Investigators at Tel Aviv University Israel fabricated pure gold nanoparticles that were two nanometers in diameter. In the unmodified state, the nanoparticles were not recognized by immune cells. Thus, the effect of any change in the surface of the particles could be detected. The investigators manufactured a series of gold nanoparticles that presented a linear increase in hydrophobicity. These particles were then added to cultures of immune spleen cells (splenocytes) and injected into mice. Results revealed that increased hydrophobicity of the nanoparticle headgroups, caused a linear increase in immune activity in the cell cultures. Consistent behavior was observed with *in vivo* mouse models, demonstrating the importance of hydrophobicity in immune system activation.

Ch.Parimala

EDUCATION

PhD/Post Doctoral Programs

Admission to Ph.D. Programme for 2012 - 2013: Applications are invited for admission to Ph.D programmes in National Institute of Plant Genome Research, New Delhi-110 067, India in the frontier areas of Plant Biology such as, Computational Biology, Genome Analysis and Molecular Mapping, Molecular Mechanism of Abiotic Stress Responses, Nutritional Genomics, Plant Development and Architecture, Plant Immunity, Transgenics for Crop improvement, Molecular Breeding and other emerging areas based on plant genomics. The selection is made on the basis of interview only. The selected candidates will be enrolled for Ph.D. program in academic affiliation with the Jawaharlal Nehru University, New Delhi. NIPGR will provide shared

accommodation to selected candidates. The project consists in the detailed analysis and characterization of changes of gene expression in primates due to structural variation such as duplications or inversions. Candidates who have completed or will be completing M. Sc. or equivalent degree (by August 31, 2012) in Life Sciences and/or in related disciplines with minimum 55% marks, and have cleared the CSIR-UGC NET exam / DBT-JRF (selected through BET/ BINC) / ICMR NET exam for JRF (with a valid Junior Research Fellowship as on September 1, 2012) are eligible. Application should be typed on A-4 size plain paper in the format given (Form can be downloaded from website www.nipgr.res.in). Completed application must be accompanied with a crossed Demand Draft for 300/- in favor of Director, National Institute of Plant Genome Research, payable at New Delhi. A demand draft of 100/- is acceptable from SC/ ST candidate provided relevant documentary proof is enclosed. Application along with Demand Draft should be sent to "The Director, National Institute of Plant Genome Research, Aruna Asaf Ali Marg, Post Box No. 10531, New Delhi-110 067" so as to reach on or before May 25, 2012.

OPPORTUNITIES

Post Doctoral Applied Bioinformatics Programme: Applications are invited for admission to Postdoc in Applied Bioinformatics / Biostatistics for medical research programme at Swiss Institute of Bioinformatics, Genopode, Lausanne, Switzerland in biomolecular data mining with high throughput data. The candidate should have enthusiastic interest for research at the crossroads of experimental and computation methods and of basis and clinical research and have excellent interpersonal skills for working within a team of multiple disciplines (medicine, molecular biology, modern profiling technologies, computer sciences, statistics), strong team attitude. He should have proficiency in the application of bioinformatics and statistics methods, in particular use of the R software and Bioconductor data analysis packages and should have completed Ph D (Statistics, Computer Science, Engineering, Mathematics, Physics, Bioinformatics, Biology) or equivalent title and research experience. The application should include a cover letter, with summary of motivation, research interests, publications and past accomplishments; curriculum vitae including description of skills and experiences; names and email addresses of at least two referees. Eligible candidates can send applications to Dr. Mauro Delorenzi, Head BCF, Swiss Institute of Bioinformatics (SIB), Quartier Sorge - Batiment Genopode, CH-1015 Lausanne Mauro.delorenzi@isb sib.ch, http://bcf.isb-sib.ch.

Department of Biochemistry, PGIMER, Chandigarh, India: Applications are invited from eligible candidates for One position of Research Assistant in the research project "Identification and molecular characterization of cystic fibrosis transmembrane conductance regulator mutations and microdeletions in human Y chromosome AZF candidate gene in male in fertility: Diagnostic and genetic implications." funded by ICMR, New Delhi and One post of Junior Research Fellow in the research project "Identification and molecular characterization of spectrum of mutations in congenital adrenal hyperplasia (CYP2 1) gene from CAH patients: Diagnostic & genetic implications" funded by DST, New Delhi on consolidated fellowship of Rs.19,482/- per month for Research Assistant and fellowship of Rs. 16,000 pm + HRA @ 20% for JRF. Candidates with M.Sc. Biochemistry/Biophysics/Biotechnology/ Human genetics with First Division and those who had qualified NET/ICMR/GATE are eligible. Eligible candidates may apply on plain paper to Dr. Rajendra Prasad, Professor, Department of Biochemistry, PGIMER, Chandigarh and appear for Interview on April 16, 2012 along with the duly filled in application form supported by Bio-data and one set of attested photo copies of Certificates of educational qualification, age, experience, caste (in case of SC/ ST/OBC candidates), latest passport size photograph.

Indian Council of Medical Research, Ansari Nagar, New Delhi – 110029, India. Applications are invited from eligible candidates for the posts of Research Scientists II Research Associate and SRF at the Medicinal Plants Unit, ICMR Headquarters, New Delhi. For the post of Research Scientists II candidates with Ph.D. in Botany / Chemistry / Pharmacy with three years of research experience are eligible. For the post of Research Associate candidates with M.D / Ph.D. in Pharmacology / Chemistry / Pharmaceutical Sciences / Botany with are eligible. For the post of SRF candidates with M.Sc. in Botany / Chemistry / Medicinal plants / Pharmacology with two years of research experience or M.Pharma are eligible. The interested candidates possessing the above qualifications along with six copies of biodata for each position, certificates and

testimonials in original and one attested copy of the same may report for the interview to the Head, Medicinal Plants unit. Date of Interview: May 7th 2012.

National Institute Of Animal Biotechnology (NIAB), Hyderabad, India. Applications are invited from eligible candidates for the Scientific Positions of Scientist – H (1 Post), Scientist – G (1 Post), Scientist – F (2 Posts), Scientist –E (2 Posts), Scientist – D (2 Posts), Scientist C/B (4 Posts) on regular basis in the priority areas like Genetic Epidemiology, Bioinformatics, Transgenic Technology and Infectious Diseases at the National Institute Of Animal Biotechnology (NIAB), Visiting Scholars House, Lake View Guest House, University of Hyderabad Campus, Prof. C.R. Rao Road, Gachibowli, Hyderabad-500 046, Andhra Pradesh, India. For further details visit the website at: www.niab.org.in; Tel: +91 40 2301 2425; Telefax: +91 40 2301 0745.

SEMINARS/WORKSHOPS/CONFERENCES

Biodiversity Asia 2012: Science, Policy, and Governance: An International conference on "Biodiversity Asia 2012: Science, Policy, and Governance" was going to be held on August 7-10, 2012 at JN Tata Auditorium, Indian Institute of Science (IISc) Campus, Bengaluru (Bangalore), India organized by Society for Conservation Biology-Asia Section, (SCB-Asia), Ashoka Trust for Research in Ecology and the Environment (ATREE), Indian Institute of Science (IISc), Bengaluru (Bangalore), India. Abstract can be submitted online through E-mail: SCBasiasecretariat @atree.org on or before May 15th, 2012. For further details contact: Secretary, Biodiversity Asia 2012, Ashoka Trust for Research in Ecology and the Environment (ATREE), Royal Enclave, Srirampura, Jakkur PO, Bangalore 560064, India. Email: SCBasiasecretariat@atree.org. Phone: +91-80-23635555.

International Conference on Recent Trends in Atherosclerosis and 25th Annual Conference of The Indian Society for Atherosclerosis Research (ISARCON2012): An International Conference on Recent Trends in Atherosclerosis and 25th Annual Conference of The Indian Society for Atherosclerosis Research [ISARCON2012] was going to be held on September 1-3, 2012 at Annamalai University, Chidambaram, Tamilnadu, India organized by Department of Biochemistry and Biotechnology, Annamalai University, Chidambaram – 608 002, Tamilnadu, India. Abstract can be submitted online through E-mail: isarcon2012@rediffmail.com on or before July 10, 2012. For further details contact: Dr. N. Nalini, Chair Person, ISARCON-2012, Department of Biochemistry and Biotechnology, Faculty of Science, Annamalai University, Chidambaram- 608 002, Tamil Nadu, India.E-mail: isarcon2012@ rediffmail.com. Phone: 04144-239141 Mobile: (0) 94432 71346. Annamalai University/Conference web site: http://annamalaiuniversity.ac.in.

World Congress on Biotechnology 2012: World Congress on Biotechnology 2012 was going to held on 4th- 6th May, 2012 at Leonia Holistic Destination, Hyderabad, India organized by Bright International Conferences & Events, Hyderabad, India. Abstract can be submitted online through Email: biotechnology2012@brightice.org/ info.brightice@ gmail.com / info@brightice.org on or before April 6th, 2012. For further details contact: Organizing Comittee-Biotechnology2012, Bright Technologies, Plot No.109, Chanikya Puri, IDA Mallapur, Hyderabad-500076, India. Website: www.brightice.org.

Workshop on Basics of Bioinformatics and its applications: A 3-day workshop on Basics of Bioinformatics and its applications was going to held on June 5th -7th, 2012 at National Institute for Research in Reproductive Health, Jehangir Merwanji Street, Parel, Mumbai- 400 012, India organized by Biomedical Informatics Centre of ICMR. Faculty from colleges/university departments and research institutes are eligible. Preferences will be given to those teaching undergraduate/ post-graduate course in Biological Sciences. Interested applicants can fill the online form available at www.bicnirrh.res.in/ workshop.php. The last date for receipt of applications is 30th April, 2012. The list of the selected candidates will be intimated by email on or before May 7, 2012. For further details contact Dr. Smita Mahale, Scientist 'E', National Institute for Research in Reproductive Health, Jehangir Merwanji Street, Parel, Mumbai- 400 012, India. Website: www.bicnirrh.res.in/ workshop.php.

6th Annual Convention of Association of Biotechnology and Pharmacy -

International Conference on Environmental Impact on Human Health and Therapeutic Challenges

December 20-22, 2012

6th Annual Convention of ABAP and International Conference on Environmental Impact of Human Health and Therapeutic Challenges is being organized at Sri Venkateswara University, Tirupathi, India during 20-22 December, 2012-01-15

Broad Areas of Focus

- Biodiversity and its Conservation
- Conservation Techniques (*in situ* and *ex situ*)
- Biotechnology in Biodiversity & Bar-coding
- Ecotoxicology impact on Biodiversity
- Water, Soil and Air Pollution & diseases
- Environmental Pollution and Control
- Environmental Impact Assessment
- Toxicology and Human Health
- Bioremediation and Biodegradation
- GM Crops & Ecofriendly Technologies
- Integrated Pest Management & Organic farming
- Nanotechnology & Drug Discovery
- Novel Therapeutics & other related areas

For further details contact

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Head, Department of Virology

Sri Venkateswara University

Tirupathi – 517 502, A.P., India

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