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Biomarkers Clinical Relevance in Cancer: Emphasis on Breast Cancer and Prostate Cancer

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Abstract

Cancer remains a major health problem in the world due to the dramatic increase in the number of cancer-related deaths in recent years. Biomarkers are biological candidates that are altered due to disease condition and can serve as indicators of the disease process. Identification of clinically useful biomarkers is very critical in cancer therapy especially for the early detection of disease which can reduce mortality and increase survival of life. Despite the serious concern and urgent need for the discovery of candidate tumor markers, the outcomes in developing credible biomarkers are remarkably low. Due to recent emergence of novel technologies, scientists are extensively conducting comprehensive analyses using multiple approaches including genomics and proteomics to address the issues related to low success rate and more disappointments in developing tumor markers with clinical significance. Both the government and public sectors are using their resources to focus on multiple approaches which could foster developing new biomarkers for diagnosis and early detection of cancer, thereby facilitating better understanding of cancer pathogenesis and advanced cancer care in a reasonable time frame. This review will focus on the relevance of biomarkers in cancer with a special emphasis on breast cancer and prostate cancer.

Key words: Biomarkers, Breast cancer, Prostate cancer, Disease therapy

Introduction

Cancer is one of the leading causes of deaths in the world. According to World Health Organization (WHO) cancer is responsible for 13% (~30% in the United States) of deaths worldwide in 2005. Based on the current projections, the number of worldwide cancer deaths may rise to 11.4 million in 2030 (1). Breast cancer is one of the five main types of cancers associated with a high incidence of mortality with an estimated 502,000 world-wide deaths in 2005(1). Breast cancer is the foremost cause of cancer related deaths among women in the United States and in the current year it is expected to record more than 240,000 new cases and more than 40,000 deaths (2). Prostate cancer is among the most common type of cancer in men in the United States and it is the second largest cancer (after lung) responsible for more male deaths. It is estimated that 230,000 new cases and 30,000 deaths due to prostate cancer may occur in the United States in the current year (3). The onset of prostate cancer may be multi-factorial; however genetics and diet are considered as key players in the development of this disease. The current remedies in treating these cancers include surgery, radiation, chemotherapy, hormonal, and proton therapy (individual or in combination). The

age and underlying health of the patient as well as the extent of tumor spread, appearance under the microscope, and the response to initial treatment are important factors in determining the outcomes of these malignancies.

Biomarkers are biomolecules that serve as indicators of biological and pathological processes, or physiological and pharmacological responses to a drug treatment. Early detection of cancer can prevent the majority of cancer related deaths by employing less extensive treatment. Since biomarkers can be used to measure the progress of disease or the response to treatment, they have very significant roles in diagnosis and prognosis of disease. The continuous increases in the incidence of cancer related deaths push for the urgent need to adopt preventive interventions which can be obtained by developing effective tools for the early detection of disease. Many classes of biological molecules including lipids (4, 5), carbohydrates (6, 7), and nucleic acids (4, 8, 9) have been tested as potential biomarkers; however the scope of protein-biomarkers seems to be relatively more beneficial, thereby leading to the accumulation of voluminous data in this area of research. Serum has rich source for proteins and can serve as an excellent non-invasive source in the biomarker discovery arena (10). However, hundreds of molecular candidates have been tested for the identification of protein-based biomarkers in serum (11), and only a limited number of biomarkers such as HER2/neu, estrogen receptor (ER), and progesterone receptor (PR) have been used in clinical practice during the treatment of breast cancer (11) and prostate specific antigen (PSA) for prostate cancer. The United States Food and Drug Administration (FDA) approved biomarkers for breast cancer and prostate cancer are shown in Table 1. Even though biomarkers have several implications, here we focus on important appli-

Table 1: List of United States Food and Drug Administration (FDA) approved biomarkers for breast cancer and prostate cancer (15).

Breast Cancer			
Biomarker	**Type**	**Source**	**Clinical Use**
CA15-3	Glycoprotein	Serum	Monitoring
CA27-29	Glycoprotein	Serum	Monitoring
Cytokeratins	Protein (IHC)	Tumor	Prognosis
Estrogen/progesterone receptors	Protein (IHC)	Tumor	Selecting therapy
Her2/neu	Protein	Serum	Monitoring
Her2/neu	Protein (IHC)	Tumor	Selecting therapy/prognosis
Her2/neu	DNS (FISH)	Tumor	Selecting therapy/prognosis
Prostate Cancer			
Biomarker	**Type**	**Source**	**Clinical Use**
PSA (total)	Protein	Serum	Screening/monitoring
PSA (complex)	Protein	Serum	Screening/monitoring
PSA (free %)	Protein	Serum	Disease classification

CA: Cancer antigen; Her2: Human epidermal growth factor receptor 2; IHC: Immunohistochemistry; FISH: Fluorescent *in-situ* hybridization; PSA: Prostate-specific antigen.

cations such as early detection, disease staging, drug therapy/prognosis and drug discovery.

Early detection:

The traditional method for cancer diagnosis was based on the biopsy of tissues followed by a histopathological examination. Diagnostic power was further improved using the imaging tools such as X-rays, ultra-sound and mammograms in certain types of cancers. Even though therapeutic agents are available for several types of cancers, it is very important to start cancer treatment at an early stage in order to improve prognosis. Government, academia and national foundations recognized this need and are using their resources to identify reliable markers for early detection of cancer. The National Cancer Institute (NCI) of the United States of America launched an investigator-driven network, Early Detection Research Network (EDRN), in 2000 to identify markers for the early detection of cancer and cancer risk. With more than 300 investigators representing divergent disciplines, including genomics, proteomics, metabolomics, bioinformatics and public health and 40 public and private institutions, this network is engaged in developing and validating the biomarkers (12). EDRN is bringing visionary people together through research collaborations that inspire innovative approaches to early detection, prevention and treatment of cancer. This network brings together both the clinical and basic scientists for a better coordination between discovery and validation. Even though FDA has approved several biomarkers in cancer care, none of them have been validated as early detection markers of any cancer (Table 2). Investigators from this network are currently studying over 120 biomarkers for their potential use in early detection of cancer. These markers are investigated for their application either alone or in combination with other therapies (Table 3).

Disease staging:

Disease staging is a mandatory step in cancer care. Since its inception in 1958, medics follow the TNM (T: tumor size; N: lymph node spread; M: status of metastasis) staging system which is based on vigilant anatomical examination and describes the extent of disease in the patient (13). Despite the extensive benefits of this anatomically based method, levels and patterns

Table 2: FDA approved early detection tests for various types of cancers (12).

Organ Site	Test
Bladder	None
Breast	Mammogram
Cervix	Pap smear
Colorectal	Fecal occult blood test, sigmoidoscopy, colonoscopy, double contrast barium, enema, digital rectal exam,
Esophageal	None
Kidney	None
Liver (primary)	None, but two molecular tests are approved for risk assessment
Lung	Imaging
Ovary	None proven to decrease mortality
Pancreatic	None
Prostate	None proven to decrease mortality

Table 3: Early detection biomarkers in study from selected cancer sites during 2003-2007 (12).

Cancer type	Number of biomarkers
Bladder	3
Breast	7
Cervical /Endometrial	2
Colorectal	21
Esophagus	7
Hepatocellular	9
Kidney	1
Lung	12
Mesothelium	2
Ovarian	5
Pancreatic	16
Prostate	15

of certain molecular markers are highly important in disease classification and patient stratification for therapy (14). The additional screening of individual or patterns of a few biomarkers associated with disease will enhance the power of assessment of disease staging (15). Estrogen receptor (ER), progesterone receptor (PR) and Her2/neu are widely considered for such assessment in breast cancer. Initially, high expression of Her2/neu was considered a negative prognostic indicator. And now, the therapeutic agent, trastuzumab (Herceptin), is extensively used as a targeted therapy for Her2/neu positive breast cancer patients (15). In addition to routine screening for PSA levels, the analysis of PSA velocity (measurement of PSA concentrations over time), ratio of free to total PSA, and human kallikrein (kK) 2 are also implicated in the diagnosis of prostate cancer (16, 17).

Drug therapy and prognosis:

Biomarkers serve as powerful tools in drug therapy. These markers help the physicians to 1). choose treatment options, 2). determine whether the tumor is responding to therapy, 3). adjust the treatment dose and 4). assess patient prognosis. For example, in the treatment of breast cancer, patients who have tumors expressing high levels of h estrogen progesterone receptors, ER and PR, are treated with hormonal therapy, whereas, patients who have Her2/neu- positive tumors, require a completely different treatment regimen, Herceptin. The markers used in the diagnosis of prostate cancer (see above) are also used to assess the response of patient to therapy and prognosis. In prostate cancer serum proteomic profiling has been emerging as a sensitive method for diagnosis, therapy and prognosis; however its applications in clinical practice have not been adequately validated (18-20).

Drug discovery:

The field of biomarkers research is gaining huge ground in the world of drug discovery. The integration of biomarkers in drug development will enable pharmaceutical companies to evaluate the efficacy of their new therapeutic agents, resulting immediate decisions as to whether or not market a drug. These “go/no-go” decisions are cost-effective and will save millions of dollars for drug development projects. Biomarkers can also be used in determining the cytotoxic effects of the candidate therapeutic agents, which help drive the decision on treatment dose, schedule and overall, the drug’s future. The FDA has recently recommended that validated or investigational biomarker data be included in the application for an investigational new drug (IND) and drug application (NDA) packages, which made it mandatory for pharmaceutical industries *not to* ignore the applications of biomarkers in their drug discovery projects. As shown in Table 4, the total biomarker market was $ 5.6 billion in 2007 and current projections suggest that it is growing at ~18% per year (21).

Table 4: The total revenue of the global biomarkers is expected to cross 12 billion by 2012. The forecast on global biomarkers revenue ($ million) and growth rate (%) among various segments is presented in the table (21).

Market segments	2005	2006	2007	2012	CAGR% 2007-2012
Biomarker Discovery	2044	2339	2677	5843	16.9
Clinical Trials	450	525	612	1761	23.5
Molecular Diagnostics	1698	1950	2300	5156	17.5
TOTAL	4192	4814	5589	12760	18

Complications in biomarker discovery and future directions:

Proteomics is highly used in biomarker discovery. Proteomics is the study of the proteome, which in was first used as a description of all proteins present in a cell, tissue or organism. Since proteins are involved in almost all biological activities, the proteome analysis will provide crucial information on the physiological condition of an organism. Serum has rich source for proteins and serves as an excellent non-invasive source in the biomarker discovery (10). However, hundreds of molecular candidates have been tested for the identification of protein-based biomarkers in serum (11), and only a limited number of biomarkers are currently used in clinical practice. The complex nature and heterogeneity of the tumor-host microenvironment limits the ability to easily identify new biomarkers and therefore, ultrasensitive techniques, which are limited, are required. For example, there is a need for a more sensitive detection system for measuring concentration of such candidate markers far lower than those currently achieved by widely used SELDI-TOF units (22, 23). Due to tumor formation and interference by high-abundant molecules present in the secretion of liver and other organs, the alterations in some biomarkers may not necessarily reflect the real changes and may lead to misrepresentation of the results (24-26). The issue of removing abundant/high molecular proteins, such as albumin and immunoglobulin, is also an important issue that needs to be addressed properly (27). Fortunately, recent advances in technology have opened new avenues for employing proteomics in cancer-related biomarker discovery. Availability of automated liquid handling systems, fractionation techniques coupled with tools for greater sensitivity and resolving power, are helping scientists in analyze more samples over a period of time and provide a more confident data report. In addition, application of quantitative proteomic methods, with the help of high-sensitivity mass spectrometry (MS) and protein chips, and using advanced bioinformatics for data handling and interpretation, makes it possible to discover biomarkers that reliably and accurately predict outcomes during cancer treatment and management.

Conclusion:

The inability to uniformly assess the causes of cancer combined with the lack of credible preventive interventions are cause for serious concerns in cancer care. The identification of biomarkers will potentially provide solutions for cancer treatment, as they address risk, cancer staging, prognosis and assessment of patient response to certain therapeutic agents and therefore, will dramatically improve the survival and quality of life of cancer patients. Fortunately, the government, alongside industry,

academic institutions and national foundations has dedicated portions of their resources to the area of biomarker research. In the end, both the researchers and clinicians must adopt the use and better understand the advantages of biomarkers. Apart from proteomics and genomics, the application of other latest tools including nanotechnology, artificial intelligence could foster biomarker discovery and we can envision for a future with reduced cancer mortalities and improved clinical care to cancer patients with the help of biomarkers.

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Current trends in biotechnological production of xylitol and future prospects

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Abstract

This review describes recent research developments on biological conversion of hemicellulosic biomass towards production of xylitol by taking advantage of power of biotechnology. Xylitol is a five-carbon sugar alcohol with established commercial uses in different healthcare sectors and especially as an alternative sweetener for diabetic persons. Xylitol can be synthesized either by chemical hydrogenation of xylose or by fermentation. The precursor xylose is produced from biomass by chemical or enzymatic hydrolysis and can be converted to xylitol primarily by yeast strains which offer the possibilities of economic production by reducing required energy when compared to chemical production. Biomass hydrolysis under an acidic environment is the most commonly used practice and is influenced by various process parameters. Several microbial growth inhibitors are produced during chemical hydrolysis that reduce xylitol production from xylose, a detoxification step is therefore essential. Enzymatic hydrolysis has advantages over chemical conversion although more research is necessary to reduce inhibition due to structural variation from different substrates or plant species. Enzymatic xylitol production is mostly an integral process of microbial species belonging to the *Candida* genus. Extensive research has been performed to screen for xylitol producing microbial strains as well as to understand microbial metabolism, the xylitol metabolic pathway, cofactor requirements, development of robust recombinant strains, optimization of bioconversion parameters and xylitol production strategies using free and immobilized cells. The imperative role of hydrolysis of xylose containing biomass and subsequent process parameters has major impact on economis of bioconversion. The review identifies ways forward for improved enzymatic xylitol production to compete with current chemical processes.

Key words: *Candida*, Detoxification, Hemicellulosic material, Hydrolysis, Bioconversion, Xylitol, Xylose.

Introduction

Xylitol is a polyol and a C_5 sugar, also known as wood or birch sugar, obtained from the reduction of xylose. It is a rare sugar that exists in low amounts and is the constituent of many fruits and vegetables, such as raspberries, strawberries, yellow plum, lettuce and cauliflower. Xylitol was first produced from birch trees in 19th century in Finland. It has attracted global interest due to its sweetening power similar to that of sucrose; equivalent to 2.4 kcal.g^{-1} and laxative nature (145 J.g^{-1}caloric content) (104, 22, 33). Xylitol has applications and potential for at least three types of industries namely food (for dietary especially in confectioneries and chewing gums), odontological (for its anticariogenicity, tooth

rehardening and remineralization properties) and pharmaceutical (for its toothfriendly nature, capability of preventing otitis, ear and upper respiratory infections and its possibility of being used as a sweetener or excipient in syrups, tonics and vitamin formulations). However the major use is for the prevention of dental caries as xylitol inhibits growth of microorganisms responsible for tooth decay (44, 69, 70, 157). In addition, xylitol is accepted for consumption for diabetics and helps in treatment of hyperglycemia as its metabolism is independent of insulin (157). The xylitol market is increasing and at present is estimated to be $340 million yr^{-1} and priced at $4–5 kg^{-1}.

Currently, xylitol is manufactured at the industrial level by a chemical hydrogenation of the five-carbon sugar D - xylose, in the presence of nickel catalyst at elevated temperature and pressure. This chemical process is laborious, cost and energy intensive. In addition, the process needs expensive refining treatments necessary for xylose production. In order to produce this xylitol in economically and eco-friendly manner, research was initiated for alternative strategies. One of the alternatives is bioconversion of renewable biomass sources which requires hydrolysis followed by bioconversion of xylose from crude hydrolysate to xylitol employing specific microbial strains for fermentation (132, 129, 130).

Photosynthetic biomass as raw material for xylitol production

In view of the disadvantages associated with the chemical production of xylitol process such as conversion efficiency, environment impact and energy input parameters research has identified alternative raw materials and production processes. One of the potential alternative raw materials is xylo-oligosaccharides (hemicellulosic materials) from plant biomass; as the annual growth of plant-derived biomass is estimated to be 73.9 terra grams per year on a dry matter basis (54) of which 20-35% is xylose. Biomass material is widespread, abundant, renewable, cost-effective and inexpensive source of polysaccharides which can be used for production of wide variety of biotechnological products including xylitol, these sources include forests, agricultural and agro-industrial residues (Table 1).

Table 1: Lignocellulosic biomass produced annually in dry mass basis (54)

Crop	Lignocellulosic biomass (Tg)
Barley	058.45
Corn	203.62
Oats	010.62
Rice	731.34
Wheat	354.35
Sorghum	010.32
Sugarcane	180.73
Rye grass*	20.00[$]

*Source: Booth et al. (5)
$ tons.hectar^{-1}

According to estimates, hemicellulose is the second most common polysaccharide available in nature (105) consisting of heterogeneous polymers of hexoses (glucose, mannose and galactose) and pentoses like xylose and arabinose (58). In order to use these materials they must be hydrolyzed into simple monomeric sugars either by chemical or enzymatic methods for fermentation using microorganisms. Several studies on hydrolysis of xylose-rich hemicellulosic materials (Table 2)

have been performed for utilization as substrates for biotechnological xylitol production (78, 129). A variety of plant biomass materials were evaluated as source of raw materials such as corn cobs (129), sugar cane bagasse (14, 129), eucalyptus (146), brewery's spent grain (12, 78), olive tree pruning (102), soyabean hull (114), palm oil empty fruit bunch fiber (96), and rice straw (65). Residue particle size reduction was performed by grinding for all the pretreatments of lignocellulose residue as it reduces cellulose crystallinity, especially in case of photosynthetic biomass as raw material (134). However, utilization of these resources mainly depends on the degradation of these polymeric materials to simple sugars, with hemicelluloses being important in the overall conversion process (102, 129, 146).

Table 2: Xylan content in different materials

Feed stock material	Xylan content (%) dry weight
Corn stover	22.4
Corn fiber	16.8
Wheat straw	21.2
Switch grass	20.4
Office paper	12.4

Hydrolysis methodologies

Photosynthetic biomass mainly composed of cellulose (34-50%), hemicellulose (19-34%), lignin (11-30%) and smaller amounts of pectin, protein, extractives and ash. Composition of these components differs with the source of plant species, age and growth conditions (4). Among these, cellulose (a homo-polysaccharide of consisting of polymerized D-glucose up to 10 000 or more linked by â-1, 4-glucosidic bonds) forms a skeleton. Hemicellulose is a complex heterogeneous polysaccharide consisting of 200 degree of polymerization composing of glucose, galactose, mannose, xylose, arabinose, glucuronic acid with acetyl side chains. Cellulose is interlinked by hemicellulose to build a structural matrix. This structure is further encrusted with lignin. Lignin, polymer of phenyl propane, is non-polysaccharidic in nature consisting of ñ-coumaryl-, coniferyl- and sinapyl alcohol units bonded by alkyl-, aryl, and combination of both ether bonds. In fact, cellulose, hemicellulose, and lignin are closely associated with covalent cross-linkages, hence, biomass can be regarded as a composite material, in which the lignin serves as a protective layer. In addition, the composition of lignocellulosic materials varies with the biomass material such as hard wood, soft wood and grasses. Because of this, the plant biomass exhibits a remarkable stability against chemical and biological attack and can rarely be converted into simple sugars under normal conditions. Therefore pretreatment is necessary in order to alter the structural integrity, remove the lignin and increase the surface area to make this material available as fermentable sugars (45). Performance of pretreatment depends on selected material harvesting nature, lignin and other components composition, storage type and time, temperature and chemicals used. In general, processes used to produce xylo-oligosaccharides from xylan-rich materials are essentially hydrolytic in nature and can be performed either by chemical means using basic or acidic media, or catalyzed by enzyme sources (78). Since, the scope of this review is limited to xylitol production, detailed information on pretreatment methodologies are delt very limited.

Chemical hydrolysis is a simple and rapid method for hemicellulosic material however treatment conditions vary with agro-industrial material and with respect to chemical agent type and concentration, incubation temperature and time (129, 134). When aged or fully grown agricultural residues or hardwoods are used as raw materials, xylose is the most abundant sugar

in hydrolysates in addition to small fractions of other sugars. For acid hydrolysis different mineral acids such as sulfuric (102, 134, 147), hydrochloric (40), nitric, hydrofluoric (25), acetic acid (17) and phosphoric (27) acids are used at high temperature and pressure (commonly 160^{o}C) and (10 atm). In general, acid hydrolysis performed under concentrated (50–70%) or diluted (below 2%) conditions. Preferences are for diluted acid conditions and high temperatures due to high reaction rates with less microbial growth inhibitors, which is a low cost technology compared to other chemical approaches (46, 101, 102).

The chemical hydrolysis reaction is a complex process (27) that is a multi-step reaction that occurs in following sequence (i) diffusion of protons through the wet lignocellulosic matrix; (ii) protonation of the oxygen of a heterocyclic ether bond between the sugar monomers; (iii) breaking of the ether bond; (iv) generation of a carbo-cation as intermediate; (v) solvation of the carbo-cation with water; (vi) regeneration of the proton with cogeneration of the sugar monomer, oligomer or polymer depending on the position of the ether bond; (vii) diffusion of the reaction products in the liquid phase. All these process steps are influenced by pH of the hydrolysis medium, solid-liquid ratio, incubation temperature and time (65, 129). Sun and Cheng (134) and Cara et al. (10) concluded that acid hydrolysis with the use of concentrated acids is toxic, corrosive and hazardous.

Auto-hydrolysis is an alternative method for the chemical depolymerization of hemicelluloses with limited solubilization of lignin (29) and reduced quantities of sugar derivatives (furfurals and hydroxymethylfurfurals) (78). In addition auto-hydrolysis presents some technical and environmental advantages too as no chemicals (acid or alkali) are used other than water. Auto-hydrolysis performed at mild temperatures yields a high mass of xylo-oligosaccharides without modifying the cellulose and lignin structure substantially, allowing improved recovery during further processing (76, 117). The xylo-oligosaccharides produced are associated with a significant fraction of acetyl and uronic acid groups which has the characteristic of very high water solubility unlike that of chemical hydrolysis. The auto-hydrolysis process efficiency and hydrolysate chemical composition depends on incubation temperature and time, solid to liquid ratio, structural integrity of raw material employed. Nabarlatz et al. (81) working with six agricultural residues namely corncobs, almond shells, olive stones, rice husks, wheat straw and barley straw as feedstocks for the production of xylo-oligosaccharides by auto-hydrolysis, reported that the yield of xylo-oligosaccharides depended on the content of xylan and its accessibility, and was proportional to the acetyl content of the raw materials. In fact, by regulation of auto-hydrolysis conditions, it is possible to influence characteristics of the xylo-oligosaccharides (the acetyl content and the molar mass distribution), but the nature of the raw material also has an influence (81). Hydrolysate analysis revealed that partially acetylated oligomeric and polymeric xylan fragments were attached with acetyl groups at 2 and 3 positions and some monosaccharides and partially O-acetylated 4-O-methylglucuronoxylan in addition to degradation products were present (80).

Biological or enzymatic hydrolysis has been proven as an alternative hydrolysis method offers conceptual edges like low chemical and energy use, but depends on enzyme accessibility to the heterogeneous biomass structure. The rate and extent of enzymatic hydrolysis of lignocellulosic biomass is dependent on catalytic properties of enzymes, their loadings concentrations, the hydrolysis period, reaction parameters employed, biomass type, pretreatment method employed and compounds produced during pretreatment process (159). Reduction of

hemicellulosic crystallinity improves the enzymatic hydrolysis rate and time in addition to the enzyme loading. Among all biomass components, lignin is identified as a major deterrent to enzyme attack on cellulose indicating the importance of reducing the structural integrity caused by lignin before hydrolysis. Cellulase and xylanases are the major enzymes employed in most of the pretreatment studies (95, 159). Biomass digestibility by enzymes is found to be regulated by the surface area of the material and an increase in surface area by pretreatment or decreasing particle size improves biomass hydrolysis (95).

Use of xylanase alone may not be sufficient in view of the complex nature of photosynthetic biomass material. Xylanases catalyzes the â-1, 4 bond in the xylan backbone yielding short xylooligomers. They are group of enzymes work synergistically and differ with microbial origin. The selection of critical xylanase blend consisting of xylosidase, Mannanases, arabinofuranosidaes, glucuronidases, esterases (ferulic and cumaric acid, acetyl-mannan, acetyl-xylan, etc.) and hemicellulolytic esterases is one of the important factors for effective production of xylose from hemicellulose fraction. This selection again related with the nature of xylan structure which vary with type of biomass (soft, hard wood, grass, etc). Pre-hydrolysis either by mild chemical treatment at elevated temperatures and/or by other specific enzyme treatment would offer the better hydrolysis process for the efficient production of xylose. Use of non catalytic proteins such as expansins and swollenins decreases the crystallinity structure thereby increases the accessibility to enzymes may be novel approach. However, the applicability and feasibility is yet to require further study. Our laboratory studies indicated that xylanase from certain specific microbial strains could be used as an efficient xylose production from palm seed fibre (95). However, enzyme treatment parameters have to be optimized for maximization of xylose production. Although enzymatic hydrolysis results in high yields in bioconversion of sugars from pretreated photosynthetic biomass, the cost of enzymes is a key aspect and needs to be costed. Use of hemicellulosic hydrolytic enzyme blend is another alternative; however, one has to identify and optimize process environment of the specific enzyme blend for each material. Wet oxidation pretreatment process proven to be efficient for lignocellulosic materials as crystallity decrease was noticed along with lignin degradation to CO_2 and H_2O and carboxylic acids. Recently use of ionic liquids such as 1-butyl-3-methylimidazolium caution for biomass pretreatment revealed optimistic results but indeapth studies are essential for its after effects like microbial/enzyme inhibitor production, process environment, etc. In nutshell, upstream to pretreatment, the choice of source material structure is an important in selection of effective pretreatment methodology.

Components of biomass hydrolysate

A range of products such as glucose (mainly from cellulose and hemicellulose), xylose, mannose, galactose and acetic acid (from hemicellulose) and phenolic compounds (from lignin) are produced during the hydrolysis process. In addition, other compounds are also produced during hydrolysis especially when chemical hydrolysis is employed. Without exception, all sugar liquors obtained by chemical hydrolysis contain furan derivatives, aliphatic acids and phenolic compounds. Furan derivatives commonly known as furfurals and hydroxymethylfurfural (HMF) are produced from the degradation of pentoses and hexoses, respectively. Further degradation of furfurals leads to the production of formic acid. HMF is normally produced in less concentration compared to furfurals by hexoses degradation mainly due to the low quantities of hexose in hemicellulose. This is because the conditions employed in the

hemicellulosic material hydrolysis process do not degrade hexoses in large quantities. Acetic acid, the major aliphatic acid present in chemical hydrolysates, is mostly released from the hemicellulosic acetyl groups. During the acid hydrolysis, a minor part of lignin is also degraded to a wide range of aromatic compounds including low molecular mass phenolics (90). With the use of strong alkali solutions, depolymerized xylan may be extracted from lignocellulosics, but the product obtained is completely deacetylated and has very limited solubility in water hence is not the preferred hydrolyzing reagent. In addition other compounds such as acidic resins, tannic, terpene, syringic, vanillic, caproic, caprylic, pelargonic, and palmitic acids are reported to be produced during chemical hydrolysis (6, 78).

Microbial fermentative inhibitors of biomass hydrolysates

The major disadvantage of chemical hydrolysis is the reduction of available monosaccharides and production of their derivatives (furans, hydroxymethylfurfurals and other phenolic toxic compounds which are microbial growth inhibitors and hinder further biotransformations (78). However, the type and concentration of microbial fermentative inhibitory compounds mainly depend on raw material as well as the operational parameters. Microbial toxicity is also associated with fermentation variables like microbial physiological growth conditions, dissolved oxygen concentration and pH of the medium. In general, biomass hydrolysate inhibitors can be categorized as sugar or lignin degradation products, derived from lignocellulosic structure and heavy metal ions (78).

Furfurals derived from pentose are the major microbial growth inhibitor compounds present in chemical hydrolysates for xylitol bioconversion. They inhibit the growth of microbe ranging from 25 – 99% relative to the furfural concentration (0.5 – 2.0 g/l) and cell mass yield per ATP by interfering with the respiration process (90, 94). Delgenes et al. (20) and Martinez et al. (71) reported that *Pitchia stipitis* and *Saccharomyces cerevisiae* growth was reduced by 100% when the HMF in the concentration of 1.5 and 1.0 g/l was supplemented in the growth medium indicating the inhibitory effect varies with the type of microbial strain. Presence of low concentrations of these compounds in the fermentation medium showed better microbial growth (94) indicating the microbial strain properties role during bioconversion of hydrolysates. In addition, the antagonistic effect of furfural and HMF along with acetate, formic and levulinic acid on microbial growth was also reported with *P. tannophilus* and *P.stipitis* during xylose fermentation (148).

A variety of lignin degradation products that included aromatic, polyaromatic, phenolic and aldehydic compounds present in hydrolysate also cause inhibitory effects on microbial growth by integrating into biological membranes and affecting the membrane permeability. Villa et al. (145) reported that phenolic compounds at more than 0.1 g/l concentration affect the xylose consumption, cell growth and xylitol production in *C. gluilliermondii*. Acetic acid toxic effect is mainly associated with its pKa property as at this value acetic acid is liposoluble, diffuses across the plasma membrane and discharges protons resulting in cell death due to dropping the internal pH. However, presence of acetic acid at low concentrations (1.0 g/l) in the fermentation medium reported to improve the xylose-to-xylitol bioconversion (24) probably due to more diffusion of internally pooled xylitol during xylose metabolism because of limited acetic acid effect at cell membrane. Heavy metals (iron, chromium, nickel and copper) produced during hydrolysis mainly originate from corrosion of hydrolysis equipment causes cell toxicity by inhibiting metabolic pathway enzymes (93).

Detoxification methodologies

In order to remove the microbial growth inhibitors and increase the hydrolysate fermentability, several detoxification treatments, including chemical, physical and biological methods have been developed. However, the needs for detoxification must be evaluated in each case since it depends on the chemical composition of the hydrolysate and is strain specific. The effectiveness of a detoxification methodology depends on raw material, type of hydrolysis process and microorganism employed (129). Taherzadeh et al. (136) reported four different approaches for minimizing the inhibitory effect of hemicellulosic hydrolysates; (1) use of bioconversion friendly hydrolysis methods; (2) detoxify the hydrolyzate before fermentation; (3) use of inhibitor resistant microorganisms; (4) convert toxic compounds into non-toxic. Since detoxification increases the cost of the process, it is important either to overcome detoxification steps or to develop cheap and efficient methods. Development of a new metabolically engineered microbial species which tolerate inhibitors could be the better option which can eliminate detoxification.

Vacuum evaporation is the best physical detoxification method with limited scope and helps to reduce volatile toxic compounds that include acetic acid, furfural, hydroxymethylfurfural and vanillin. Mussantto and Roberto, (78) reported that more than 90% these compounds are removed from wood, rice straw and sugarcane bagasse hemicellulosic hydrolysates by employing a vacuum evaporation method. However, this process enhances the concentration of non-volatile toxic compounds and reduces volumes of the hydrolysate (61). Neutralization, over-liming, sulfite treatment, extraction with organic solvents, treating with ion-exchange resins and adsorption into activated charcoal or diatomaceous earth reduce the ionization properties of inhibitory compounds by precipitation of toxic compounds. pH adjustment is effective and the most cost-effective chemical detoxification method among available treatments. Calcium hydroxide and sulfuric acid are commonly used for treatment of hemicellulosic hydrolysates for removal of phenolic compounds, ketones, furfurals and hydroxymethylfurfurals (84, 129). Activated charcoal is the other process attracting much attention because of its low cost and a high capacity to absorb pigments, free fatty acids, n-hexane and other oxidation products (98, 129). The effectiveness of activated charcoal treatment depends on different process variables such as pH, temperature, contact time and solid-liquid ratio. Acidic pH favours removal of the neutral or non-ionized phenolic molecules while alkaline pH for organic bases during activated charcoal treatment. Increase of contact time is reported to influence the clarification process. The absorption process increases at elevated temperatures during charcoal treatment basically due to a faster rate of diffusion of absorbate molecules from the solution to the absorbent and temperature induced orientation of charcoal surface (77). Comparative evaluation of different chemical detoxification methodologies indicated that anion exchange resins remove high percentages of toxic compounds such as acetic acid (96%), phenolic compounds (91%), furfural (73%), HMF (70%) in addition to substantial removal of aldehydes and aliphatic acids from hydrolysates compared to cation-exchange resins (78, 129). Grzenia et al. (35) reported use of hollow fibre based liquid extraction system for removal of acetic acid from corn stover hydrolysate using two different LiquiCel Membranes.

Biological detoxification can be done either by using specific enzymes or microorganisms. Laccases and peroxidases are generally employed for detoxification (78). The probable enzymatic detoxification mechanism involved is oxidative polymerization of low

molecular weight phenolic compounds (49) whereas, microbial detoxification of hydrolysate involves utilization of toxic compounds for microbial growth or adaptation of specific microbe for hemicellulosic hydrolysate (129). Schneider (116) reported that acetic acid in the hydrolysate can be removed more than 90% by *S. cerevisiae* mutant from wood hydrolysate. Silva and Roberto (118) and Sreenivas Rao et al. (129) successfully demonstrated that adaptation of *C. tropicalis* as an effective and inexpensive approach to alleviate the inhibitory effect of toxic compounds on xylose utilization for xylitol production from rice straw and corn cob hydrolysates.

Sreenivas Rao et al. (129) working on xylitol production from sugarcane bagasse and corncob hydrolysate reported that various chemical and biological detoxification methodologies i.e., more than one method, were effective compared to single treatment processes. The authors reported pH adjustment followed by activated charcoal and resin treatment only helped up to certain level and adaptation of microbial strain would be the better option for effective and efficient use of sugar compounds from hemicellulosic hydrolysates. In summary, each detoxification method is specific to certain types of compounds. Choosing detoxification methods (more than one) and their sequence was important for improved yields, however identification of inhibitory compounds and their concentrations in the hydrolysate was necessary.

Xylitol producing microbial strains

In the last few decades, several papers published on xylitol production using bacteria (157, 158), fungi (19), and yeasts (55, 62, 119, 126, 132). Among the microorganisms, yeasts are considered as the best xylitol producers (Table 3). *Candida* strains have been extensively studied for the production of xylitol as they have an advantage over the metabolically engineered *S. cerevisiae* for being natural D-xylose consumers and maintaining the reduction– oxidation balance during xylitol accumulation.

Table 3: Some of the best xylitol producing yeasts

Yeast	References
Candida boidinii	106
Candida guilliermondii FTI-20037	118, 119
Candida intermedia	28
Candida maltosa	36
Candida mogii	122
Candida parapsilosis	55
C. tropicalis HXP 2	32
C. tropicalis	129
Debaromyces hansenii	107
Hansenula polymorpha	135
Pachysolen tannophilus	110
Pichia caribica	126
Pichia miso	88

Screening programmes for xylitol production from D-xylose

Xylitol is an intermediate metabolic compound produced in all microbial strains whose xylose metabolism occurs in a sequential catalytic activity of xylose reductase and xylitol dehydrogenase enzymes. Keeping this in view, several scientific researchers have been involved in microbial screening programs to isolate efficient microbial strains for xylitol production. Hiroshi and Toshiyuki (41) tested 58 strains and *P. miso* emerged as the best xylitol producing strain with an yield of 3.77g of xylitol from 8.50g of D-xylose was consumed. Ojamo (88) screened 30 yeast strains for a xylitol metabolizing pathway and reported that *C. gluilliermondii* and *C.*

tropicalis were the highest yielding strains. While, Sirisansaneeyakul et al. (122) reported xylitol yield of 0.62g.g^{-1} of xylose using *C. mogii* from 11 strains they tested for xylose utilization. Suryadi et al. (135) tested four methanol-utilizing yeasts for xylitol production from D-xylose. *H. polymorpha* was found to be the better strain out of 4 strains tested with 43.2 g.l^{-1} xylitol production from 100 g.l^{-1} D-xylose after 4 days of cultivation. Whereas, Yablochkova et al (155) tested 13 strains and noticed only 6 strains emerged from *Candida* genus as the best xylitol producers in the range of 0.50 to 0.65 g.g^{-1} xylitol production. After screening 274 yeasts for xylitol production Guo et al. (36) selected 5 strains for further production and observed that *C. gluilliermondii* and *C. maltosa* were the best xylitol produces. Recently Sreenivas Rao et al. (126) tested a total of 35 yeasts isolated from the gut of beetles collected from Hyderabad city, India. Twenty of these yeasts utilized xylose as a sole carbon source but only 12 of these strains converted xylose to xylitol. The authors also reported that the ability to convert xylose to xylitol varied among the isolates and ranged from 0.12 to 0.58 g.g^{-1} xylose. Out of these strains *Pichia sp*. was the best xylitol producer (0.58 g xylitol.g^{-1} from xylose). In another study, Sampaio et al. (107) tested 270 yeast isolates for xylitol production using xylose as the sole carbon source. The authors reported that *D. hansenii* UFV-170 was the best isolate with production capacity of 5.84 g.l^{-1} xylitol from 10 g.l^{-1} xylose after 24 hours incubation. A report with xylose transport capacity as a screening parameter was reported by Gardonyi et al (2003) to isolate xylose-utilising yeasts.

Molecular characterization of xylitol producing yeasts

The approach to yeast identification has significantly changed in just a few decades due to the rapid increase in basic biological knowledge, increased interest in the practical applications and biodiversity of this important microbiological group, and technological advances. The development of molecular techniques has significantly widened the tools available for understanding and documenting species designations and phylogenetic relationships. Analyzing ribosomal DNA (rDNA) is now standard in molecular techniques and have made it possible to construct phylogenetic trees of all known species, with the capacity to better understand interspecific and intergeneric relationships. As a result, it is a common practice to deposit the sequences of key molecular regions, such as the 600-nucleotide variable region D1/D2 of LSU (large subunit) (26S) rDNA and the ITS1 and ITS2 (internal transcribed sequences) of 18S rRNA, with database servers such as Genebank. From D1/D2 sequence analysis, greater than a 100 species have been assigned to the genus *Pichia* and *Candida* which are distributed across the *Saccharomycetales* (59) and there is a specific distinct clade that contains xylose utilizing species (The xylose-fermenting clade). Molecular characterization studies help to understand the relationship between xylose utilizing yeasts that fall in this specific clade. Sreenivas Rao et al. (126), reported that phylogentic analysis helped to characterize the xylitol producing yeasts (Table 4).

The best xylitol producer YS54 based on their D1/D2 domain sequence, showed similarity with *Pichia caribbica* and this strain is identified within the xylose utilizing clad in the phylogenetic tree. Suh et al. (133) isolated several xylose utilizing strains and demonstrated that the LSU rDNA sequence data helped to identify the xylose fermenting yeasts and noticed that they also in to specific xylose fermenting clade. Similar trend was also observed by Nguyen et al. (83) who isolated two yeasts which ferment xylose, and

Table 4: Tentative identification of xylitol producing yeasts from insect guts based on D1/D2 domain sequence of the 26S rRNA gene (126)

Yeast isolate	Accession no. of D1/D2 domain	Identification	Isolated from	Xylitol yield (g^{-1} of xylose)	XR activity (U/mg protein)
YS 5	AM159103	*Issatchenkia* sp.	*Euetheola* sp.	0.14	1.5
YS 6	DQ358865	*Candida* sp.	*Nicrophorus* sp.	0.30	4.4
YS 21	AM159101	*Candida* sp.	*Strategus* sp.	0.54	8.0
YS 24	AM159108	*Candida* sp.	*Diplotaxis* sp.	0.40	6.2
YS 43	AM159105	*Candida* sp.	*Calligrapha* sp.	0.26	2.6
YS 44	DQ358867	*Candida* sp.	*Blepharida* sp.	0.48	6.5
YS 47	DQ358868	*Candida* sp.	*Copris* sp.	0.52	8.0
YS 54	AM159106	*Pichia* sp.	*Megalodacne* sp.	0.58	9.1
YS 60	AM159102	*Clavispora* sp.	*Epicauta* sp.	0.12	1.4
YS 27	AM420304	*Candida* sp.	*Anoplophora* sp.	0.36	5.4
YS 34	AM420306	*Candida* sp.	*Pseudomorpha* sp.	0.26	2.4
YS 19	AM420305	*Candida* sp.	*Calosoma* sp.	0.16	1.6
YS 5	AM159103	*Issatchenkia* sp.	*Euetheola* sp.	0.14	1.5
YS 6	DQ358865	*Candida* sp.	*Nicrophorus* sp.	0.30	4.4
YS 21	AM159101	*Candida* sp.	*Strategus* sp.	0.54	8.0

based on molecular characterization the authors reported that these strains belong to novel species and named as *Spathaspora passalidarum* gen. sp. nov. and *Candida jeffriesii* sp. nov.

Construction of recombinant yeasts for xylitol production

Screening of different xylitol producing microbial strains confirmed that xylitol production metabolic process is mostly associated with yeast in general and particularly with the *Candida* genus. Among different species in this genus, *C. tropicalis* is the best strain for xylitol production due to its high xylose uptake rate and xylitol production capacity (33, 132) and has application potential at industry level. In addition, this genus has an advantage, due to the lack of sexual stage (33), for further development of recombinant strains with high xylitol production potential. In fact, the major genetical differences of *Candida* and *Saccharomyces* genera are that the latter species is more tolerant in terms of their xylose fermentation, toxicity and growth tolerance in the presence of inhibitors of hemicellulosic hydrolysates. This has created new horizons to develop recombinant strains of *Saccharomyces* sp. with *Candida* sp. XYL1 gene for improved biological production of xylitol (82, 151). In order to make *S. cerevisiae* an efficient xylose-utilizer for the production of xylitol, an efficient enzyme

system for the conversion of xylose to xylitol should be introduced into the *S. cerevisiae*. Cloning of XYL1 gene from *C. tropicalis* to *S. cerevisiae* improves the latter yeast for utilization of xylose from hemicellulosic material and conversion of xylose to xylitol (82, 130).

Several investigators have cloned the necessary genes responsible for xylose metabolism in *S. cereviceae* and constructed the recombinant strains for production of xylitol (16, 38, 39, 73, 138). In the construction of a xylose metabolizing *S. cerevisiae* the gene encoding XR was cloned from the xylose metabolizing yeasts and transferred to *S. cerevisiae*. The authors of this review observed that these transformants could not produce xylitol for prolonged periods due to an imbalance of the redox potential in the cell (130).

One of the main possible limitations of utilization the XYL1 gene recombinant strains during continuous production of xylitol was the lack of reducing cofactors for the xylose to xylitol catalyzing enzyme, NADPH. The redox balance on substrate uptake in the yeast xylose metabolism has therefore been studied (38, 48). Different co substrates were evaluated, as generators of reduced cofactors for xylitol production by recombinant *S. cerevisiae* expressing the XYL1 gene, encoding xylose reductase. Glucose, mannose, and fructose, which are transported with high affinity by the same transport system as xylose inhibit xylose conversion rates by 99, 77 and 78 respectively. Competitive inhibition of xylose transport was indicated and xylitol yields varied widely with different co-substrates (48). Galactose as co-factor generator gave the highest xylitol yield, 5.6 times higher than that for glucose. This may be attributed to the observed difference in redox metabolism of glucose and galactose and subsequent enhanced availability of reduced cofactors for xylose reduction with galactose (130). Granstrom et al. (33) evaluated formate as a co-substrate to increase the intracellular concentration of NADH and based on the results the authors have hypothesized that excess NADH would result in higher oxygen and xylose consumption and correspondingly increase xylitol production by inhibiting xylitol dehydrogenase enzyme.

In this context, addition of cofactor in the growth medium may be a possible solution. Experimental evidence of 25% enhanced XR activity in galactose supplemented xylose media further supported that cofactor limitation is an important drawback for enhanced production of xylitol in recombinant strain studies (130). Similar observations are also noticed by Granstrom et al. (33) where the authors reported the metabolism (Metabolism Flux Analysis (MFA)) of xylose by *C. tropicalis* in oxygen-limited chemostat conditions. Furthermore, in vitro enzyme assay indicated that glycolytic and gluconeogenetic enzymes are expressed simultaneously, facilitating cofactor recycling. Moreover, enhancing the redox imbalance by co feeding of formate increased xylose and oxygen consumption rates and ethanol, xylitol, glycerol and CO_2 production rates at a steady state. MFA indicated that fructose 6-phosphate is replenished from the pentose phosphate pathway in sufficient amounts without contribution of the gluconeogenetic pathway (33). Overall, the observed enhanced XR activity in galactose supplemented xylose medium by transformant *S. cerevisie* suggested the cofactor availability importance for xylose metabolism in recombinant strain and improved xylitol production.

Metabolic pathways for xylose utilization

In 1960, Chiang and Knight found that the filamentous fungus *Penicillium chrysogenum*

converted D-Xylose to D-xylulose through a two-step reduction and oxidation and noticed xylose utilizing enzyme in the bacteria was different. This finding, as well as some further investigations (15) led to the conclusion that the two-step conversion of D-xylose to D-Xylulose is specific for yeasts and fungi, whereas in bacteria the same conversion is catalyzed by xylose isomerase in a single step. The detection of xylose isomerase in the yeasts *Rhodotorula* (42) and *C. boidinii* no. 2201 (149) is one of the few exceptions to this generalization.

In xylitol producing yeasts, xylose is reduced to xylitol either by NADH- or NADPH-dependent xylose reductase (aldose reductase EC 1.1.1.21). The produced xylitol is either secreted from the cell or oxidized to xylulose by NAD- or NADP-dependent xylitol dehydrogenase (EC 1.1.1.9). These two reactions are considered to be limiting for D-Xylose fermentation and xylitol production. The ratio of xylose reductase and xylitol dehydrogenase in addition to cofactor regenerating system is the major metabolic regulator for xylitol production. However, certain strains of yeast are known to utilize xylose as a carbon source via the phosphorylation of xylulose to xylulose-5-phosphate which is catalyzed by xylulokinase (EC 2.7.1.17) (60, 124). A detailed study of biochemistry and physiology of the yeasts metabolizing xylose was published by Hahn-Hagerdal et al. (37). In fact, the conversion of D-xylose to xylitol in yeasts cannot be separated from the conversion of D-Xylose to other metabolic products such as carbon dioxide, ethanol, acetic acid and polysaccharides.

Coenzyme specificity

The first two enzymes, D-xylose reductase (XR) and xylitol dehydrogenase (XDH), of xylose utilization in xylitol producing microbial strain, are regulated by the ratio of cellular pools of NAD(P)H/NAD(P). These two enzymes require pyridine nucleotide cofactors and their specificity which differ with different yeast strains. It was reported that XR from, e.g. *Candida utilis* can utilise only NADPH (8), the XR from *Pachysolen tannophilus* CBS4044 and *Pichia stipitis* can use either NADH or NADPH as a cofactor (143, 144). The dual cofactor dependence of XR on NADH and NADPH may prevent a complete regeneration of NAD^+ which is needed for the XDH reaction (47, 57), and hence xylitol is secreted into the medium. Xylitol may also be formed due to the action of unspecific reductases, like *GRE3* (139).

Under anaerobic or oxygen-limited conditions, the difference in the cofactor requirements of these enzymes causes a redox imbalance which influences xylitol production in yeasts. In general xylitol formation is favored under oxygen-limited conditions because of the NADH accumulation and subsequent inhibition of NAD-linked xylitol dehydrogenase. Cell growth depends on some of the above metabolic products and it is also necessary that the cofactors be regenerated through different steps in the metabolic pathway. Therefore, for obtaining good yields of xylitol, the amount of xylose being converted to xylitol and the amount of xylitol which is available for further metabolism have to be well balanced (130).

Process regulatory factors on xylitol production

Bioconversion of xylose to xylitol using microbial strains is generally influenced by, nutritional composition (substrate, nitrogen source and micro nutrients and their concentrations), culture and process conditions (temperature, pH, aeration, inoculum concentration, immobilization and reactor conditions) as well as genetic nature of the microorganisms (native isolates, mutants and recombinant strains).

Impact of nutritional composition

Among all nutritional parameters xylose concentration (51, 107, 108, 125, 132) yeast extract, urea, corn steep liquor, casamino acids, hydrolysate composition play a vital role on cellular metabolism and subsequent xylitol production. In general, in the presence of glucose, xylose utilization was strongly repressed and glucose followed by xylose sugar utilization was observed.

Kim and Oh (56) demonstrated a chemically defined medium with urea (5 gl^{-1}) as a nitrogen source and various vitamins supplementation as a substitute for a complex medium containing yeast extract (10 g l^{-1}) in the production of xylitol by *C. tropicalis*. *C. gluilliermondii* VTT-C-71006 growth on rare pentoses and their implications for production of pure xylitol was studied by Granstrom et al. (33) and observed that this yeast strain grew on all the tested pentoses like L-arabinose, L-ribulose, D-ribose and D-xylose and gave the fastest growth. Suryadi et al. (135) working with methanol-utilizing yeasts reported that *H. polymorpha* produces 43.2g/l xylitol from 100g/l D-xylose after 4 days of cultivation with 1% (v/v) methanol supplementation and further additions of urea, $(NH_4)_2SO_4$, and NH_4NO_3 proved to be effective for an increase of xylitol yield this yeast. The effect of different nitrogen sources on xylitol production from D-xylose by *Candida* sp.L-102 was also reported by Lu et al. (67) and maximum xylitol production of 87 % was obtained with urea as the nitrogen source. Yeast extract at a maximum concentration of 10 g/l was found to be optimum for xylitol production by *C. tropicalis* DSM 7524 and concentrations higher than 15 g/l blocked the conversion of D-xylose to xylitol (120).

Increase in concentration of yeast extract from 5 and 10g/l increased the biomass production but a sharp decrease in xylitol productivity was identified for *C. gluilliermondii* FTI 20037 (121). Similarly the addition of yeast extract and peptone to the chemically defined medium enhanced cell growth of *C. mogii* ATCC 18364 but had little impact on the yield and specific productivity of xylitol (122). However, in some yeasts, special nutrient supplementation improved xylitol production. Lee et al. (63) reported that high-biotin containing medium favored ethanol production over that of xylitol with *P. tannophilus* NRRL Y-2460, while in *C. gluilliermondii* FTI 20037, xylitol formation was favored under similar conditions.

Role of temperature and pH on xylitol production

In general, the most suitable temperature for xylitol production in yeasts is 30°C. However, the xylitol yield was temperature-independent when the yeast was cultured in a temperature range between 30°C and 37°C but above 37°C the xylitol yield decreases sharply (120). Exceptions to this were observed by Sreenivas Rao et al. (132) where a variation of 3°C influenced (27%) on xylitol production in *C. tropicalis*. No variation in xylitol formation in *C. guilliermondii* FTI 20037 was noticed in temperature range of 30 and 35°C but decreased when the temperature increased to 40°C (Barbosa et al., 1988). The conversion of D-xylose to xylitol by *Candida sp.* B-22 was relatively constant over the temperature range of 35-40°C and further increase in temperatures to 45°C and higher, the conversion was sharply reduced (Cao et al., 1994). This was probably due to loss of the activities of both NADPH and NADH-dependent xylose reductase associated with the temperature increase (123). Sampaio et al. (108) reported a significant observation, that xylitol production with *D. hansenii* UFV-170 was hardly affected either at lower (10–20 °C) or higher (40–45 °C) temperatures. Wilkins et al., (2008) reported higher xylitol production at above 45°C with thermotolerant yeasts.

The yeasts are generally cultivated at pH values between 4 and 6. However, variation also reported in literature. For example, *C. parapsilosis ATCC* 28474 (86) and *C. guilliermondii* NRC 5578 (75, 86) revealed the maximum growth at pH 6.0 while, *Candida mogii* ATCC (122) and P. *stipitis* NRRL Y-7124 show optimum at pH 5 and 5.5, respectively whereas, pH 4 was optimum for *C. tropicalis* IFO 0618 (43). In general, the optimum initial pH value for the best xylitol yield in *C. boidinii* was 7.0 (142, 150), whereas under controlled conditions, a pH of 5.5 (142). Batch culture of *C. parapsilosis* ATCC 28474 (64) showed higher performance in xylitol production at pH 6 while for continuous culture a pH of 4.5 was found to be effective (26). Variation of pH from 4.5 to 5.5 did not show any influence on xylitol production by isolated *C. tropicalis* (130, 132). In contrast Silva and Afschar (120) reported that *C. tropicalis* DSM 7524 was not very sensitive to pH and attained a maximum xylitol yield at pH 2.5. Increasing the pH from 2.5 to 4.0 led to an increase in xylitol productivity but a decrease in xylitol yield. Sampaio et al (108) noticed that the percentage of xylose consumed for xylitol production progressively increased with pH and decreased reaching nearly constant values at pH 4.0. This process is associated with both biomass growth and catabolic reaction through the TCA cycle.

Inoculum

Conflicting reports are available in the literature on xylitol production versus innoculum loading. On studying the effect of initial cell concentration of *Candida sp.* B-22 on xylitol production from D-xylose, Cao et al. (9) reported that the rate of xylitol production was linear and the fermentation time was dramatically reduced over an initial biomass concentration range of 3.8 to 26 $g.l^{-1}$. The authors noticed 210$g.l^{-1}$ of xylitol with an initial yeast cell concentration of 26 $g.l^{-1}$ and using 260 $g.l^{-1}$ D-xylose indicating a high initial cell mass concentration is beneficial for xylitol production by *C. boidinii* NRRL Y17213. In another study, Vandeska et al., (142) reported a doubled xylitol yield and specific productivity with the increase of inoculum level from 1.3 to 5.1 $g.l^{-1}$ using initial D-xylose concentration of 50 $g.l^{-1}$. Use of very high inoculum observed to improve the xylitol formation under nitrogen limitation environments. In addition, xylitol formation was simultaneously influenced by the physiological state of the culture and the concentration of biomass (109). However limited variation in xylitol production was observed by *C. tropicalis* with the use of inoculum concentration in the range of 6%-10% (132).

The effect of inoculum size on the microbial production of xylitol from hemicellulose hydrolysates was also investigated. A high initial cell density did not show any positive effect when *C. guilliermondii* FTI 20037 when grown on rice straw hemicellulose hydrolysate since increasing the initial cell density from 0.67 $g.l^{-1}$ to 2.41 $g.l^{-1}$ decreased biomass formation, xylose utilization and xylitol accumulation (99). On the contrary *D. hansenii* NRRL Y-7426 grown on wood hydrolysate produced more xylitol at higher initial cell densities (91). Overall, the relationship between biomass and xylitol production was observed to be dependent the microbial strain physiological growth and metabolic properties.

Aeration

With respect to aeration, the oxygen supply rate is a key parameter for D-xylose metabolism in xylitol producing yeasts and determines whether D-xylose will be fermented or respired. It is very important, therefore, for an effective process to determine the oxygen flux that will enable balanced utilization of carbon both for growth and xylitol production.

Xylitol production by yeasts is always associated with micro aerobic conditions. Several authors reported aeration and agitation effects

on yeast growth and xylitol production (7, 14, 132, 152). In general, under strict aerobic and anaerobic conditions, xylitol is not produced extracellularly (115). Kastner et al. (50) reported that the growth of the xylitol producing organism, *Candida shehatae*, is drastically affected when the culture was incubated under anaerobic conditions and a step change from aerobic to anaerobic improved product formation. Walther et al. (152) reported that oxygen limitation and initial xylose concentration had considerable influences on xylitol production by *C. tropicalis* ATCC 96745. Under semi-aerobic conditions, the maximum xylitol yield was 0.62 $g.g^{-1}$ substrate, while under aerobic conditions, the maximum volumetric productivity was 0.90 $g.l^{-1}.h^{-1}$. Granstrom et al. (33) studied the metabolism of xylose by *C. tropicalis* in oxygen-limited chemostat and reported glycolytic and gluconeogenetic enzymes are expressed simultaneously facilitating substrate cycling based on an *in vitro* enzyme assay. The authors were able to enhance the redox imbalance by co-feeding of formate which increased xylose and oxygen consumption. Santos et al. (112) working with immobilized cells of *C. guilliermondi* on porous spheres reported xylitol production in fluidized bed reactor using sugarcane bagasse hemicellulose hydrolysate and reported a maximum xylitol (17.0 $g.l^{-1}$) yield with an aeration rate of 75ml/min.

To determine the specific oxygen uptake rate at which *C. boidinii* NRRL Y-17213 begins to produce xylitol, Winkelbausen et al. (154) cultivated yeast continuously under oxygen-limited conditions and noticed that xylitol secretion was triggered at 0.91 $mm.g^{-1}.h^{-1}$. No xylitol production was observed at specific oxygen uptake rates above this value. Upon a shift to lower specific oxygen uptake rates, as expected, xylitol production rates and yield increased more rapidly than those of ethanol. Branco et al. (7) studied the influence of the aeration on ca alginate immobilized *C. guilliermondii* cell concentration and reported the highest conversion efficiency (41%) using 1.33 vvm aeration rate and 40% immobilized system. Whereas, Roseiro et al., (103) reported a combinatorial influence of substrate concentration and aeration rate on xylitol formation in yeasts. The authors noticed a maximum xylitol productivity of 2.67 $g.l^{-}1$ when the initial $k_L a$, D-xylose and yeast extract concentrations were 172, 21 $g.l^{-1}$ and 452 $l.h^{-1}$, respectively.

Reports are also noticed in the literature on relationship between co-factor generation and aeration. The general characteristic of most xylose-fermenting yeasts is that their xylitol dehydrogenase uses predominantly NAD and very rarely the NADP cofactor (30, 31, 60, 64). The varying ratio of NADH- to NADPH-linked D-xylose reductase activity with aeration conditions was first found in *P. tannophilus* and similar variations were observed in the yeasts *C. parapsilosis* ATCC 28474 (86) and *C. boidinii* NRRL Y17213 (141). It has been noticed that oxygen may lower the ratio of NADH linked D-Xylose reductase and NAD-linked xylitol dehydrogenase activities and consequently minimize xylitol accumulation in D-xylose-fermenting yeasts (123). This was also observed in *C. boidinii* NRRL Y-17213 (141). The NADH/NAD ratio decreased 2-fold with increasing oxygen availability from 10 - 30 mmol/h.

It is very difficult to compare data from different studies because oxygenation is measured and reported differently. Yet it is evident that yeasts producing xylitol require small amounts of oxygen that is specific for each yeast strain. It is observed that *D. hansenii* has the highest demand for oxygen compared to other yeasts (107).

Optimization studies

Nutritional, physiological, operational, genetical and metabolic parameters are important for the economic xylitol production by microbial strains at industrial scale. The scientific community has performed elaborate optimization studies using several statistical approaches (127). When optimizing the xylitol production rate of C. *tropicalis* ISO 0618 by employing the Box-Wilson method, Horitsu et al. (43) found that the interaction between D-xylose concentration and aeration rate is related to cell biomass concentration. Rodrigues et al. (100) used response-surface methodology for xylitol production optimization from sugarcane bagasse hydrolysate in a fed-batch process and reported the best experimental parameter for achieving a maximum of 0.78g of xylitol per g of xylose by using *C. guilliermondii*. Whereas, Carla et al. (11) used a fractional factorial design for selection of important variables on xylitol biosynthesis from rice straw hydrolyaste by *C. guilliermondii*. The authors noticed that all four selected factors such as xylose concentration, inoculum level, agitation speed and nutrient supplementation have played a critical role in the xylitol fermentation and the most important factor is initial xylose concentration. Genetic algorithms coupling neural network was used for optimization of six medium components for xylitol production by *C. mogii* by Baishan et al. (2) and noticed 0.65g xylitol production per g of xylose utilized. In another study, Sreenivas Rao et al. (132) optimized incubation temperature, pH, agitation, inoculum size, corn steep liquor, xylose, yeast extract and KH_2PO_4 requirements for maximum xylitol production using Taguchi methodology and achieved 78.9% conversion at optimized environment with isolated *C. tropicalis*. Optimization studies are also reported for fed-batch fermentation based xylitol production by *C. tropicalis* ATCC 13803 by Kim et al. (53) and noticed 0.75% xylitol conversion rate per gram of xylose utilization.

Xylitol production by immobilized yeasts

Another way to improve the process parameters is the use of immobilized cells since it allows obtaining high cell concentration in the reactor, with the increase in the efficiency and productivity of the process. In addition, the use of immobilized cell systems make possible the recovery of cells for later use in repeated batch operations. A good performance of an immobilization system depends on immobilization matrix properties, procedures employed, reactor configuration and bioconversion conditions (93, 97). Reports on use of different matrices have been evaluated for immobilization of cells and for xylitol production. Carvalho et al. (13) and Branco et al. (7) working with alginate immobilized *C. guilliermondii* cells reported repeated use of these cells for bioconversion in stirred tank reactor with average productivity value of 0.43 g/l and 0.21 g/l/h, respectively. In another study, Santos et al. (111) observed more than 70% bioconversion of xylose to xylitol with *C. guilliermondii* cells immobilized on natural sugarcane bagasse fibers. The maximum yield was 0.73 g of xylitol per gram of xylose consumed was noticed by Liaw et al. (65) with *Candida subtropicalis* immobilized in polyacrylic hydrogel thin films whereas, Cunha et al., (18) reported increased productivity with increase in recycling of polyvinyl alcohol immobilized *C. guilliermondii*. Silva and Afschar (120) immobilized the cells of *C. tropicalis* DSM 7524 on a porous glass and used them in a fluidized bed reactor. The authors intended to reuse the immobilized cells several times by repeating the batch fermentation with substrate shift. However the yeast was degenerated after completion of the first cultivation and addition of fresh medium. Under continuous conditions, the immobilized cells of *C. guillermondii* converted D-xylose into xylitol with a high productivity of 1.35 $g.l^{-1}.h^{-1}$.

Co-immobilization of different microbial strains and their use in xylitol bioconversion

revealed improved productivity values. The highest conversion rate was observed when benzene-treated cells were co-immobilized in the photo-crosslinkable resin prepolymers ENT 2000 and 4000 (85). Almost 100% of the D-xylose (4.5 $g.l^{-1}$) was converted into xylitol after 33 h of incubation when the volume ratio of immobilized methanogen to immobilized *Candida pelliculosa* was 1:2. In the co- immobilized cell system, the degree of conversion and the conversion rate of D-xylose were higher than those in the separately immobilized cell system. Co-immobilized cells were stable for about 2 weeks with approximately 35% conversion. Lohmeier-Vogel et al. (66) studied the glucose and D-xylose metabolism in agarose-immobilized *C. tropicalis* ATCC 32113 by nuclear magnetic resonance. NMR studies showed that neither glucose nor xylose metabolism was enhanced by use of an immobilization process. Attempts to improve the rate of D-xylose metabolism by increasing the oxygen delivery to the entrapped cells were not successful.

Bioreactor process strategies

Most of the xylitol bioconversions by employing the microbial strains are associated with batch culture methods either at flasks or lab batch stirred tank reactors with the use of free or immobilized cell systems and pure xylose or xylose containing hydrolysates (3, 31, 75, 79, 86, 87, 103, 126, 129, 132, 142, 150, 153) with productivity values ranging from 0.55 to 0.78 gram substrate per gram xylitol. Application potential of these batch processes at industrial scale is time consuming as batch processes are associated with preparatory activities such as regular inoculum development, sterilization of the reactor, etc involving considerable input of labour, energy and time leading to decreased productivity. Efforts have been made to improve the product volumetric productivity values using different reactor configurations and varying the process parameters. In this context, continuous culture techniques often provide better productivities and yields. Santos et al. (111) working on the development of a bioprocess for the continuous production of xylitol from hemicellulosic hydrolysate using *C. guilliermondii* immobilized cells reported 70% xylose to xylitol bioconversion. Similar xylitol productivity values with *C. guilliermondii* FTI20037 under continuous fermentation using sugarcane bagasse hydrolysate have been reported by Martinez et al. (72) however, the authors noticed little impact of k_La on volumetric productivity which is interesting phenomenon in xylitol production process. Faria et al. (23) evaluated the role of membrane bioreactor in a view to achieve the simultaneous separation of xylitol during continuous bioconversion process and noticed the best performance (86% conversion) with 0.2 µm pore diameter containing membrane at a dilution rate of 0.03 per hour. An improvement of 30% on xylitol production/conversion under continuous cultivation of *D. hansenii* was observed with the supplementation of small amounts of glucose and at lower aeration environments (137). In fact production of xylitol from hemicellulosic hydrolysate may be more effective with the use of mixed culture as in continuous process and process efficiency depends on removal of other monosaccharides from the hydrolysate by the co-microbial culture (21). In most of the continuous reactor configurations, a substantial improvement in productivity values can be achieved only by using low dilution rates of xylose with high residence time, which is very difficult to achieve in practice for bulk production.

Research has therefore focused on xylitol production by fed-batch mode where substrate concentration can be maintained at a suitable level throughout the course of fermentation, i.e., a level sufficient to induce xylitol formation but not to inhibit microbial growth. In addition, these processes generally operate with high initial cell density which normally leads to an increase in volu-

metric productivity. The yeast *C. boidinii* NRRL Y17213 gave better results when cultivated in a fed-batch fermentor compared to other ways of cultivation. The highest xylitol yield was 75% of the theoretical yield, compared to 53% in the batch culture. The productivity of 0.46 $g.l^{-1}. h^{-1}$ was twice as high as the highest obtained under batch conditions (140). Olofsson et al. (89) reported xylitol production yield of 0.67% under fed-batch condition using recombinant *S. cerevisiae* strain with wheat straw hydrolysate. Whereas, Oh et al. (87) working with glucose-limited fed-batch cultivation of recombinant yeast strain observed an 1.9 fold increase in specific xylitol productivity over a control strain containing only xylose reductase enzyme. In order to improve the volumetric productivity and to overcome loss of xylitol producing biocatalysts in repeated fed-batch reactors, cell recycle attachment with hollow fiber membrane was employed and 3.8-fold increases were observed compared with the corresponding values of batch-type xylitol production parameters (1). Xylitol productions by other reactor configurations are also reported in the literature. Branco et al. (7) reported only 41% conversion of xylose to xylitol in bubble column bioreactor using immobilized *C. guilliermondii* and sugarcane bagasse hydrolysate. More than 70% xylitol yield was reported with the use of semi-continuous process in stirred tank reactor by alginate immobilized yeast cells (13).

Future prospects and conclusions

Xylitol is gaining the commercial importance due to its application potential in health and pharmaceutical sectors. Xylose is the raw substrate used for xylitol production either by chemical hydrogenation or by bioconversion with certain microbial species. Chemical production of xylitol is cost-intensive, energy consuming process and production economics depend on purity of the xylose and the main source of xylose is xylan from hemicellulosic biomass. Hemicellulosic xylan can be converted to xylose either by chemical or enzymatic hydrolysis which is depend on the parameters related to biomass, hydrolysis and enzyme. Chemical hydrolysis of biomass produces microbial growth inhibitors and needs detoxification. Detoxification of hydrolysate can be performed by physical, chemical and biological methods. However, the major challenge is for economic pretreatment technology with energy efficiency, in addition to optimum convertibility associated with reduced formation of degradation products. Development of species specific hydrolyzing enzymes would offer selective hydrolysis of xylan from renewable biomass as well as eliminate or reduce the inhibitory effects of some hydrolysates and xylose utilization in presence of other monomeric sugars. However, combination of all these detoxification methods is most suitable and cost effective approach but adaptation is necessary according to the microbial metabolic pathways. Many scientific groups have screened for xylose utilizers and noticed that *Candida* genus is the best for xylitol production. Molecular characterization of xylose utilizing yeast strains revealed the presence of a xylose utilizing clade in the phylogenetic tree. Xylitol production by any microbial strains is related to the balance of xylose reductase and xylitol dehydrogenase. Xylitol production depends on the nutritional, fermentation and physiological growth factors associated with micro-aerophillic conditions. Several studies have investigated the optimization of xylitol production using free or immobilized cells in batch or in continuous fermentation conditions using different reactor configurations. Considering the limitation of microbial conversion of xylose to xylitol, especially with the use of the necessary high dilution rates and residence time, it is important to focus on the development of xylose reductase dependent enzymatic bioconversion of xylose from hemicellulosic hydrolysate. The development of an independent microbial

metabolic cofactor regeneration system needs special attention. One of the other alternatives is to develop robust microbial systems by cloning the xylose reductase gene by recombination along with reduced cofactor generation system, however this has not been successful due to lack of continuous cofactor regeneration system. Use of co-substrates such as galactose for cofactor regeneration increased xylitol production indicating the need for further understanding and exploitation of this approach at the genetic level for successfull development of recombinant starins. Screening and development of robust and novel microbial strains with hydrolysate inhibitor tolerance play a pivotal role in xylitol production at the industrial scale. A focus should be maintained on a common platform of understanding of the hydrolysate material, hydrolysis procedure, microbial performance, bio-conversion environment and downstream processing is one of the most essential aspects for development of integrated technological solution for production of second generation biorefinary products like xylitol via biotechnological process at an economic industrial scale.

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Plants as source of novel Anti-Diabetic Drug: Present Scenario and Future Perspectives

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Abstract

For thousands of years plants and their derivatives are being used for treatment of diabetes. Application of modern science to traditional system of medicine has also given birth to compound like Metformin. More than 400 plants incorporated in approximately 700 recipes are used to treat diabetes mellitus in almost two thirds of the world population. A large number of animal studies to test the claimed activity have demonstrated the hypoglycaemic property of many of these plants. In addition, clinical trials have shown some plants as useful antidiabetic agents, but the pure chemical compounds isolated from the crude extracts of these plants do not bear structural resemblance to the antidiabetic drugs in current clinical use nor have they similar mechanisms of action. But still the search for a novel antidiabetic drug advocates the utilization of plants as a potential source and can be achieved by application of modern scientific technology and recent knowledge on the physiological changes in case of Diabetes.

Key words: - Hypoglycaemic, Natural Product, New Chemical Entity, FDA, Toxicity

Introduction

Since olden days, plants are used to treat many ailments and India has about 45,000 plant species and several thousands have been claimed to possess medicinal properties (1). It is also well known that certain foods may have the potential to prevent diseases (2, 3). For instance, the Mediterranean diet is helpful to lowering the risks of coronary heart disease, cancer and cognitive impairment (4-6). Consumption of green tea is beneficial for preventing cancer and Alzheimer's disease (AD) (7-9). It is also reported that adherence to vegetables (including cruciferous vegetables, green leafy vegetables, yellow vegetables, allium vegetables, tomatoes and others) and legumes (including soybean, peanut, *etc.*) is inversely associated with the risk of type 2 diabetes (T2D) in a large Chinese population (10, 11). Plants have been the basis of many traditional medicine systems throughout the world for thousands of years and continue to provide mankind with new remedies. Many plant based medicines now serve as the basis of novel drug discovery (12). The active principles of many plant species are isolated for direct use as drugs, lead compounds or pharmacological agents (13).

Diabetes mellitus is a major endocrine disorder (14) responsible for renal failure, blindness or diabetic cataract (15), poor metabolic control (16), increased risk of cardiovascular disease including atherosclerosis and AGE (advanced glycation end) products (17). The earliest recorded

attempt to treat diabetes mellitus dates back more than 3,500 years and the treatment used was of plant origin. Nowadays, insulin and other oral blood-glucose lowering agents are used in the clinical management of diabetes mellitus (18). The prevalence of this disease continues to rise worldwide and little, so far, can be offered to prevent delay its secondary complications. Thus, the search for new antidiabetic drugs with novel mechanisms of action should still be pursued.

Medical plants play an important role in the management of diabetes mellitus especially in developing countries where resources are meager. Medicinal plants used to treat hyperglycemic conditions are of considerable interest for ethno-botanical community as they are recognized to contain valuable medicinal properties in different parts of the plant and a number of plants have shown varying degree of hypoglycemic and anti-hyperglycemic activity (1). Several species of medicinal plants are used in the treatment of diabetes mellitus, a disease affecting large number of people world-wide. Traditional plant medicines or herbal formulations might offer a natural key to unlock diabetic complications (19). This review has summarized the plants/plant products as source of antidiabetic agents, the present status of herbal antidiabetic therapies, and future direction in the field of research and evaluation of plants, which may increase the chance of getting new antidiabetic drugs from existing herbal antidiabetic therapies.

Plants as source of antidiabetic drugs

Plants, as folk remedies, are widely used to treat diabetes mellitus. In modern allopathic medicine, however, their role is limited to the use of guar gum as an adjunct therapy (20). The therapeutic benefit of guar gum resides in its ability to reduce the calorific value of consumed diet by reducing absorption of carbohydrates from the gastrointestinal tract (21). Searching for a novel antidiabetic drug from plants should be advocated, since plants are well recognized as an important source of providing new drugs (22).

Natural products as source of antidiabetic agents

According to the review published by Newman and Cragg, nearly 32 New Chemical Entities has been filed with FDA for treatment of Diabetes, both types I and II in last 25years. These drugs include a significant number of biologics based upon varying modifications of insulin produced in general by biotechnological means. In addition to these well-known agents, the class also includes a very interesting compound (approved by the FDA in 2005) Extenatide (the first in a new class of therapeutic agents known as incretin mimetics), a Natural Product Derivative. The drug exhibits glucose lowering activity similar to the naturally occurring Incretin hormone glucagon-like peptide-1 (GLP-1), but is a 39-residue peptide based upon one of the peptide venoms of the Gila monster, *Heloderma suspectum* (23). Metformin created by Bristol-Myers Squibb Company is an oral anti-diabetic drug from the biguanide class. It is the first-line drug of choice for the treatment of type-2 diabetes, particularly in overweight and obese people and those with normal kidney function, and evidence suggests it may be the best choice for people with heart failure (24-27). The biguanide class of anti-diabetic drugs, which also includes the withdrawn agents phenformin and buformin, originates from the French lilac or Goat's Rue (*Galega officinalis*), a plant known for several centuries to reduce the symptoms of diabetes mellitus (28-30).

Present status of herbal antidiabetic agents

Approximately 80% of the populations of third world countries are dependent on traditional therapies for their health care (31), and has been substantiated by the WHO

recommendation to include traditional medicines in the primary health-care level of these countries (32). Most of the traditional therapies are constituted of plants. When tested using modern methods of evaluation, only 18% were found to exhibit some kind of pharmacological activity (33). According to the review compiled by Bnouham *et al* (34), the families of plants with the most potent hypoglycaemic effects include: Leguminoseae (11 species), Lamiaceae (8 sp.), Liliaceae (8 sp.), Cucurbitaceae (7 sp.), Asteraceae (6 sp), Moraceae (6 sp.), Rosaceae (6 sp.), Euphorbiaceae (5 sp.) and Araliaceae (5 sp.). The most commonly studied species are: *Opuntia streptacantha* Lem, *Trigonella foenum graecum* L, *Momordica charantia* L, *Ficus bengalensis* L, *Polygala senega* L. and *Gymnema sylvestre* R.

Published data

Plants are being used heavily to treat diabetes mellitus, an effort that resulted in having more than 700 recipes containing more than 400 plants reputed for their antidiabetic activity (18, 35-39). The comprehensive review on antidiabetic medicinal plants has been compiled by Atta-ur-Rahman and Zaman (36) provides information regarding nearly 343 plants reputed for their blood glucose lowering activity has been reviewed and classified according to their botanical name, native name, country of origin, part used and the nature of the active principle, if known. Ajay Babu *et al* has created DiaMedBase, a diabetes literature database of medicinal plants with abstract, plant parts, objective and a 'disease link' to diseases other than diabetes for each medicinal plant. DiaMedBase is constructed using html. Data are collected from various literature sources viz. PubMed, ScienceDirect, Mary Ann Libert, BlackWell Scientific, IngentaConnect, Scirus, Bentham Publishers, Wiley journals and others. Currently, DiaMedBase includes 742 records, constituting about 309 genus and an overall 389 species of plants described to possess medicinal properties against diabetes (40). The review by Ivorra *et al* (37) included details of the experimental data of 45 plants published in the original articles. Review done by, Bailey and Day (18) includes some of the published work on antidiabetic plants to evaluate their scientific merit as candidates for new antidiabetic drugs and classified the cited plants into groups like,

- Plants with tested hypoglycaemic activity where an active principle has been isolated
- Plants with hypoglycaemic activity but without characterized active principle
- Plants with disputed hypoglycaemic activity

Animal studies of herbal antidiabetics

Most of the works done for determining the antidiabetic or hypoglycemic property of plants include works done on animals (mice, rats, rabbits and dogs) where as very less experiment has been conducted on humans. Animal work comprised *in vivo* and *in vitro* (such as skeletal muscle, epididymal fat and liver) preparations. The animal models used for the *in vivo* work were either normoglycaemic or rendered diabetic by depriving the animals of their functioning beta-cells using chemicals (alloxan or streptozotocin) or surgery (pancrea-tectomy). The list of some plants tested on different animal experimental models for their potential hypoglycemic/blood glucose lowering activity in recent times has been presented in Table 1 (41-70).

In most of the reports, the mechanism of action was not included and all suggested mechanisms of action can be related, generally to the ability of the plant or its active principle to lower plasma glucose level by interfering with one or more of the processes involved in glucose homeostasis. However, a few reports on the benefit of medicinal plants in treatment of diabetes mellitus that are not directly related to blood

Table 1. Plants Tested in Animal Models for their Blood Glucose Lowering Activity

Sr. No.	Name of the Plant	Family	Part Tested
1	*Andrographis paniculata*	Acanthaceae	Root
2	*Barleria lupulina*	Acanthaceae	Aerial Part
3	*Amaranthus spinosus*	Amaranthaceae	Stem
4	*Ichnocarpus frutescens*	Apocynaceae	Root
5	*Catharanthus roseus*	Apocynaceae	Flower, Leaves, Stem & Root
6	*Hemidesmus indicus*	Asclepiadaceae	Roots
7	*Helichrysum plicatum ssp. Plicatum*	Asteraceae	Capitulum
8	*Centratherum anthelminticum*	Asteraceae	Seed
9	*Berberis aristata*	Berberidaceae	Stem Bark
10	*Adansonnia digitata*	Bombacaceae	Stem Bark
11	*Capparis sepiaria*	Capparaceae	Leaves
12	*Tridax procumbens*	Compositae	leaves
13	*Kalanchoe crenata*	Crassulaceae	Whole Plant
14	*Momordica dioica*	Cucurbitaceae	Fruit
15	*Cucurbita ficifolia*	Cucurbitaceae	Fruit
16	*Securinega virosa*	Euphorbiaceae	Leaf
17	*Mallotus Roxburghianus*	Euphorbiaceae	Leaves
18	*Butea monosperma*	Fabaceae	Bark
19	*Prunella vulgaris*	Labiatae	Spikes
20	*Rosmarinus officinalis*	Lamiaceae	Leaves
21	*Caesalpinia bonducella*	Leguminosae	seed
22	*Hibiscus rosasinensis*	Malvaceae	Flower
23	*Ceiba pentandra*	Malvaceae	Roots
24	*Nymphaea stellata*	Nymphaeaceae	Flower
25	*Picea glauca*	Pinaceae	Needle, Bark, and Cone
26	*Cynodon dactylon*	Poaceae	Whole Plant
27	*Talinum cuneifolium*	Protulaceae	Leaves
28	*Eriobotrya japonica*	Rosaceae	Leaves
29	*Aegle marmelos*	Rutaceae	Leaf and Callus
30	*Solanum xanthocarpum*	Solanaceae	Fruit

glucose level lowering activity have also been reported. *Trigonella foenumgraecum* was reported to be useful in reducing hypercholesterolaemia in alloxan- diabetic dog (71, 72) and humans (73). The water extract of *Poupartia birrea* (74) and a compound isolated from *Glycyrrhizae* radix (75) exhibited enzyme-inhibiting activity on aldose reductase *in vitro*. The majority of the experiments confirmed the benefits of medicinal plants with hypoglycaemic effects in the management of diabetes mellitus. Numerous mechanisms of actions have been proposed for these plant extracts. Some hypotheses relate to their effects on the activity of pancreatic ß cells (synthesis, release, cell regeneration/revitalization) or the increase in the protective/inhibitory effect against insulinase and the increase of the insulin sensitivity or the insulin-like activity of the plant extracts. Other mechanisms may involve improved glucose homeostasis (increase of peripheral utilization of glucose, increase of synthesis of hepatic glycogen and/or decrease of glycogenolysis acting on enzymes, inhibition of intestinal glucose absorption, reduction of glycaemic index of carbohydrates, reduction of the effect of glutathione. All of these actions may be responsible for the reduction and or abolition of diabetic complications. The proposed mechanisms can be summarized as follows:

- Stimulation of insulin secretion (76-78)
- Enhancement of glucose utilization by with insulin mimetic action both *in vivo* (79-81) and *in vitro* (82,83)
- Alteration of activity of some enzymes, involved in glucose utilization (84)
- Diminishing the release of some hormones like glucagons, that counteract insulin action (85)
- Actions, such as inhibiting lipolysis (86,87) or reducing intestinal glucose transport (88,89)

Clinical trials on herbal antidiabetics

Clinical trials employing normal subjects, type I and type II diabetics also have been cited in the literature (73, 89-109). The reported clinical usefulness of the tested plants is largely ascribed to their ability to decrease hyperglycaemia (100, 103), to reduce fasting plasma glucose after chronic administration (101, 103) and/or to improve glucose tolerance (73, 96, 99). As hypoglycaemic agents, two plants *(Momordica charantia* and *Gymnema sylvestre)* have been extensively tested both in animals and human. The hypoglycaemic activity was demonstrated in both types of diabetes mellitus implying the presence of an active principle(s) with insulin like action. A polypeptide called "vegetable insulin" was isolated from *Momordica charantia* (94, 98), whereas a glucoside was isolated from *Gymnema sylvestre* (84, 100). Along with the insulin-like effects attributed to these plants, a possible regeneration of Islet tissue has also been claimed in case of *Gymnema sylvestre* (110). Apart from *Momordica charantia* and *Gymnema sylvestre*, the other herbs that had been tested on Human Subjects are, *Allium sativum, Aloe vera, Artocarpus heterophyllus, Asteracanthus longifolia, Bauhinia forficate, Coccinia indica, Ficus carica, Panax quinquefolius, Myrcia uniflora, Ocimum sanctum, Opuntia streptacantha.*, Silymarin, *Trigonella foenum, Asteracantha longifolia, Hordeum vulgare, Ginkgo biloba, Withania somnifera* etc (111-114).

Chemistry of compounds derived from plants with antidiabetic activity

Ivorra *et al* (36) had studied the structure of 78 different compounds isolated from plants with attributed hypoglycaemic activity and classified these compounds as follows according to their chemical groups. Bailey and Day (18) also listed 29 compounds that contained 14 poly-saccharides, 5 alkaloids, 4 glycosides and 6 other

compounds. Study reveals that hundreds of components were identified from vegetables and legumes that are recorded in the Traditional Chinese Medicine Database (TCMD) and according to the pharmacological activity annotations, some of those components are directly associated with prevention and/or treatment of T2D, because of their aldose reductase inhibitory or hypoplycemic activity, besides the functions of many other vegetable and legume components (*e.g.*, antiatherosclerotic, antihypertensive, antilipemic, antithrombotic, lipase inhibitory, lipid peroxidation inhibitory, lipoxygenase inhibitory and platelet aggregation inhibitory) (Table 2) (115-119). The comparison of the structures of these components with those recorded in the Comprehensive Medicinal Chemistry (CMC) database (120) and the MDL Drug Data Report (MDDR) database (121), it is evident that some of these agents have been recognized by modern Western medicine (Table 3). Although some activities annotated in CMC and MDDR are not the same as displayed in TCMD, they are also associated with combating T2D. Taken together, the analysis clearly indicates that vegetables and legumes also contain many antidiabetic components, which provide new clues to understanding the beneficial effects of vegetable and legume consumption on the risk of T2D (10, 11).

The classification done by Ivorra *et al* is as follows:

- Polysaccharides and proteins
- Steroids and terpenoids
- Alkaloids
- Flavonoids and related compounds

It can be concluded that the majority of plants with blood glucose lowering activity contain polysaccharides. Day has cited 66 plant fractions (38) that contained hypogly-caemic polysaccharides, which lowers blood glucose level by impeding glucose absorption from the gastrointestinal tract and thus reducing postprandial hyperglycaemia. Guar gum is an example of this chemical class. Proteins and polypeptides don't add a great advantage to the current antidiabetic therapy in spite of their ability to act like insulin. The reason is that they have to be administered parenterally, so no novel advantage can be seen in exchanging insulin with an "insulin-like" agent if both have to be given parenterally. The other chemical groups include: alkaloids, flavonoids, terpenes, glycosides and related compounds. Clearly with such diverse chemical formulae, no clear resemblance can be observed to the oral blood glucose lowering agents in current clinical use, namely sulphonylureas and biguanides. Moreover, no common structure-activity relationship can be found to correlate with these chemical groups. In recent times, new drugs are being developed on basis of structure-activity relationship, no pharmacological sense can be extrapolated out of the chemical data presently available on compounds isolated from medicinal plants and shown to have hypoglycaemic activity.

Approved herbal products for treatment of diabetes

Despite the importance of plant-led discoveries in the evolution of medicine, herbal remedies are yet to get acceptance by the regulatory authorities throughout the world. The acceptance and recognition of herbal medicine has been in part due to the acknowledgement of the value of traditional and indigenous pharmacopoeias, the incorporation of some medicines derived from these sources into pharmaceuticals (122), the need to make health care affordable for all and the perception that pharmaceutical drugs are increasingly overprescribed, expensive and even dangerous. Another important perception fomenting this interest is that natural remedies are somehow safer and more efficacious than remedies that are pharmaceutically derived (123, 124).

The Department of Indian Systems of Medicine & Homoeopathy has taken initiative in this direction and the concerted efforts of various experts of Ayurveda and departmental technical staff members have resulted in bringing out the document – "Essential Ayurveda Drugs for Dispensaries and Hospitals" which is notably different from Essential Drugs List of Allopathic System of Medicine. The publication aims at providing ready reference for selection or procurement of Ayurvedic drugs for dispensaries and hospitals of various levels. Its utility is much higher for the learners and practitioners of Ayurveda as it will provide a window to peep into the wide range of Ayurvedic medicines required for setting up their professional establishments (125). Many herbal extracts or derivatives have been documented in traditional Chinese medicine (TCM) as having clinical effectiveness in treating sugar imbalances in diabetes mellitus. Thus far, there are seven antidiabetic drug products of plant origin that have been approved for clinical use in China. Table 4 lists all these approved products and the composition of the formulas (126).

Recent regulatory developments

WHO has recently defined traditional medicine (including herbal drugs) as comprising therapeutic practices that have been in existence, almost for several hundreds of years, before the development and spread of modern medicine and are still in use today. The traditional preparations comprise medicinal plants, minerals, organic matter, etc. Herbal drugs constitute only those traditional medicines, which primarily use medicinal plant preparations for therapy.

In recent years the FDA and the EMEA have reviewed the regulatory frameworks governing the development and use of botanical drug and provided a significant fillip to the Natural Products Industry have significantly lowered the entry barriers for botanicals vis-à-vis chemicals and biologicals in these regions. These new guidelines more importantly also provide for unique guarantees of market exclusivity for botanicals as well as the acceptance of synergistic combinations of bioactives. So, the acceptance of Herbal remedies by the regulatory authorities has given a certain fillip to research in this field. India and countries like China, with their vast library of natural compounds - some actively used in traditional systems and many still not codified - has clearly a natural advantage over the others (127, 128).

Future perspectives

If plants are to be used according to their original traditional method, the WHO guidelines (129) on their use should be applied to rationalize that use, and to ensure consistency, efficacy and safety of these products. In spite of the various challenges encountered in the medicinal plant-based drug discovery, Natural products compounds discovered from medicinal plants (and their analogues thereof) have provided numerous clinically useful drugs and still remain as an essential component in the search for new medicines. So, these traditionally used plants can be exploited effectively in order to find New Chemical Entity for treatment of diabetes.

There are many ways to approach for getting new biologically active principles from higher plants. One can simply look for new chemical constituents and hope to find a pharmacologist who is willing to test each substance with whatever pharmacological test is available, but can not be considered to be a very valid approach. A second approach is random collection and broad screening, which means simply to collect every readily available plant, prepare extracts, and test each extract for one or more types of pharmacological activity. This is a reasonable approach that eventually should produce useful drugs, but it is contingent on the availability of adequate funding and appropriate predictable bioassay systems. During random

selection process prior information on the following three key factors will be helpful in choosing a suitable plant for getting a new drug for diabetes.

- Information of Traditional/Folkloric Use of the Plant
- Information on Chemical constituents of the plant
- Taxonomical Position of the plant in the Plant kingdom and information on any other Plants of same Taxonomical Family/ Genus known to have antidiabetic Activity

To achieve success in this field following measures are being proposed,

Restoring the ethnomedical knowledge

The body of existing ethnomedical knowledge has led to great developments in health care. With the rapid industrialization of the planet and the loss of ethnic cultures and customs, some of this information will no doubt disappear. An abundance of ethnomedical information on plant uses can be found in the scientific literature but has not yet been compiled into a usable form. Collection of ethnomedical information remains primarily an academic endeavor of little interest to most industrial groups. The use of ethnomedical information has contributed to health care worldwide, even though efforts to use it have been sporadic. Are we loath to continue plant-derived drug discovery efforts because we anticipate that current industrial technology, i.e., mass screening, will provide novel drugs at a greater rate than will the ethnomedical information already at hand? "Those who cannot remember the past are condemned to repeat it" (130).

Establishment of mechanism of action

There has been insufficient in-depth investigation into the possible mechanism of action for majority of the plants with claimed "antidiabetic" properties. The value of a plant product as a novel antidiabetic agent can be appraised if its mechanism of action, among other factors, is assessed in comparison with current antidiabetic agents in clinical use. A plant that is anticipated to yield a new antidiabetic drug is more likely to be ultimately utilized if it reveals a novel mechanism of action. Thus, testing crude extracts of plants would prove fruitful if it is associated with a scheme to isolate pure compounds.

Toxicity and safety profiling

Acute Toxicity, Sub-acute Toxicity, Chronic Toxicity and Pharmacological Safety profiling of plant products is as important as getting the mechanism of action of the products. As is well known, liver toxicity alters the activity of enzymes that control body metabolism. Thus, it is imperative to rule out that the claimed antidiabetic activity was a result of specific or nonspecific toxicity. In depth animal toxicity studies of the compounds are required prior to clinical testing.

Focusing on the antidiabetic activity

Testing of plants for "antidiabetic" rather than the "hypoglycaemic" effect is required, as a drug that may retard or prevent diabetic complications would have great clinical implications. In such an effort, one would compile the search for hypoglycaemic agents with that for agents which may counteract diabetic complications, a therapeutic goal that is of immense importance. Investigating other remote possibilities, such as regeneration of islet tissue as has been proposed with *Gymnema sylvestre* (110), may be included under this category.

New trends

Current knowledge on altered body metabolism during diabetes mellitus can be utilized for development of new trends in herbal antidiabetic research. Amylin, a polypeptide that is co-secreted with insulin have demonstrated to

Table 2. TCMD-documented vegetable and legume functional components associated with ameliorating Type-2 diabetes

Compound	Activity	Source
Aframodial	Antilipemic	Zingiber officinale Rosc.
Agavasaponin C	Platelet aggregation inhibitory	Allium sativum L.
Allicin	Antihypertensive, Antithrombotic	Allium fislulosum L. Allium sativum L.
Alliin	Antithrombotic, Platelet aggregation inhibitory	Allium cepa L. Allium sativum L.
Bergapten	Antihypertensive	Lycopersicon esculentum
beta-Sitosterol	Antilipemic	Glycine max (L.) Merr.
Camphene	Antilipemic	Zingiber officinale Rosc. Mentha haplocalyx Briq.
Daidzein	Lipase inhibitory	Glycine max (L.) Merr.
Ferulic acid	Platelet aggregation inhibitory	Allium cepa L.
Genistein	Lipase inhibitory	Glycine max (L.) Merr.
Glycitein	Lipoxygenase inhibitory	Glycine max (L.) Merr.
Isoeruboside B	Platelet aggregation inhibitory	Allium sativum L.
Isorhamnetin	Antilipemic	Oenanthe javanica (B1.)DC.
Kaempferol	Ä-5-lipoxygenase inhibitory	Vicia amoena Fisch. ex DC.
Leucocyanidin	Platelet aggregation inhibitory	Arachis hypogaea L.)
Lycopene	Antiatherosclerotic	Lycopersicon esculentum Momordica charantia L.
Methyl allyl trisulfide	Platelet aggregation inhibitory	Allium sativum L.
Myristicin	Platelet aggregation inhibitory	Apium graveolens L.
p-Coumaric acid	Antilipemic	Solanum tuberosum L.
Proto-iso-eruboside B	Antithrombotic	Allium sativum L.
Rosmarinic acid Platelet aggregation inhibitory	Antithrombotic, Mentha haplocalyx Briq.	
6-Shogaol	Antihypertensive, Platelet aggregation inhibitory	Zingiber officinale Rosc.
Solasonine	Platelet aggregation inhibitory	Capsicum *annuum L.* *Solanum melongena L.*
Soyasaponin A1/A2	Antilipemic	Glycine max (L.) Merr.
Soyasaponin A3/A4/A5/A6	Lipoxygenase inhibitory	Glycine max (L.) Merr.
Soyasaponin V	Lipoxygenase inhibitory	Glycine max (L.) Merr. Phaseolus vulgaris L.
Stigmasterol	Antilipemic	Glycine max (L.) Merr. Lablab purpureus (L.)Sweet Arachis hypogaea L. Phaseolus vulgaris L.
Tomatine	Antihypertensive	*Lycopersicon esculentum*
2-Vinyl-4H-1,3-dithiin	Platelet aggregation inhibitory, Antithrombotic, 5-lipoxygenase inhibitory	*Allium sativum L.*

Table 3. CMC- and MDDR-documented vegetable and legume functional components associated with ameliorating type 2 diabetes

Compound	Activity
Allicin	Hypolipidemic (CMC/MDDR); Hypocholesterolemic (CMC); Platelet aggregation inhibitory (MDDR)
6-Shogaol	Cyclooxygenase inhibitory (MDDR); Lipoxygenase inhibitory (MDDR)
beta-Sitosterinum (beta-Sitosterol)	Hypolipidemic (CMC)
Stigmasterin (Stigmasterol)	Antiatherosclerotic (CMC)

Table 4. Drug products and their ingredients approved in China for Treatment of Diabetes

Sr. No.	Drug product	Ingredients
1	Yi-jin	Panax ginseng (Ginseng) Atractylodes macrocephala (Largehead Atractylodes Rhizome) Poria cocos (Indian Bread) Opuntia dillenii (Cactus)
2	Ke-le-nin	Radix Astragalus (Milkvetch Root) Rehmannia glutinosa (Chinese Foxglove Root)
3	Yu-san-xiao	Radix Astragalus (Milkvetch Root) Scrophularia ningpoensis (Figwort Root) Anemarrhena asphodeloides (Common Anemarrhena Rhizome) Rehmannia glutinosa (Chinese Foxglove Root)
4	Qi-zhi	Radix Astragalus (Milkvetch Root) Rehmannia glutinosa (Chinese Foxglove Root) Hirudo nipponia (Leech)
5	Shen-qi	Panax ginseng (Ginseng) Radix Astragalus (Milkvetch Root) Dioscorea opposita (Common Yam Rhizome) Coptis chinensis (Coptis Root) Rehmannia glutinosa (Chinese Foxglove Root) Cornus officinalis (Asiatic Cornelian Cherry Fruit) Hirudo nipponia (Leech)
6	Jin-qi	Lonicera japonica (Honeysuckle flower) Radix Astragalus (Milkvetch Root) Coptis chinensis (Coptis Root)
7	Xiao-ke-an	Radix Astragalus (Milkvetch Root) Pueraria lobata (Lobed Kudzuvine Root) Ophiopogon japonicus (Dwarf Lilyturf Tuber) Hirudo nipponia (Leech)

inhibit insulin release and muscle glycogenesis. Amylin is thought to play a major role in the disturbed metabolism associated with diabetes mellitus. The search for drugs that may antagonize amylin, and thus improve metabolic control in diabetic patients, is considered as a frontier in the search for novel antidiabetic agents (131). Medicinal plants that have been shown to improve the diabetic state without apparent enhancement of insulin secretion may be tested for amylin antagonism. Randle (132) has reviewed the role of disturbed free fatty acid metabolism, as a major factor in the development of diabetes mellitus. A selective approach testing the effects of plants on lipid metabolism would appear to be fertile.

Conducting clinical trials

Clinical trials are the proof of efficacy in humans for both crude and pure forms of herbal therapies. At present, relatively few clinical studies to test herbal therapies have been published. The justification of the use of herbal therapies in any form (crude or pure) can only be established with clinical trials.

Conclusion

Plants are being heavily utilized as antidiabetic therapies by many patients where traditional systems of medicine are in operation or as folk remedies and the use is justified in countries where modern health-care facilities are not readily available. Plants can also be utilized as a source of novel antidiabetic agents. For achieving the latter objective, it is suggested to enforce the ongoing research effort in this field as well as developing new areas where the likelihood of identifying new compounds may be increased.

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Biochemical characterization of a recombinant derivative (*Ct*Lic26A-Cel5) of a cellulosomal cellulase from *Clostridium thermocellum*

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Abstract

The truncated cellulase derivative (*Ct*Lic26A-Cel5) from *Clostridium thermocellum* cloned earlier into an expression vector pET21a was over-expressed using *Escherichia coli* cells (Taylor et al. 2005). The recombinant cellulase derivative (*Ct*Lic26A-Cel5) was purified by immobilized metal ion affinity chromatography. The purified enzyme on SDS-PAGE showed a single homogeneous band of molecular mass of 64 kDa. The enzyme derivative *Ct*Lic26A-Cel5 showed catalytic activity with soluble substrates such as lichenan, β-glucan and carboxymethyl cellulose. It also hydrolyzed insoluble substrates such as acid swollen cellulose, avicel and steam exploded bagasse. The cellulase derivative *Ct*Lic26A-Cel5 gave an optimum temperature of 50°C and an optimum pH of 4.3 for maximum activity when assayed with carboxymethyl cellulase as a substrate. Analysis of the thermal stability of enzyme derivative (*Ct*Lic26A-Cel5) revealed that the enzyme is maximally stable at 50°C. The Mg^{2+}, Ca^{2+}, Ni^{2+} and Na^{+} ions enhanced significantly (1.8-2.3 fold), the enzyme activity of *clostridial* recombinant derivative.

Key words: *Clostridium thermocellum*, cellulase, cellulose, carboxymethyl cellulose, bagasse

Introduction

Microorganisms produce a wide variety of cellulolytic enzymes that can hydrolyze the glycosidic bond of cellulose. Cellulose is composed of linear chains of β-1,4-linked D-glucosyl residues. Cellulases or Glycoside Hydrolases (GHs) differ in protein architecture, endo/exo specificity, and inverting/retaining reaction mechanism, but all hydrolyze the glycosidic linkage via general acid catalysis (1). Cellulases and hemicellulases are important enzymes that are widely used in the paper, animal feed, fruit juice, detergents, textiles and alcohol fermentation industries (2). By understanding the mechanism by which these enzyme systems degrade their complex insoluble and highly recalcitrant substrates, the efficiency of biomass conversion to bioethanol or other products can be improved.

Most of the glycoside hydrolases (GHs) have modular structures and comprising catalytic modules connected via linker sequences to one or more non-catalytic modules called carbohydrate-binding modules (CBMs). Glycoside hydrolases, are grouped into 113 families based on primary sequence similarities (3) and have been listed in the carbohydrate active enzymes database (CAZy), website (http:/

/afmb.cnrs-mrs.fr/~cazy/CAZY/index.http://www.cazy.org/) based on primary sequence similarities. The full length cellulosomal cellulase (*Ct*Lic26A-Cel5E) enzyme from *Clostridium thermocellum* displays a modular architecture containing an N-terminal family 26 GH module (Lic26A), internal family 5 GH (Cel5E) and family 11 carbohydrate binding module (*Ct*CBM11) modules and a C-terminal dockerin. The bifunctional cellulase from *C. thermocellum* has hydrolysing activity for soluble as well as insoluble cellulose substrates (4). The associated family 11, carbohydrate binding module (CBM11) does not enhance the degrading action of enzyme Lic26A (4). The crystal structure of CBM11 has been determined, and it was shown that CBM11 binds to ligands such as lichenan and β-glucan that are substrates for both Lic26A and Cel5E catalytic modules (5,6). Both the catalytic domains Cel5E or GH5 (7) and Lic26A (8,9) displayed activity towards lichenan and β-glucan. Interestingly, Lic26A was found to bind glucomannan but did not hydrolysed it, whereas it hydrolysed lichenan but did not bind it (10). In the present study the recombinant bifunctional cellulase derivative (*Ct*Lic26A-Cel5) from *C. thermocellum* (4) was over-expressed and purified by immobilized metal ion affinity chromatography. The bifunctional cellulase was analyzed for various substrate specificities and was functionally characterized.

Materials and Methods

Over-expression and purification of cellulase derivative *Ct*Lic26A-Cel5

The *Escherichia coli* strain used in this study was BL-21 (Novagen) for protein expression. The recombinant plasmid was the construct from the expression vector pET21a (Novagen) containing the bifunctional cellulase (*Ct*Lic26A-Cel5) gene insert from *Clostridium thermocellum*. The recombinant gene also contained a sequence for a His_6 tag (4). The substrates were purchased from Sigma Chemical Company, Kolkata, India. β-Glucan was donated by Dr. S. Charnock, Megazyme International Ltd., Dublin, Ireland and Steam Exploded Bagasse (SEB) was donated by Dr. A.J. Varma, National Chemcial Laboratory, Pune, India. The *E. coli* (BL-21) cells harbouring the recombinant plasmid containing *Ct*Lic26A-Cel5 gene were cultured in Luria Bertani medium supplemented with 100μg/ml ampicillin at 37°C and grown to mid-exponential phase (A_{550} 0.6) at which point isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the cultures were incubated for further 8h for protein induction (4). The cells were collected by centrifugation and the cell pellet was resuspended in 50 mM sodium phosphate buffer pH 7.0. The cells were sonicated and centrifuged at 4°C. The supernatant containing soluble protein was collected and passed through a 0.45 μm filter membrane. The protein was purified to homogeneity by immobilized metal ion affinity-chromatography (5) using 1 ml prepacked affinity columns (HiTrap Chelating HP, GE Healthcare, India). The purified protein was dialysed against 20 mM sodium phosphate buffer pH 7.0 with at least three changes using the same buffer. The concentration of the purified protein was determined by the method of Bradford, 1976 (11) and the purity was analysed by SDS-PAGE (8).

Activity assay of cellulase derivative *Ct*Lic26A-Cel5

The enzyme assay was carried out by incubating the enzyme with substrate for 10 min at 50ºC. The reaction mixture (100 μl) contained 10 μl of enzyme and 2% final carboxymethyl cellulose (CMC) in 100 mM sodium acetate buffer pH 4.3. The 100 μl reaction mixture was analyzed for the release of reducing sugar using the methods of Nelson, 1944 (12) and Somogyi, 1945 (13). The activity of cellulase against various polysaccharides was determined by incubating the

enzyme with the polysaccharide. The enzyme (0.83 mg/µl) was incubated with substrates in 100 mM sodium acetate buffer (pH 4.3) at 50°C and the activity was determined as described earlier.

Characterization of cellulase derivative *Ct*Lic26A-Cel5

The effect of pH on the activity of cellulase derivative *Ct*Lic26A-Cel5 was determined at 50ºC using different buffers. To explore the pH profile 100 mM sodium acetate for pH 4-6 and sodium phosphate/citrate buffer for pH 6-7.5 were used in enzyme assays that employed 2.0% (w/v) CMC as the substrate. The optimum temperature for maximum activity of enzyme was determined by assaying the enzyme at various temperatures ranging between 40ºC to 90ºC in 100 µl reaction mixture containing 2% (w/v) CMC in 100 mM sodium acetate buffer (pH 4.3). The thermal stability was determined by incubating the 50 ml enzyme (0.83 mg/µl, 3.0 U/mg) at various temperatures ranging from 40ºC to 90ºC for 30 min. 10 µl aliquots of the enzyme were withdrawn and assayed at 50ºC using 2% CMC in 100 mM sodium acetate buffer pH 4.3. The enzyme assay was carried out by estimating the released reducing sugar using the Nelson, 1944 (12) and Somogyi, 1945 (13) method. The kinetic parameters were determined by carrying out the reactions with varying CMC and SEB concentrations under optimized conditions. The kinetic parameters were determined by GraphPad Prism software. The effects of certain salts like $MgCl_2$, $CaCl_2$, $NiSO_4$, NaCl and EDTA on the activity of enzyme were determined using CMC as substrate. The enzyme (10 µl) was incubated with 2% (w/v) CMC in 100 mM sodium acetate buffer (pH 4.3) in 100 µl reaction mixture volume containing varying concentrations of salts. The reaction mixture was incubated at 50ºC for 10 min. The released reducing sugar was analysed by the method given by Nelson, 1944 (12) and Somogyi, 1945 (13).

Results and Discussion

Purification and substrate specificity of the cellulase derivative *Ct*Lic26A-Cel5

The recombinant cellulase derivative *Ct*Lic26A-Cel5 of *Clostridium thermocellum* was purified by a single step immobilized metal ion affinity chromatography (IMAC) using 1 ml affinity columns. The enzvme was expressed as soluble protein and that appeared in the cell free extract. The Fig. 1 shows the SDS-PAGE analysis of purified enzyme from the cell free extract mainly as a single homogeneous band of molecular mass of 64 kDa with some minor contaminant protein bands. The purified enzyme was analyzed for specificities for various substrates. The enzyme showed high catalytic activity with soluble substrates such as lichenan, β-glucan and carboxymethyl cellulose as well as insoluble substrates such as acid swollen cellulose, avicel and steam exploded bagasse (Table 1). The bifunctional cellulase *Ct*Lic26A-Cel5 exhibits low

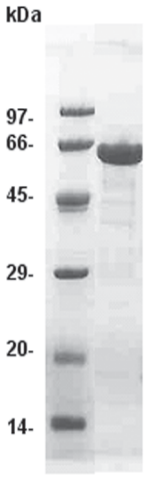


Fig. 1. SDS-PAGE analysis of purification of bifunctional cellulase derivative *Ct*Lic26A-Cel5. Lanes: (1) Mol. wt. marker and (2) purified cellulase derivative (64 kDa).

Table 1. Substrate specificity of bifunctional cellulase (*Ct*Lic26A-Cel5)[a].

Ligand	Enzyme activity[b] (U/mg)
Carboxymethyl cellulose (1%, w/v)	++++
Hydroxyethyl cellulose (0.5%, w/v)	++
β-Glucan (0.5%, w/v)	++++
Galactomannan, locust bean (1%, w/v)	++
Carob galactomannan (1%, w/v)	++
Glucomannan (1%, w/v)	-
Laminarin (1%, w/v)	-
Lichenan (1%, w/v)	++++
Xylan, oat spelt (0.5%, w/v)	++
Acid swollen cellulose (0.5%, w/v)	++++
Avicel cellulose (1.0%, w/v)	++++
Steam exploded bagasse (1.0%, w/v)	++++

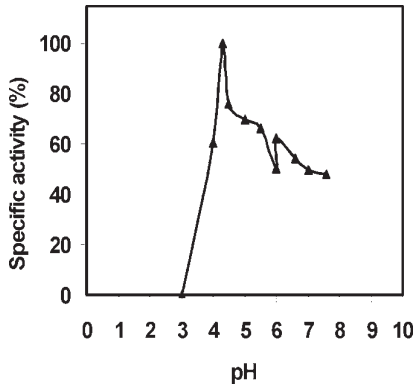
[a] The enzyme (0.83 mg/ml) was incubated with substrates in 100 mM sodium acetate buffer (pH 4.3) at 50°C.

[b] (-) No activity (below 0.2 U/mg); (++) Low activity (between 0.2 -1.0 U/mg); (++++) High activity (above 1.0 U/mg)

activity towards hydroxyethyl cellulose and xylan and no activity towards mannans and laminarin. The substrate specificity displayed by this is in agreement with the specificities shown by individual catalytic modules Cel5E (5) and Lic26A (4, 8) and thus is a bifunctional cellulase.

Biochemical characterization of cellulase derivative *Ct*Lic26A-Cel5

The *Clostridium thermocellum* cellulase derivative *Ct*Lic26A-Cel5 showed an optimum



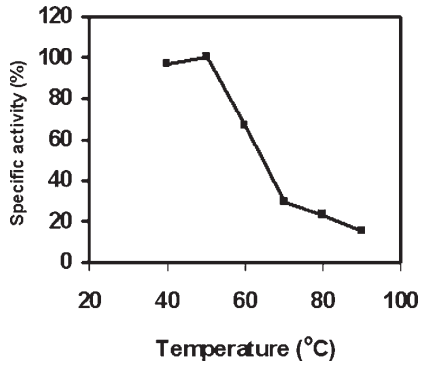


Fig. 2. Effect of pH (A) and temperature (B) on reaction of *Ct*Lic26A-Cel5 derivative with CMC. The reaction mixture (100 µl) contained 10 µl of enzyme (0.83 mg/ml, 3.0 U/mg) and 2.0% (w/v) CMC in 100 mM sodium acetate buffer (pH 4.3) and incubate d 50°C for 10 min. The enzyme activity was calculated as described in methods.

pH of 4.3 (Fig. 2A) and an optimum temperature in the range of 40-50°C (Fig. 2B) and when assayed with carboxymethyl cellulase as the substrate. All subsequent assays were performed using 100 mM sodium acetate buffer of pH 4.3 at 50°C following the optimum pH and the optimum temperature of cellulase derivative. A study of thermal stability of cellulase enzyme showed that, it is fairly stable up to 50°C (Fig. 3). The enzyme stability decreases drastically and

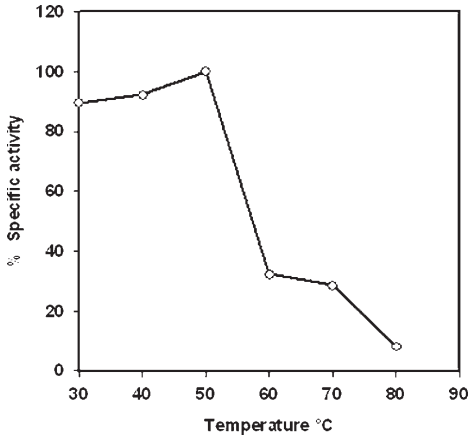


Fig. 3. Thermostability of cellulase derivative *Ct*Lic26A-Cel5. The enzyme (0.83 mg/ml, 3.0 U/mg) was incubated at different temperatures for 30 min. An aliquot (10 µl) of enzyme was taken in 100 µl reaction mixture containing 2% (w/v) CMC in 100 mM sodium acetate buffer (pH 4.3). The enzyme activity was determined as described in methods.

the activity drops by 80% as the temperature was raised to 60°C.

The kinetic parameters of cellulase derivative *Ct*Lic26A-Cel5 were determined using the soluble substrate CMC (Fig. 4A) and insoluble substrate SEB (Fig. 4B). The enzyme activity determined at increasing concentration of CMC or SEB was plotted and the data were analyzed using GraphPad software. The data analysis gave the values of V_m of 5.2 U/mg, K_m of 1.1% (w/v)

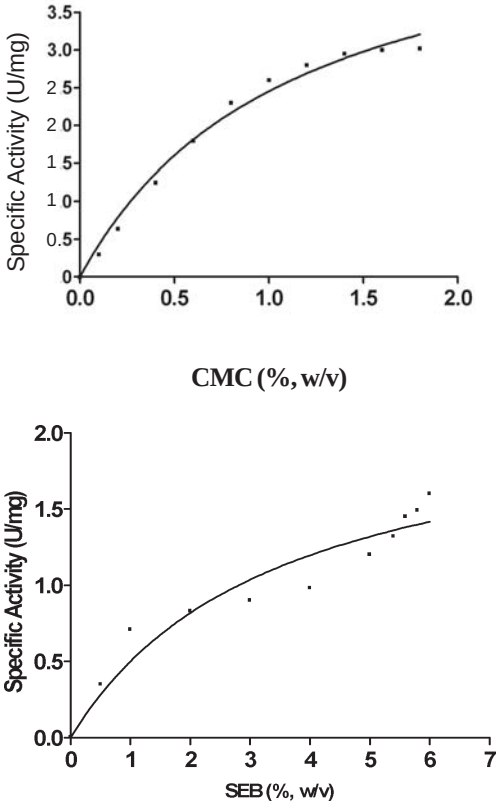


Fig. 4. Kinetics of cellulase derivative *Ct*Lic26A-Cel5 with CMC (A) and SEB (B). The reaction was performed with varying concentrations of CMC or SEB in 100 mM sodium acetate buffer (pH 4.3) at 50°C as mentioned in methods.

for CMC and 2.1 U/mg and 3.0% (w/v) for SEB, correspondingly.

The effects of Mg^{2+}, Ca^{2+}, Ni^{2+}, Na^{+} ions and EDTA on the activity of cellulase derivative *Ct*Lic26A-Cel5 were studied. All the three divalent metal ions and Na^{+} ions displayed a significant increase of the activity of recombinant cellulase derivative *Ct*Lic26A-Cel5 from *Clostridium thermocellum*. A concentration of 2 mM Mg^{2+} ions caused 180 percent increase of the enzyme activity (Fig. 5A). And a Ca^{2+} ion concentration of 6 mM also displayed the same enormous increase (180%) of CMCase activity (Fig. 5B). The increase in activity of enzyme

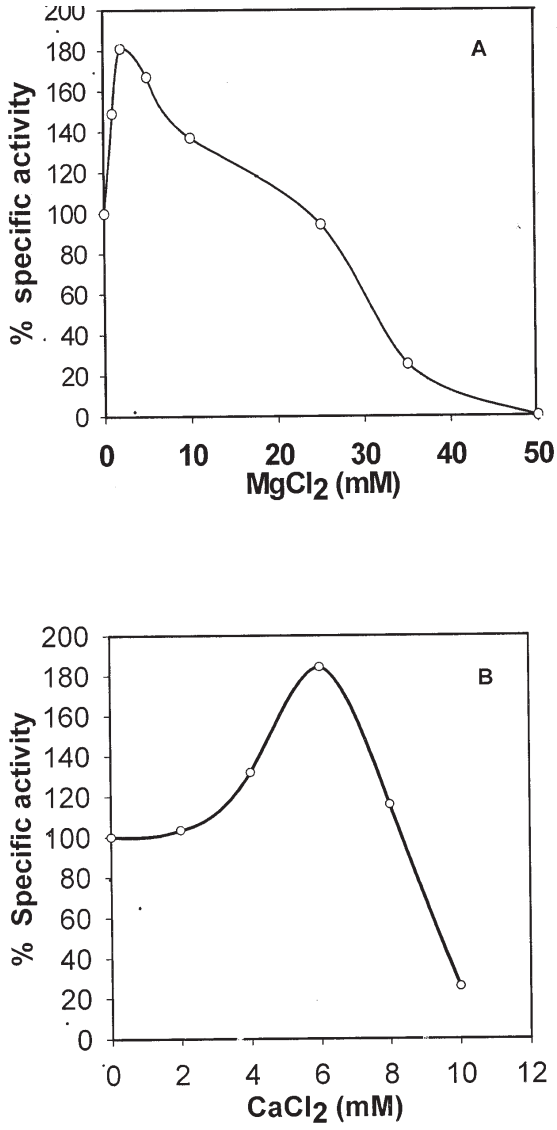


Fig. 5. Effect of $MgCl_2$ (A) and $CaCl_2$ (B) on cellulase derivative *Ct*Lic26A-Cel5. The assays were carried out in reaction mixture (100 µl) containing 10 µl of enzyme (0.83 mg/ml, 3.0 U/mg) with 2.0% CMC as substrate at 50°C and 0.1 M sodium acetate buffer (pH 4.3) and the enzyme activity was determined as described in the methods.

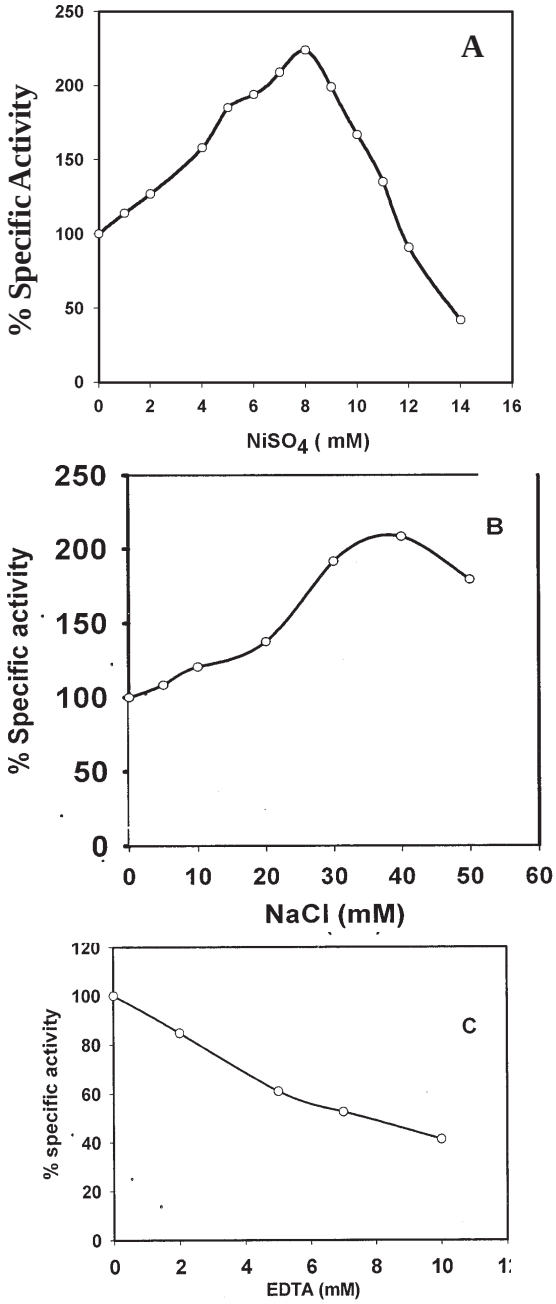


Fig. 6. Effect of $NiSO_4$ (A), NaCl (B) and EDTA (C) on cellulase derivative *Ct*Lic26A-Cel5. The assays were carried out in reaction mixture (100 µl) containing 10 µl of enzyme (0.83 mg/ml, 3.0 U/mg) with 2.0% CMC as substrate at 50°C and 0.1 M sodium acetate buffer (pH 4.3) and the enzyme activity was determined as described in the methods.

however was even much higher, around 225% at 8 mM Ni^{2+} ion concentration (Fig. 6A). It is possible that Ca^{2+} ions are required by the enzyme for its activity. The increase of enzyme activity of Cel5E or GH5 (5) and of Lic26A (6) by Ca^{2+} ions was also reported earlier though the increase was not so significant. A 40 mM concentration of NaCl also showed significant effect and it doubled the activity of enzyme (Fig. 6B). Unlike the above salts, EDTA significantly reduced the CMCase activity and a 10 mM concentration of EDTA resulted in 60% reduced activity of the enzyme (Fig. 6C). This further corroborates the fact that the enzyme is a metalloenzyme and has metal ions at catalytic centers and requires the metal ion(s) for its catalytic activity.

Conclusions

The bifunctional cellulase derivative *Ct*Lic26A-Cel5E from *Clostridium thermocellum* displayed high activity with both soluble (lichenan, b-glucan and carboxymethyl cellulase) as well as insoluble (acid swollen cellulose, avicel and steam exploded bagasse) substrates. The optimum pH of the enzyme was 4.3 and the optimum temperature was 50°C for maximum activity with carboxymethyl cellulose as substrate. The bifunctional cellulase derivative was thermally stable up to 50°C. The cellulase derivative gave the V_m of 5.2 U/mg, K_m of 1.1 % (w/v) for CMC and 2.1 U/mg and 3.0% w/v for SEB correspondingly. The *in vitro* cellulase activity of the enzyme was significantly increased by Mg^{2+}, Ca^{2+}, Ni^{2+} and Na^{+} ions. Further studies are going on for enhancement of activity and characterization of the enzyme derivative and for exploring its potential for conversion of biomass or the processed substrate such as steam exploded bagasse to simple sugar.

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Rapid HPLC Determination of Venlafaxine in Microbial Biotransformation Studies

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Abstract

Venlafaxine is a novel antidepressant, which acts by inhibition of the reuptake of presynaptic noradrenalin and serotonin. In humans, it is metabolized by CYP450 2D6 to an active metabolite O-desmethyl venlafaxine, which has antidepressant activity similar to that of parent drug. Microorganisms have recently been successfully used as models for drug metabolism studies and for obtaining metabolites that could be developed as new drug entities. In the present investigation venlafaxine was used for producing an active metabolite by microbial model using different microorganisms. For estimation of venlafaxine and its metabolites in microbial biotransformation studies, a rapid, specific and sensitive HPLC method was developed. Linearity was observed over a concentration range of 0.5µg – 10µg/ml. Accuracy (98.15%) was achieved for all quality controls with intra-day and inter-day variation coefficient less than 8%. No endogenous interfering peaks were visible with blank culture media. A metabolite peak was found in the sample of *Saccharomyces cerevesiae* culture among five organisms used. This method was used for estimation of the venlafaxine metabolites in microbial biotransformation studies.

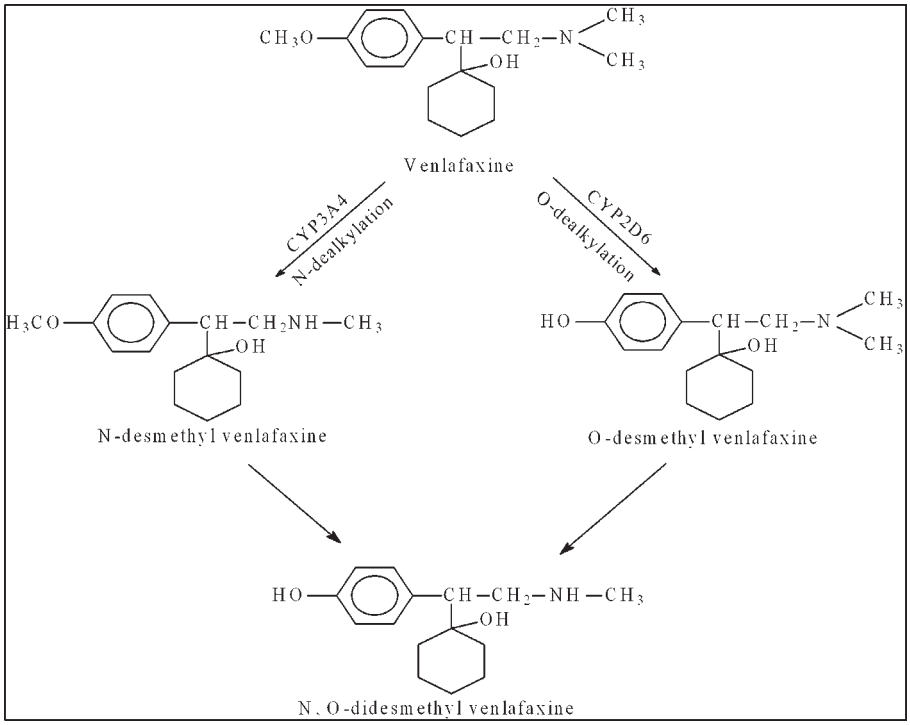
Keywords: Venlafaxine, microbial biotransformation, venlafaxine HPLC

1. Introduction

Venlafaxine is a novel phenethylamine bicyclic antidepressant (fig.1) which inhibits the reuptake of both noradrenalin and serotonin(1,2,3). In human, venlafaxine is well absorbed and is extensively metabolized to two less active metabolites N-desmethyl and N,O- didesmethyl metabolite and one active metabolite O- desmethyl venlafaxine (fig.1) has antidepressant activity profile similar to that of parent drug (4). Traditionally, drug metabolism studies were conducted on small animal models, perfused organs (5,6) *in vitro* enzyme systems and *in vitro* cell cultures. Later microbial models were developed as an alternative methods to study the metabolic fate of the drug with advantages of reducing the use of animals, in the early phases of drug development.

Microorganisms such as bacteria and fungi were used as *in vitro* models for the prediction of mammalian drug metabolism with successful applications (7,8,9). A systematic examination of microbial hydroxylation on variety of model organic compounds (10) followed by a comparison of O- and N-dealkylation reactions led Smith and Rosazza (8) to propose that a microbial transformation systems could closely mimic most of the phase I transformations of a drug observed in mammals. The use of microorganisms as models of mammalian metabolism has been well documented (11,12,13,14)

Fig. 1 : Mammalian metabolic pathway of venlafaxine



for obtaining novel metabolites as new drug entities and also for producing existing metabolites in large amounts..

In the present investigation, different microorganisms were used for evaluating their ability to metabolize venlafaxine. The aim of this study was to identify the microbes that can be used for production of an active metabolite of venlafaxine O-desmethyl venlafaxine in larger quantities for further characterization as well as pharmacological and toxicological evaluation. For that, the estimation of venlafaxine and its metabolites in microbial culture media is essential. The metabolites of drugs formed by microorganisms in culture are identified and confirmed by TLC, HPLC, LCMS or NMR techniques.(15) But the published methods for venlafaxine analysis (16) are only in biological fluids which include mainly solid phase extraction of drugs and are tedious (17). Therefore the present study is aimed at development of a simple, rapid and useful method for identification of the venlafaxine and its metabolites in the microbial culture media.

2. Materials and Methods

2.1. Microorganisms

Cultures were obtained from NCL, Pune, India. The cultures used in the present work were, *Proteus vulgaris* (NCIM 2027), *Pseudomonas aeruginosa* (NCIM 2053), *Nocardia hydrocarbonoxydans* (NCIM 2386), *Cunninghamella elegans* (NCIM 689) and *Saccharomyces cerevisiae* (NCIM 3090). These were selected from different types of microorganisms i.e. bacteria, fungi, and yeast. Based on the literature few of these were used for different substrates and found that are mimicking human metabolism.

2.2 Chemicals

Venlafaxine was obtained from Vimta labs, Hyderabad, India. All the reagents used in the analysis were of HPLC grade. Acetonitrile and sodium dihydrogen phosphate were purchased from Merck, Mumbai, India. Chloroform, Isopropanol, n-Heptane were obtained from SD. fine chemicals Ltd., Mumbai, India. Culture media was purchased from Himedia, Mumbai, India.

2.3 Fermentation procedure

The experiments were carried out using their respective growth media consisting of the following composition : For bacteria : Peptone 1 g, sodium chloride 0.5 g, beef extract 1 g, distilled water 100 ml and pH adjusted to 7.0-7.2. For fungus: Potato extract, dextrose 2 g, yeast extract 0.3 g, peptone 0.5 g, distilled water 100 ml. For yeast : Malt extract 0.3 g, glucose 1 g, yeast extract 0.3 g, peptone 0.5 g, distilled water 100 ml. pH adjusted to 6.4-6.8. Stock cultures were stored on agar slants prepared according to the above composition at 4°C, and transferred for every 2 months to maintain viability. The media were sterilized in an autoclave for 20 min. at 121°C and 15 lb/sq.in. Microbial metabolism studies were carried out by shake flask cultures in an incubator shaker, operating at 120 rpm at 32°C. The experiments were carried out in conical flask (250 ml) containing 50 ml. growth medium. Fermentations were carried out according to standard protocol. In brief, the substrate (venlafaxine) was prepared as a 1% (w/v) solution in methanol and added to the culture medium of selected organisms at a concentration of 10 µg/ml of medium in samples and incubated in shaker. The study also maintained the substrate control to which substrate was added and incubated without microorganisms and culture control consisted of fermentation blanks in which the microorganisms were grown under identical conditions without the substrate. The incubation was continued for 48 h .

2.4. HPLC analysis of extracts of microbial samples

2.4.1. Extraction procedure:

The pre incubated medium was heated on water bath at 50°C for 30 min. and centrifuged at 4000 rpm for 10 min. at 37°C (Remi instruments Pvt. Ltd., Mumbai, India). A clear supernatant liquid was collected and extracted by mixture of chloroform, isopropanol, n-heptane (HPLC grade, Ranbaxy Fine Chemical Ltd., Delhi, India) at a ratio of 60:14:26.(15). The upper organic layer was collected from two immiscible layers and was dried. The extract was reconstituted with 1ml. acetonitrile (HPLC grade, Ranbaxy Fine Chemical Ltd., Delhi, India) and centrifuged at 13000 rpm for 8 min. at 37°C in Biofuge fresco centrifuge (Hercaus, Germany). 20 µl portions were injected into the HPLC. Calibration standards were prepared in the range of 1.0 to 250µg/ml.

2.4.2. Chromatographic conditions:

High performance liquid chromatography (HPLC) analysis was conducted using a HPLC system (Shimadzu, Kyoto, Japan) consisted of LC-8A solvent delivery module and SPD-10AVP UV-Visible spectrophotometric detector and a Wakosil II5c-18rs-100a. 5UM, 4.6X 250 mm SS column (SGE, Japan). Sensitivity was set at 0.001 aufs. Mobile phase consisted of acetonitrile and 0.05 M disodium hydrogen phosphate buffer of pH 3.8 (25:75 v/v) with a flow rate of 1 ml./min. Elute was monitored using a UV/Vis detector set at 200 nm.

2.4.3. Standard solutions

Stock solution of 1mg/ml. of venlafaxine was prepared in methanol and stored at 4°C. Appropriate dilutions of venlafaxine were made in methanol to produce working stock solutions of 50.0, 10.0, 1.0 µg/ml. these dilutions were used to spike in culture media in the preparation of calibration curves. Calibration samples were prepared by spiking 200µl. of media with the appropriate amount of the drug on the day of

analysis. Samples for the determination of recovery, precision and accuracy were prepared by spiking control media in bulk at appropriate concentrations (1,10,50 µg/ml) and stored at -20°C.

3.Results and Discussion

3.1. Chromatography

Typical chromatogram corresponding to blank media and sample media obtained after adding 200µl of 10 µg/ml venlafaxine in sample of *Cunninghmela elegans* culture are shown in (figure 2 a,b) respectively. No endogenous (broth) interfering peaks were visible in blank media at retention time of venlafaxine confirming the specificity of the analytical method. System suitability parameters for the method were as follows: theoretical plates for venlafaxine were 2024, tailing factors were less than 1.25.

3.2. Quantification

A representative calibration graph of peak area versus venlafaxine concentration in the range of 0.5 µg to 10 µg resulted in regression equation y = 252773X+57656 (r^2= 0.9993) (fig.3) the lowest concentration with relative standard deviation (RSD) <20% was taken as lower limit of quantification (LLOQ) and was found to be 0.05 µg/ml. The RSD and S/N ratio at LLOQ were found to be 15% and 6% respectively.

3.3.Precision

Precision of assay was determined by analyzing media samples containing venlafaxine at three different concentrations. Samples for precision study were obtained by spiking blank media with the analytic solution at each concentration in bulk and the aliquots were stored in ependroff tubes at -4°C. The intra-day precision was determined by analyzing six spiked media samples at each concentration on the same day. For the determination of inter-day precision, fortified samples were analyzed on four different days. The inter-day relative standard deviation

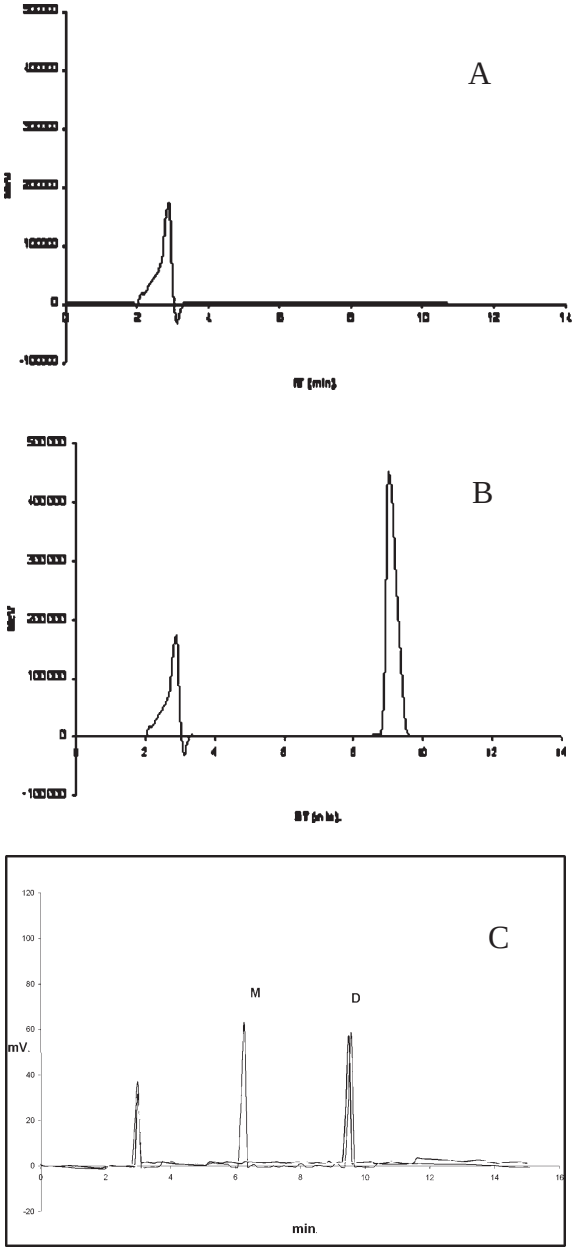


Fig. 2.0 HPLC chromatograms of venlafaxine and its metabolite
(a)Typical HPLC chromatogram of blank culture media (solvent peak)
(b) Typical HPLC chromatogram of venlafaxine (solvent peak and drug peak).
(c) HPLC chromatogram of venlafaxine and its metabolite in *Saccharomyces cerevesiae* culture media (M – Metabolite; D- Drug) (solvent, metabolite and drug peak).

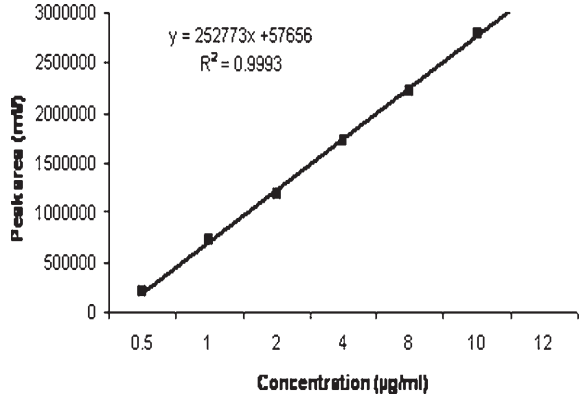


Fig.3.0 Standard graph of venlafaxine in culture media

(RSD) ranged from 1.02 to 3.73 at 1.0 µg/ml, 1.16 to 5.03 at 10.0 µg/ml. and 3.33 to 5.01 at 50.0 µg/ml. The intra-day RSD were 1.03,2.16 and 1.95 for 1, 10 and 50 µg/ml. respectively. These values are within the limits (table1) (<15%) specified for inter and intra day precision .

3.4. Recovery and accuracy

The extraction recovery of venlafaxine was estimated at 1,10, 50 µg/ml concentrations. Media samples (in six replicates) containing venlafaxine were extracted and analyzed. Six samples containing similar concentrations of the

Table 1. Inter and Intra day variation of venlafaxine analysis in culture media

Spiked concentration Inter day variation	**day**	**Measured concentration***	
µg/ml	Mean (µg/ml)	S.D	RSD
1.0 0	0.98	0.01	1.02
1	1.02	0.02	2.05
2	1.35	0.05	3.70
3	0.96	0.01	1.51
4	0.99	0.03	3.73
10.0 0	10.23	0.32	3.12
1	9.87	0.49	4.96
2	10.53	0.53	5.03
3	10.27	0.12	1.16
4	9.06	0.27	2.98
50.0 0	50.13	1.67	3.33
1	50.95	2.03	3.98
2	50.01	2.51	5.01
3	49.27	1.98	4.01
4	48.97	1.67	3.41
Intra day variation			
1.0	0.97	0.01	1.03
10.0	10.62	0.23	2.16
50.0	49.63	0.97	1.95

compound in mobile phase were directly injected and peak areas were measured. Absolute recovery was calculated by comparing the peak areas for direct injection of pure venlafaxine in methanol with those obtained by media samples containing same amount of venlafaxine. The range of absolute recoveries was from 92.49 to 99.12 (table 2). The accuracy of the method was verified by comparing the concentrations measured for venlafaxine spiked in media with the actual added concentrations. The results (table2) indicate that accuracy of the method was 97.01 to 99.67%. Thus this method is quite simple, sensitive and accurate.

Table 2 Recovery and accuracy of determination of venlafaxine in culture media

Concentration (µg/ml)	Absolute recovery (mean ± S.D. n=6)	Accuracy (%) (mean ± S.D. n=6)	Range (µg/ml)
1.0	92.49±1.34	99.67±1.97	0.92 -1.23
10.0	98.97±1.07	98.32±1.32	9.78 – 10.32
50.0	99.12±2.31	97.01±0.99	48.90 – 51.23

3.5. Metabolite identification in microbial cultures

In the HPLC analysis of the culture extracts of selected organisms, the obtained peaks were compared with controls. An additional peak at 6min. was found in sample of *Saccharomyces cerevesiae* culture extract when compared with its controls. (fig.2 c). It indicates metabolite of venlafaxine was formed by *Saccharomyces cerevesiae*.

4.Conclusions

The HPLC method developed is quite simple, sensitive and accurate and can be adopted for estimation of venlafaxine and its metabolites in the microbial culture media in metabolism studies. It was also found that the *Saccharomyces cerevesiae* is able to metabolise the venlafaxine among the tested microbes.

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Incidence of *Candida albicans* Infection in Cerebrospinal fluid - A First Report from Vidarbha, Central India

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Abstract

The candidial infection of cerebrospinal fluid is an uncommon manifestation but may have an usual occurrence in an immunocompromised patients treated with broad spectrum antibiotics in association with intravenous hyper alimentation following the surgical manipulation of mucosal surface colonized with *Candida* species. In the present study, the CSF samples of the patient were collected and the organism isolated was identified as *C. albicans.* So far, there is no report of *C. albicans* in CSF from Vidarbha region, hence an attempt has been made in the present study.

Keywords: *Candida albicans*, Cerebrospinal fluid, Immunocompromised.

Introduction

Cerebrospinal fluid is an isotonic solution and acts as a "cushion" or buffer for the cortex, providing a basic mechanical and immunological protection to the brain inside the skull. From the last decade or so, the incidence of fungal infections have been increased out of which, *Candida* species are becoming an important cause of nosocomial infection, primarily affecting immunocompromised patients (1, 2). In practice, the majority of cases of CNS candidiasis are associated with disseminated or invasive candidiasis (IC). Another form of candidal CNS infection is the one occurring as a postoperative complication of neurosurgical procedures, especially ventriculo-peritoneal (VP) shunt placement. *Candida* meningitis is the most frequent clinical manifestation of IC-related CNS Candidiasis (3).

Candida species are now the fourth most common isolated organism. The crude mortality rates ranges from 70 to 100 % in CNS candidiasis, and are increasingly isolated from surgical site and urinary tract infections (4). *Candida* remained a relatively uncommon CNS pathogen until the 1960s when use of chemotherapeutic agents, glucocorticoids, and intravenous drugs rendered increasing numbers of patients susceptible to opportunistic infections (5). Meningitis is the most common form of CNS infection caused by *Candida*. The clinical symptoms are highly variable and range from acute to chronic (6). Typically, the onset of meningitis evolves subacutely over several days to weeks with fever, headache, meningismus and diminished consciousness. More acute manifestations are often indistinguishable from bacterial meningitis. Meningitis is among the ten most common infectious causes of death and is responsible for approximately 135,000 deaths throughout the world each year (7). Oral thrush is also one of the common clinical manifestations of candidiasis, seen in both HIV seropositive as well as seronegative patients (8).

In the current study, the patient was a 23 years old female admitted to the hospital. She was suffering from fever with chills and constant

vomiting. CSF samples were collected by a procedure called lumbar puncture, processed and *C. albicans* was isolated repeatedly. So far there is no report of infection of *C. albicans* in CSF from the Vidarbha region, it is being reported for the first time here.

Materials and Methods

The pathogen was isolated on Sabouraud Dextrose Agar (40g glucose, 10g peptone, 15g agar-agar dissolved in 1 liter of distilled water and incubated at 37°C). For the identification, samples were processed for Gram staining, culture, germ tube test, production of chlamydospores, sugar fermentation and assimilation tests.

Culture

The samples were cultured on Sabouraud Dextrose Agar (SDA), incubated at 37°C and examined twice a week for growth showing cream coloured pasty colonies suggestive of *Candida* species (Figure 1 [a] and [b]).

Gram stain

Smears were prepared from the CSF samples. Gram stained smears were used for detection of gram-positive budding yeast cells with pseudohyphae (Figure 2).

Germ tube Test

C. albicans was inoculated in human serum and incubated at 37°C. After 2-4 hours, wet mount was prepared and examined under the microscope for the presence of germ tube (Figure 3).

Formation of Chlamydospores

All Candida isolates were tested for the production of chlamydospores in corn meal agar. The Candida strains were inoculated in corn meal agar (CMA) and then incubated at 25°C. After 72 hours, the plates were examined under the microscope for the presence of chlamydospores.

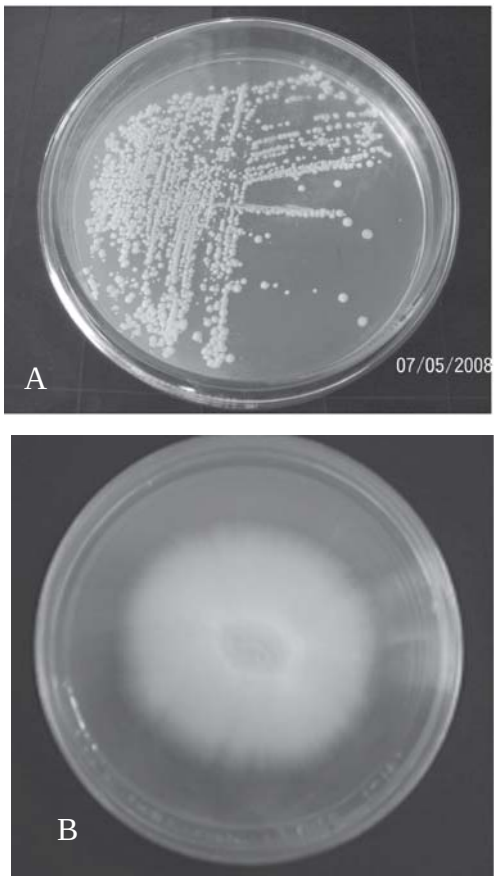

Figure 1: Growth of *Candida albicans* on the Sabouraud Dextrose Agar [a] after 3 days and [b] after 14 days. budding yeast cells of *Candida albicans*

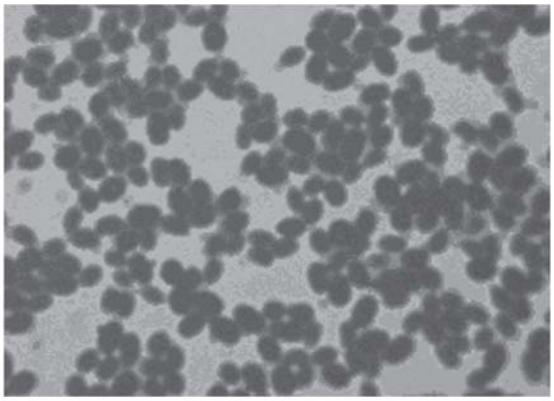

Figure 2: Micrograph showing gram positive budding yeast cells of *Candida albicans*

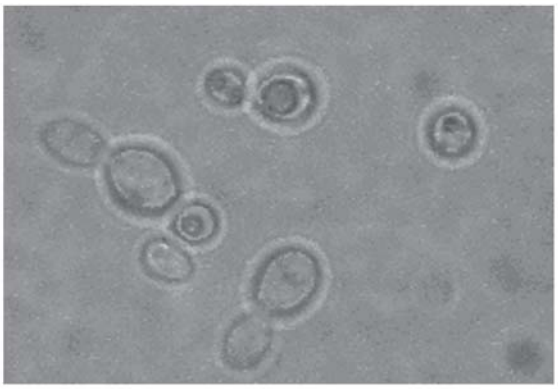

Figure 3: Micrograph showing formation of germ tube in human serum, characteristic to *Candida albicans.*

Sugar fermentation test

All Candida isolates were subjected to carbohydrate fermentation test. Carbohydrate solutions used were 6% solution of dextrose, maltose, lactose and sucrose with basal media.

Sugar assimilation test

The assessment of the ability of yeast to utilize carbohydrates is based on the use of carbohydrate- free yeast nitrogen base agar and observing for the presence of growth around carbohydrate impregnated filter paper disks after an appropriate period of incubation. Carbohydrates used were glucose, lactose, maltose, sucrose and galactose.

Results and Discussion

During the past decade, there has been increasing incidence of fungal pathogens (9). The prevalence of systemic fungal infections has increased significantly and has been reported from other parts of India. Rao *et al.* (10), reported fungal infections in neonates. Disseminated Candidiasis is a significant source of mortality and morbidity in neonatal intensive care nurseries. Nabi *et al.* (11) found a very low birth weight infant with disseminated invasive candidiasis including meningitis. In the present investigation, *Candida albicans* was repeatedly isolated from cerebrospinal fluid. Recently, a case of *Candida parapsilosis* has been reported to cause meningitis in 50 year old HIV seropositive male presented with chronic headache, altered sensorium and neck rigidity (12). Wabale *et al* (8) reported oral thrush in both HIV seropositive as well as seronegative patients. The isolation rate of *Candida* species is found to be 90% in HIV-seropositive group, as compared to HIV-seronegative group (60 %). Thus, *Candida* species has emerged as an opportunistic pathogen with severe manifestations since the last two decades. The other *Candida* species isolated by Wabale *et al* (8) were, *C. dubliniensis, C. parapsilosis, C. glabrata, C. krusei* and *C. lusitaniae*.

In the present study, the smear prepared, was stained by Gram staining method and the micrograph reveals the gram-positive budded yeast cells, characteristic to the *Candida* species. Formation of germ tube was observed which is a rapid and a presumptive test for *Candida* species. A conventional (Sabouraud dextrose agar) and a chromogenic media (HiCrome® agar) were used for the preliminary identification. HiCrome® agar is a novel, differential culture medium that is claimed to facilitate the isolation and presumptive identification of some clinically important yeast species (Figure 4). Besides, HiCrome® agar,

Figure 4: Green pigmented colonies produced on HiCrome®agar, characteristic to *Candida albicans.*

there are other chromogenic media utilized such as, CHROMagar *Candida* (13), Albicans ID2® media (14), BBL™ CHROMagar™ *Candida* (15) for the identification of *Candida* species. *Candida glabrata* can be identified more rapidly on the basis of trehalose assimilation of fermentation (16). This new differential culture medium allows selective isolation of yeasts and simultaneously identifies colony of *C. albicans, C. tropicalis, C. krusei and C. glabrata.* Also, other biochemical tests were performed which shows the characteristic features of *Candida albicans.*

Conclusion

Incidences of candidal infection have been increased drastically since the past two decades. Thus, with the advent of new technologies new diagnostic tools are now being used for the characterization of different pathogenic organisms. In general medical laboratories, characterization of *Candida* species is based on the traditional diagnostic methods. However, with the introduction of different diagnostic tools like DNA-microarrays, PCR and immunodiagnostic tools the laboratory diagnosis of *Candida* species up to the strain level can be achieved.

In the present study, characterization of *Candida* species was carried out with the traditional methods, which reveal that the organism isolated from the CSF samples was *Candida albicans*. As in past, there has been no such report of candidal infection in CSF from Vidharba region, so it is reported here.

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Formulation and Evaluation of Solid Dispersions of an Anti-diabetic Drug

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Abstract

The present work investigates the dissolution and bioavailability characteristics of an anti-diabetic drug, Glimepiride. Glimepiride, is an oral hypoglycemic drug and has problems in bioavailability and bioequivalence due to its poor water solubility. In the present study, dissolution studies were carried out by using USP XXIV apparatus, for the drug glimepiride, and its binary systems (both physical mixture as well as solid dispersions of glimepiride). Infrared (IR) Spectroscopy, Differential Scanning Calorimetry (DSC), and X-ray Diffractometry (XRD) were performed to identify any physicochemical interaction between the drug and the carrier and its effect on dissolution behavior. Tablets containing solid dispersion products were formulated and compared with the commercial product. The commercial product and the tablet formulation under investigation were than characterized for their various physicochemical properties such as weight variation, % friability, disintegration and *in vitro* dissolution profiles. IR Spectroscopy, XRD, and DSC showed no change in the crystal structure of glimepiride thus indicating the absence of any interaction between the drug and the polymer. A significant improvement in the dissolution of glimepiride in solid dispersion products has been observed (>85% in 5 minutes). Also tablets containing solid dispersion exhibited better dissolution profile than commercial tablets. Thus, the solid dispersion technique can be successfully used for the improvement of dissolution of glimepiride.

Key words: Solid Dispersion, Dissolution enhancement, Poorly soluble drugs, Antidiabetic drugs.

Introduction

Glimepiride is an oral anti-diabetic drug, which comes under the BCS (Biopharmaceutical Classification System) class 2 category drugs i.e. drugs, which are having high permeability and low solubility profiles. A drug substance is considered highly permeable when the extent of absorption in humans is determined to be $\geq$ 90% of an administered dose, based on mass-balance or in comparison to an intravenous reference dose. A drug product is considered to be rapidly dissolving when $\geq$ 85% of the labeled amount of drug substance dissolves within 30 minutes using USP apparatus I or II in a volume of $\leq$ 900 ml buffer solutions. Sparingly water-soluble drugs often exhibit low dissolution profile and oral bioavailability problems (1).

Various techniques such as micronization, solubilization, salt formation, complexation with polymers, change in physical form, use of prodrugs, drug derivitization, alteration in pH, addition of surfactants, and others (2, 3) have been employed in order to improve the dissolution and bioavailability of sparingly soluble drugs. Among the various approaches, the solid dispersion

technique has proved to be the most successful, simple and economic in improving the dissolution and bioavailability of poorly soluble drug (4).

Solid dispersion, which was introduced in the early 1970s (5) is a multicomponent system, having drug dispersed in and around hydrophilic carrier(s). It (solid dispersion technique) has been used for a wide variety of poorly aqueous soluble drugs such as nimesulide (6), ketoprofen (7), tenoxicam (8), nifedipine (9), nimodipine (10), ursodeoxycholic acid (11), and albendazole (12). Various hydrophilic carriers, such as polyethylene glycols (13), polyvinylpyrrolidone (14), hydroxypropyl methylcellulose (15), gums (10), sugar (16), mannitol (17) and urea (11) have been investigated for improvement of dissolution characteristics and bioavailability of poorly aqueous-soluble drugs. Sekiguchi and Obi (18) were the first to propose the solid dispersion technique to improve the dissolution characteristics of poorly water-soluble drugs by the use of water-soluble carriers. Chiou and Rigelman, 1971 (5) have used the solid dispersion technique for dissolution enhancement of poorly water-soluble drugs by thoroughly dispersing the drug in a water-soluble carrier by solvent-melting methods. In this method, the drug is thoroughly dispersed in a water-soluble carrier by melting, solvent, or solvent-melting methods (5). Many water-soluble carriers have been employed for preparation of solid dispersion of poorly soluble drugs. The most common are polyethylene glycols (19, 20), polyvinyl pyrrolidone (21, 22), lactose (23), β-cyclodextrin (24, 25), and hydroxypropyl methylcellulose(26).Moreover, Polyethylene glycol (PEG) is one of the most widely used carriers to prepare solid dispersions (27-29).This work investigated the possibility of developing glimepiride tablets, allowing fast, reproducible and complete drug dissolution, by using solid dispersion technique. Solid dispersions of Glimepiride in PEG 6000 were prepared by solvent evaporation method. Differential Scanning Calorimetry (DSC) curves, Infra-Red (IR) Spectroscopy and Powder X-Ray Diffraction (XRD) patterns of solid dispersions and physical mixtures were obtained using a Differential Scanning Calorimeter (DSC 60 Shimadzu Japan), FTIR (Jasco FTIR-5300 spectrophotometer (Tokyo, Japan) and XRD (Seimens D 5005 diffractometer) respectively.

Materials and Methods

Materials

For preparation of solid dispersions the following materials were used: Lactose (Sigma); PEG 6000 (BASF, India); Glimepiride (Zydus Recon) Bangalore. Chemicals used for buffer preparation were of reagent grade. All other materials used were of analytical grade.

Preparation of solid dispersions

Different ratios of solid dispersions (1:1, 1:2) were prepared by solvent evaporation technique using vacuum flash evaporator using methanol as solvent. The solvent was evaporated in the vacuum flash evaporator at 60°C until no trace of solvent was remaining. The residue was scrapped, collected and dried for 10 min. in oven at 40°C. After drying the mass was pulverized and passed through sieve no. 80 mesh. All these dispersions were then stored in the screw cap bottles for further analysis.

Physical mixtures of glimepiride were prepared by mixing glimepiride with the hydrophilic carriers for 5 min. in a mortar until a homogenous mixture was obtained. The resulting mixture was then sieved and 105-250 micron particle size fraction was obtained using 60- and 140 mesh screen. The powders were stored in screw cap bottles at room temperature until further analysis.

Estimation of drug content

Drug content of the preparations were estimated by dissolving weighed quantity of physical mixture (PM) or solid dispersion (SD) in minimum amount of methanol and then making

up the volume with water and then assayed for drug content spectrophotometrically at 229 nm.

An accurately weighed quantity of solid dispersion equivalent to 4 mg of drug were taken into 50 ml volumetric flask and then dissolved in minimum amount of methanol. This was then made up to the volume with water and was assayed for drug content by using UV double beam spectrophotometer at 229 nm. Three replicates were prepared and the average drug contents were estimated in the prepared solid dispersions (Table 1).

Table 1: Assay for the drug content in the binary mixtures

S. No.	Ratio	Binary Mixtures	
		PM	SD
1.	1:1	96.14	95.41
2.	1:2	96.58	95.85

Fourier-Transform Infrared (FTIR) Spectroscopy

FTIR spectra were recorded on samples prepared in potassium bromide (KBr) disks using a Jasco FTIR-5300 spectrophotometer (Tokyo, Japan). Samples were prepared in KBr disks by means of a hydrostatic press. The scanning range was 400 to 4000 cm^{-1} and the resolution was 4 cm^{-1}

Differential Scanning Calorimetry (DSC)

DSC analysis was performed using DSC-60 Shimadzu (Japan) on 2-4 mg samples (Sartorius BP 210 S electronic microbalance, Goettingen, Germany). Samples were heated in an open aluminium pans at a rate of 10°C per min^{-1}. Indium was taken as reference and the hold temperature was maintained at 300°C.

X-Ray Powder Diffractometry

The Powder X-Ray Diffraction (PXRD) pattern of all ingredients and all binary systems were recorded using an automated Seimens X-ray diffractometer (Seimens D5005, IISc, Bangalore).

Dissolution rate studies

Table 2 summarizes % drug dissolved in 5 minutes (DP_5), dissolution efficiency at 15 minutes (DE_{15}), and dissolution efficiency at 60 minutes (DE_{60}) for Glimepiride and its binary systems with carriers. Dissolution test was conducted using USP XXIV apparatus at 75 rpm. The dissolution medium was 900 ml of simulated gastric fluid. Solid products, (both solid dispersions as well as physical mixtures), each containing 4 mg of drug were subjected to dissolution. Samples were withdrawn at fixed time intervals, filtered (pore size 0.22 ìm) and assayed spectrophotometrically for drug content at 229 nm. Each test was performed in triplicate. T_{50} values were evaluated directly from the dissolution data. (Table 3).

Tablet preparation and characterization

Tablets each containing 4 mg of the drug in solid dispersions (in PEG-6000) were prepared by wet granulation method as per the formulae given in Table 4. The blend of powders was compressed into tablets on a multi station tablet machine (Cadmach) to a hardness of 3-4 Kg/ sq.cm. Tablets were tested for uniformity of weight (IP-1996). Prepared tablets were evaluated for hardness (Monsanto hardness tester), friability (Roche Friabilator), weight variation, and drug content.

Estimation of Glimepiride in phosphate buffered saline (pH 7.8) was accomplished spectriophotrometrically using an double beam UV spectrophotometer. The excipients used in the dissolution did not interfere in the method.In vitro dissolution studies of tablets containing solid dispersion and commercial tablet of glimepiride were carried out in 900-mL simulated gastric fluid.

Table 2. Percentage Dissolution and Dissolution Efficiency of Glimepiride from Different Binary Systems in Comparison With Original Drug*

S. No.	System	DP_5 %†	DE_{15} %†	DE_{60} %†
1.	Glimepiride	10.10±1.0	9.0±0.87	16.58±1.36
2.	PM1	25.30±2.3	22.50±1.91	36.80±2.84
3.	PM2	34.10±2.7	32.90±2.61	53.06±3.74
4.	SD1	70.10±3.8	63.45±4.34	85.62±5.37
5.	SD2	85.40±1.3	77.20±2.76	93.20±0.95

*Glimepiride is the drug, DP_5, % dissolved at 5 minutes; DE_{15} and DE_{60}, dissolution efficiency at 15 and 60 minutes).
†All values are mean of 3 readings ± SD.

Table 3: T_{50} values of the Marketed Tablets and the SD containing Tablet formulations of Glimepiride and PEG.

T_{50} values (min)	
Preparations	**T_{50} values (min)**
Marketed	60
SD containing formulation	4

Result and Discussion

Fourier Transform Infrared (FTIR) Spectroscopy

IR spectra of Glimepiride and its binary systems with PEG are presented (Figure 1). Pure glimepiride spectra has a sharp characteristic peaks at 1700, 1710, 1375, and 610 cm^{-1}. All the above characteristic peaks appears in the spectra of all binary systems at same wavenumber indicating no modification or interaction between the drug and carrier.

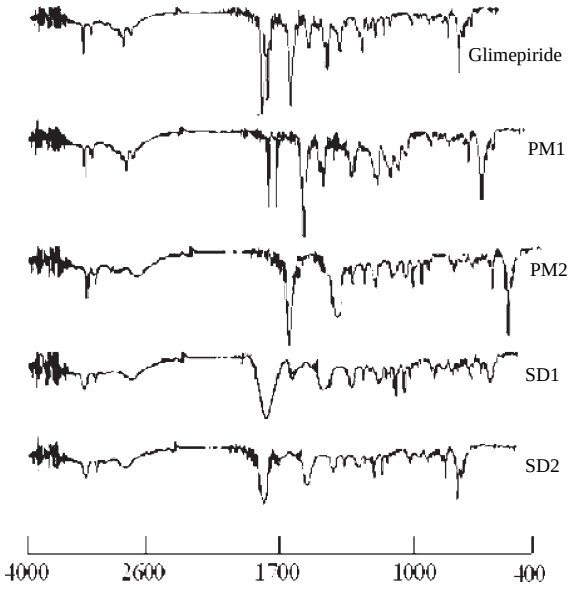

Figure 1 : FTIR Spectra of Glimepiride and various binary systems with PEG

Differential Scanning Calorimetry

Thermal behavior of pure drug and corresponding drug carrier system are depicted (Figure 2). The DSC curve of Glimepiride profiles a sharp endothermic peak (T_{peak} = 210°C) corresponding to its melting point, indicating its crystalline nature. However, the characteristic endothermic peak, corresponding to drug melting was broadened and with reduced intensity, in both physical mixtures as well as solid dispersions.

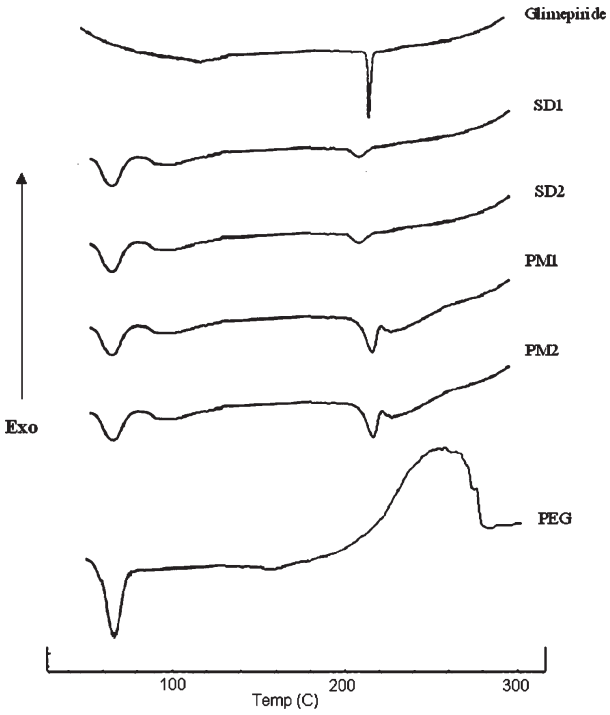

Figure 2 : DSC Curves of Glimepiride and various binary systems.

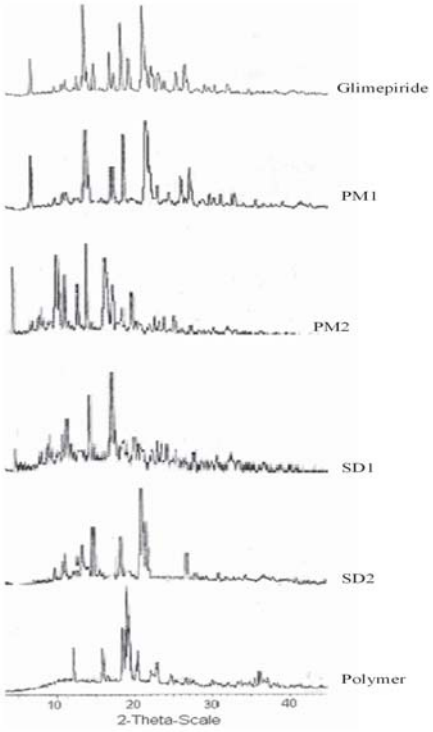

Figure 3 : XRD spectra of Glimepiride and various binary systems.

This could be attributed to higher polymer concentration and uniform distribution of drug in the crust of polymer, resulting in complete miscibility of molten drug in polymer. Moreover, the data indicate no interaction between the components of binary system. The intensity of the peaks of solid dispersions was smaller than those of the pure drug and the corresponding physical mixture at same weight ratio. These results suggested that glimepiride became partially amorphous during dispersion into PEG matrix.

X-ray Diffractometry

X-ray diffracometry (XRD) spectra of pure drug and its binary systems with carriers are presented (Figure 3). The x-ray diffractogram of Glimepiride has sharp peaks at diffraction angles (2è) 13.8°, 17.01°, 18.1°, 19.1°, 21.2° and 26.5° showing a typical crystalline pattern.

The diffraction pattern of glimepiride showed that glimepiride has high crystallinity because of the presence of numerous peaks. PEG is found to be amorphous powder having no crystalline structures. The XRD peaks of crystalline glimepiride in all the physical mixtures were similar to those in the pure drug, indicating that the crystallinity of glimepiride did not change in the physical mixtures.

The crystalline structure of glimepiride in all the solid dispersions was different from that of the pure drug and the corresponding physical mixture as indicated from the differences in their XRD patterns. The number of peaks and the peak height was reduced in all the solid dispersions as the polymer concentration increased. These findings suggest that the glimepiride crystals got converted to the amorphous form in the polymer matrix in solid dispersions with higher weight

ratios of the polymer. IR and DSC studies support the same hypothesis, as is confirmed by x-ray diffractometry.

Dissolution rate studies

Dissolution profiles of original drug crystals and drug-carrier binary systems are presented (Figure 4). As is evident from the graph that the solid dispersion (SD) technique has improved the dissolution rate of Glimepiride to a great extent, the results indicate that within the two solid dispersion ratios, SD2 (DE_{60} = 93%) showed maximum enhancement in dissolution rate than the SD1. Moreover, SD1 also produced comparable results in terms of dissolution efficiency (DE_{60} = 85%). Physical mixtures (PM) also improve dissolution rate by a significant extent as compared with drug alone ($P < 0.001$). The order of efficiencies of products based on DE values is SD2 > SD1 > PM2 > PM1 > Glimepiride. This enhancement of dissolution of Glimepiride from drug-carrier systems can be attributed to several factors. The mechanism of dissolution rate improvement from solid dispersion is reviewed by Ford (30). Lack of crystallinity,

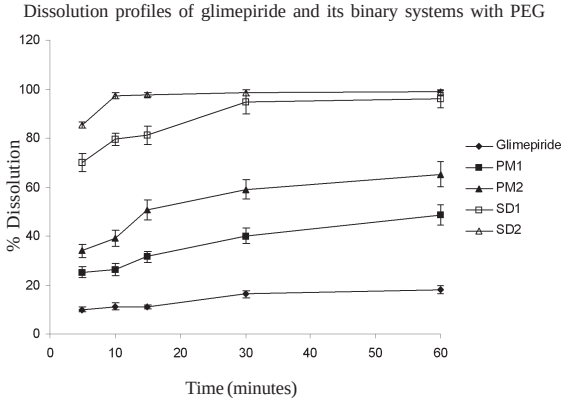

Figure 4 : Dissolution profile of Glimepiride and its binary systems with PEG

i.e., amorphization, increased wettability, dispersibility and particle size reduction are considered to be the important factors for the enhancement of dissolution rate. As indicative from the dissolution data of the physical mixtures, improvement could be attributed to higher wettability and dispersibility. Dry mixing of drugs with a hydrophilic carrier results in greater wetting and increases surface available for dissolution by reducing interfacial tension between the hydrophobic drug and the dissolution media. During dissolution studies, it was noted that drug-carrier system sinks immediately, whereas pure drug keeps floating on the surface for a longer time interval. Furthermore, kneading results in uniform distribution of drug in the polymer crust in a highly dispersed state. Thus, when such a system comes in contact with an aqueous dissolution medium, the hydrophilic carrier dissolves and results in precipitation of the embedded drug into fine particles, which increase the dissolution surface available. Moreover, other factors such as absence of aggregation and/or reagglomeration phenomenon during dissolution and particle size reduction could be attributed to a better dissolution profile (31).

Tablet preparation and characterization

On the basis of *in vitro* dissolution efficiency, the SD2 binary system was selected to formulate the tablet of glimepiride. Tablet characteristics of the optimized formulation (SD2) are tabulated in Table 4. *In vitro* dissolution studies of the optimized formulation confirmed the results obtained with the solid binary mixtures. SD2 tablets showed good dissolution efficiency (DE_{60} = 81.38%) and rapid dissolution (DP_5 = 65.13%). When compared with commercial formulation (Figure 5), tablets formulated with the binary mixture (SD2) clearly performed better and a significant enhancement in dissolution characteristics was observed ($P < 0.001$). A significant increase in DP_{60} (% dissolved in 60 minutes) was found with SD2 with respect to commercial formulation.

Conclusion

Finally, solid dispersions are known for their dissolution rate–enhancing properties of

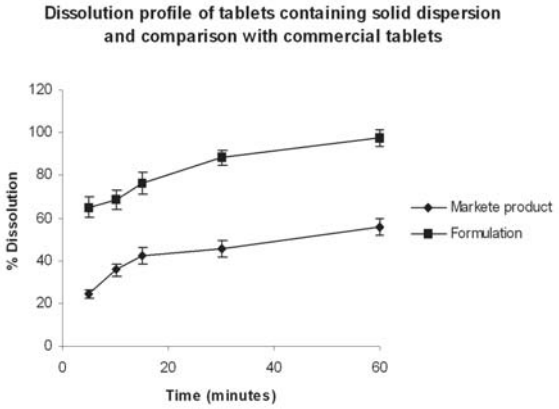

Figure 5 : Dissolution profile of tablets containing solid dispersion and comparison with commercial tablets.

poorly soluble drugs, such as CsA. (Leuner C, 2000; Sethia S, 2003; Kaushal AM, 2004) (11-13). The study shows that the dissolution rate of glimepiride may be enhanced to a great extent by solid dispersion technique using an industrially feasible kneading method. Hence glimepiride-PEG binary systems could be considered for formulation of fast-dissolving tablets of glimepiride

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Table 4 Composition for the SD2-containing Tablets:

S. No.	Ingredients	Quantity (mg)
1	Glimepiride	5
2	PEG-6000	20
3	Starch	34
4	Magnesium Sterate	2
5	Lactose	113

Table 5. Percentage Dissolution and Dissolution Efficiency of Glimepiride from Tablets Containing solid dispersion (SD2) and Commercial Formulation A

S. No.	Formulation	DP_5 %*	DP_{15} %*	DE_{60} %*
1.	SD2	65.13±4.81	76.41±4.91	81.38±2.94
2.	Marketed product	24.49±1.91	32.37±4.12	43.16±3.16

*All determinations are mean of 3 readings ± SD. DP_5 and DP_{15}, % dissolved at 5 minutes and 60 minutes respectively, DE_{60}, dissolution efficiency in 60 minutes.

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High frequency plant regeneration from callus cultures of two finger millet (*Eleusine coracana*) cultivars

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Abstract

Genotypic differences for callus induction, per cent frequency of plantlet regeneration and mean number of plants formed per 200 mg callus were observed in finger millet (*Eleusine coracana).* BAP was better, but kinetin is effective in finger millet for plant regeneration. High frequency plant regeneration was noticed upto 165 to 180 days which would pave the way for genetic transformation of finger millet.

Key Words

Callus cultures, plant regeneration, finger millet (*Eleusine coracana*).

Abbreviations: BAP, 6-benzylaminopurine; KN, kinetin; 2, 4-D, 2, 4-dichlorophenoxyacetic acid; 2,4,5-T, 2,4,5-trichlorophenoxypropionic acid.

Introduction

Finger millet or Ragi (*Eleusine coracana* (L.) Gaertn) is an important food crop grown mainly in arid and semiarid regions under rain-fed conditions. The plant is known to produce grain under hot and dry conditions. In millets, plant regeneration from callus cultures is known. Rangan (1) reported growth and plantlet regeneration in tissue cultures of some millet, including finger millet. In finger millet, plant regeneration was reported through shoot bud differentiation and by somatic embryogenesis (2). However, long-term shoot regeneration with high frequency has not been reported so far. Present study therefore, deals with the regeneration of whole plants from long-term callus cultures of two popular cultivars of finger millet (*Eleusine coracana*).

Materials and Methods

Seeds of finger millet [*Eleusine coracana* (L.) Gaertn] cultivars Himachal and Neelachal were surface sterilized with 0.1% $HgCl_2$ for 10-12 min followed immediately by washing with sterile glass distilled water. This was repeated thrice and 4-5 seeds were inoculated into each test-tube containing 15 ml of Murashige and Skoog's (MS) agar medium (3) supplemented with either (a) 2 mg/l 2,4-D (or) (b) 2 mg/l 2,4-D + 0.5 mg/l kinetin (or) (c) 2 mg/l 2,4,5-TP. Different plant growth regulator combinations were used for plant regeneration. All the cultures were incubated in diffused light (10 μ Em^{-2} s^{-1}) for regeneration of shoots or roots. Callus cultures were sub-cultured routinely on MS medium containing 2 mg/l 2,4-D and 0.5 mg/l KN and incubated at 26 ± 2 ºC. Callus initiation in finger millets was found better in MS medium fortified with 2 mg/l 2,4-D plus 0.5 mg/l KN. Therefore, callus grown on this medium was subsequently used for regeneration. MS medium incorporated with 2 mg/l BAP and 2% sucrose was used for shoot regeneration. Embryogenic callus was

characterized by compact, pale yellow cells that are smaller in size. For callus initiation and regeneration, 10-13 replicates were used and all experiments were repeated at least once and average values are given.

Results and Discussion

While the frequency of callus initiation from the seeds of finger millet cultivar Himachal was 100%, Neelachal recorded 90% in MS medium containing 2 mg/l, 2,4-D and 0.5 mg/l KN. Callus initiation took 10-12 days irrespective of the cultivar. But, the frequency of callus initiation was reduced in presence of 2 mg/l, 2, 4-D and 2,4,5-TP (70-90%) alone. Embryogenic callus was observed from both the cultivars in presence of 2 mg/l 2,4-D plus 0.5 mg/l KN, which is compact, pale or deep yellow in colour and the cells appeared smaller (observed under the microscope) compared to the non-embryogenic cells. Embryogenic callus of the finger millet cultivar Himachal and the shoot regeneration from this callus are shown in the figures 1A and 1B respectively. BAP at 2 mg/l gave the highest frequency of response (80%) with an average of 19.2 shoots per 200 mg of callus mass compared to lower or higher concentrations. BAP was found more effective than KN for shoot regeneration frequency (75% at 2 mg/l) as well as for the number of shoots generated (an average of 18.4) per 200 mg of callus (Table 1). Sucrose at 0.5% concentration did not elicit any morphogenetic response. Two per cent was found optimum for shoot organogenesis and the number of shoots formed per 200 mg of callus (Table 1). Similar results were noticed in Neelachal also (data not shown).

Table 1: Effect of kinetin, BAP and sucrose on the frequency and the number of shoots formed per 200 mg of callus in the cultivar Himachal.

MS + Growth regulators (mg/l)	Sucrose (%)	% Frequency of shoots	No. of shoots formed/ 200 mg of callus (Mean ± SE)
0.5 BAP (control)	2	20	7 (± 1.1)
1 BAP	2	40+	12.6 (± 0.6)$^{+}$
2 BAP	2	80+	19.2 (± 0.8)$^{+}$
4 BAP	2	60+	17.1 (± 0.5)$^{+}$
0.5 KN (control)	2	20	7.2 (± 0.1)
1 KN	2	40+	12.5 (± 0.5)$^{+}$
2 KN	2	75+	18.4 (± 0.7)$^{+}$
4 KN	2	65+	17.2 (± 0.5)$^{+}$
2 BAP (control)	0.5	Nil	Nil
2 BAP	1	10+	7.5 (± 2.5)$^{+}$
2 BAP	2	80+	18.8 (± 0.8)$^{+}$
2 BAP	4	70+	17.5 (± 0.7)$^{+}$

Data scored at the end of 30 days from 20 replicates using 90-day-old callus. $^{+}$Significant P = 0.01.

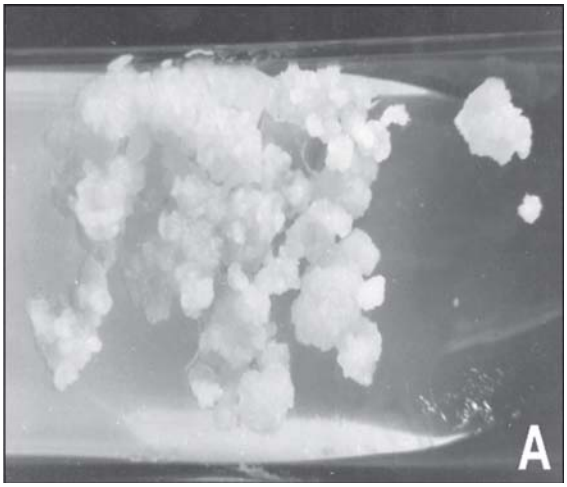

Fig.1A. Embryogenic callus of finger millet cultivar Himachal (MS + 2 mg/l 2,4-D + 0.5 mg/l KN)

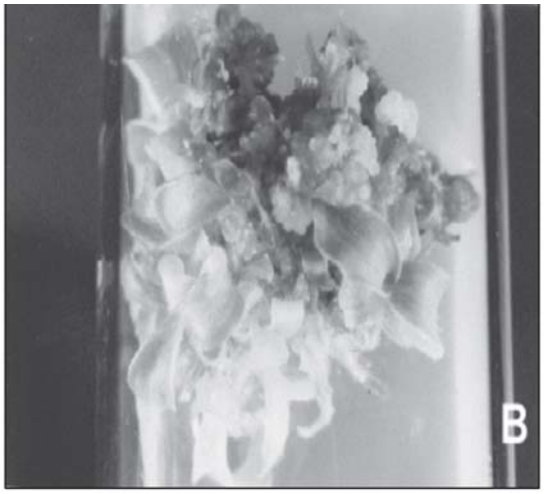

Fig. 1B. Plantlet regeneration from 150-day old callus of Himachal (MS + 2 mg/l BAP)

Table 2: Regenerating ability of callus cultures of finger millets from long-term cultures

Cultivar	Age of callus	% Frequency of shoot regeneration
Himachal	45	68
	60	68
	75	68
	90	78
	105	78
	120	63
	135	53
	150	13
	165	Nil
Neelachal	45	70
	60	70
	75	70
	90	78
	105	78
	120	78
	135	63
	150	48
	165	33
	180	Nil

Data represent an average of 20 replicates per each treatment scored at the end of 30 days.

Ability of callus cultures to differentiate shoots from the two finger millet cultivars is shown in Table 2. Increase in the age of callus decreased the shoot regeneration frequency irrespective of the cultivar (Table 2). The potentiality of the callus to regenerate shoots was lost by 165-180 days in Himachal and Neelachal respectively. Well formed shoots were rooted with 100% frequency in 7-10 days on MS basal medium devoid of growth regulators. Plantlets were later transferred to pots containing sand and soil mixture in a ratio of 1:3. Plants were covered with glass beakers to maintain humidity and watered with Hoagland nutrient solution at 3-4 day intervals. Glass beakers were removed after two weeks of transfer to the pots. The frequency of survival was 60-90% in both cultivars and about 10% of the plants showed morphological variations.

While Rangan (1) observed plant regeneration in finger millet via organogenesis on MS medium containing NAA (6 pico moles) and coconut water (15%), Eapen and George (2) obtained somatic embryos on the medium supplemented with picloram and kinetin. Immature embryos and immature inflorescences were earlier used in other cereals like *Paspalum* (4), *Panicum* (5), *Pennisetum* (6) and *Sorghum* (7). While 2,4-D has been the most frequently used for callus induction and somatic embryogenesis in large number of cereals, other auxins like picloram and para-chlorophenoxyacetic acid (6), ethylene inhibitors also like silver nitrate (8) improved the formation of embryogenic callus considerably. Embryogenic callus was observed in the present study in presence of 2,4-D, but not when 2,4,5-TP was added. Cytokinins are known to influence the shoot formation in several plants including cereals (9, 10). Concentration of sucrose seemed to play a vital role during organogenesis in finger millet since lower (0.5 and 1%) or higher (4%) concentration could not promote optimum organogenesis (Table 1). The ability of callus tissues of both the cultivars of finger millet for shoot regeneration was extremely high up to 135 to 150 days, but was lost by 165 to 180 days in culture in the present study. Consistent shoot formation in graminaceous species was obtained mainly from callus induced from very immature material in particular immature zygotic embryos and young inflorescences (10). Plant regeneration in the gramineae was reported to occur by shoot morphogenesis and through the germination of somatic embryos (2, 10, 11). It was observed in the present study that somatic embryogenesis in the finger millets was low and the regeneration was mainly via organogenesis. However, the protocol developed is now being used for genetic transformation of finger millet. Also, morphologically variant plants that are obtained in the present study are now being assessed for any possible somaclonal variation.

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Production of Alkaline Xylanase by an Alkalo-thermophilic Bacteria, *Bacillus halodurans*, MTCC 9512 isolated from Dung

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Abstract

An alkalo-thermophilic bacteria from dung has been isolated using Emerson medium in the agar plates. The bacteria has growth at the pH 10 and temperature 55°C. The bacteria was screened for the xylanase activity using Congo red dye followed by wash out by 1 mM sodium chloride. A clear zone around the colony in the replica plate was considered to have xylanase activity. The suspected colony in another replica plate was grown in Emerson broth and extracellular xylanase enzyme activity was analyzed by the colorimetric method using dinitro salicylic acid for estimation of reducing power. The morphological study of the bacteria was done after Gram stain and using 40x amplification in the phase contrast microscope. The isolated bacteria retained violet color after washing with acetone. Therefore, it is gram positive. Further characterization using various morphological, physiological and biochemical tests confirmed the bacteria as *Bacillus halodurans* and was given Accession number MTCC 9512 by IMTECH, Chandigarh. Growth conditions for the bacteria were optimized for maximum production of xylanase. The maximum amount of xylanase activity was found at the pH 9.5 and temperature 55°C. The growth of the bacteria and enzyme production were monitored up to 52 hours and it was found that the bacteria grew logarithmically up to 30 hours. Different carbon sources viz. xylan, sucrose, glucose, starch individually at 0.5% concentration were used in the Emerson growth medium. Maximum biomass growth was found with xylan whereas xylanase was maximally produced with glucose as carbon source. Therefore, glucose was considered to be the best inducer followed by xylan among the various carbon sources used. The enzyme was enriched by using 0-80% ammonium sulfate precipitation followed by desalting through Sephadex-G-25 gel filtration. The results indicated inhibitory nature of ammonium sulfate.

Key words: Alkaline xylanase, Alkalo-thermophilic bacteria, *Bacillus halodurans*, Gram positive, glucose inducer

Introduction

Xylanase (E.C 3.2.1.8) acts on β-1,4 xylan and cleaves β-1,4 glycosidic linkage randomly (6). The products are xylose, xylobiose and xylo-oligosaccharides. These products are useful feedstock for food and fine chemicals (5). It is of industrial importance and is used in paper manufacturing to degrade xylan to bleach paper pulp, increasing the brightness of pulp, improving the digestibility of animal feed and for clarification of fruit juices (3). Use of xylanase avoids the use of chemical processes that are very expensive and cause pollution (10, 11, 12). Bajpai et al. (2) showed that chemical extraction of lignin from pulp may be improved by treatment with xylanases. The enzyme has been mostly isolated from microbial and fungal sources (1, 4, 6, 9, 10 , 13).

Xylan is the most abundant noncellulosic polysaccharide present in both hardwoods and annual plants, and accounts for 20–35% of the total dry weight in tropical plant biomass. In temperate softwoods, xylans are less abundant and may comprise about 8% of the total dry weight. Xylan is found mainly in the secondary cell wall and is considered to be forming an interphase between lignin and other polysaccharides. It is likely that xylan molecules covalently link with lignin phenolic residues, and also interact with polysaccharides, such as pectin and glucan. In simplest forms, xylans are linear homopolymers that contain D-xylose monomers linked through β-1,4-glycosyl bonds

Microorganisms are rich sources of xylanase enzymes, which are produced by diverse genera and species of bacteria, actinomycetes and fungi. While several *Bacillus* species secrete high levels of extracellular xylanase, filamentous fungi secreting high amounts of extracellular proteins, xylanase secretion often accompanies cellulolytic enzymes – for example as in species of *Trichoderma, Penicillium*, and *Aspergillus.* To use xylanase enzymes for pulp treatment, it is preferable not to have any accompanying cellulolytic activity, since the cellulase may adversely affect the quality of the paper pulp. Some of the initial approaches for overcoming cellulase activity in xylanase preparations included treatment with mercurial compounds to selectively inhibit cellulase, or cloning and selective expression of xylanase genes in heterologous host systems. But perhaps the most practical approach has been in the screening for naturally occurring microbial strains that are capable of secreting cellulase-free xylanases under optimized fermentation conditions. A summary of such organisms and their enzyme characteristics has been presented by Srinivasan and Rele (11). Besides overcoming cellulase activity and conferring stability to xylanases at high temperatures (usually 60–70°C, which is also the temperature of the incoming pulp for the bleaching operation), the highly alkaline conditions prevailing in the pulp would also require that xylanases remain active and stable under the high alkaline pH conditions.

In the present study, a bacteria has been isolated from dung and its conditions of growth were optimized for production of xylanase. The produced xylanase has also been partially characterized.

Materials and Methods

Screening for the bacteria

Emerson medium (yeast extract, 0.55%; peptone, 0.5%; $MgSO_4$, 0.02%, K_2HPO_4, 0.1%, pH adjusted to 10 with the help of 0.1N NaOH) was used for the growth of the microbes. Semi-dried soil from a farm house of Kasturbagram, Indore where dung was abundant, was used as a source of the microbe. Using autoclaved water, 10^2 to 10^7 times dilutions were made from a suspension of the soil. Serially diluted soil sample was plated on Luria Broth (LB) nutrient agar plates and incubated at 55°C for 24 hours. The LB medium was consisted of bacto-tryptone, 10 gm; yeast extract, 5 gm; sodium chloride, 10 gm, agar 15 gm per litre and pH adjusted to 10 using 0.1N NaOH. Few single colonies with distinct morphology were isolated and transferred to Petri plates having Emmerson medium, pH 10.0 and incubated at 55°C for 24 hours.

Screening for Xylanase activity

The colonies so obtained were screened for xylanase activity using Congo red dye method**.** A replica plate was made and incubated at 55°C for 24 hours. Thereafter, one of the replica plate was flooded with 0.5% Congo red dye and then flooded with 1 mM solution of sodium chloride. A clear zone around the colony was taken as indication of xylanase activity.Corresponding colonies from another replica plate were

inoculated individually in Emerson medium broth, allowed to grow overnight and xylanase activity was analyzed in the medium.

Morphological Study of the bacteria

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

Production of Xylanase
Organism

Bacteria isolated from dung containing soil was used for the production of xylanase. It was maintained by routine monthly subculturing on Emerson medium slants.

Inoculum

The growth from 24 hours old Emerson slant was scrapped into sterile Emerson medium contained in a 500 ml capacity Erlenmeyer flask. and allowed to grow at 55°C for 24 hours on an orbital shaker with a speed of 200 rpm. 1 ml of this suspension was used as an inoculum for 100 ml medium. The flasks were incubated at 55°C for 72 hours on an orbital shaker with a speed of 180 rpm.

Medium for growth

Various synthetic and semi-synthetic growth media were tested for their suitability, viz. TGY media (tryptone , 0.5%; yeast extract, 0.5%; glucose, 0.1%; K_2HPO_4, 0.1%), Emerson media (yeast extract, 0.55%; peptone, 0.5%; $MgSO_4$, 0.02%, K_2HPO_4, 0.1%). Growth medium was then further studied for the effect of different carbon sources on the growth of the bacteria as well as increase in enzyme activity per ml of culture media. It was studied by supplementing Emerson media with 0.5% of different carbon sources viz. sucrose, starch, glucose and xylan.

Growth

From the growth media, culture samples were drawn at intervals of 4 hours. The growth was estimated in terms of wet weight of the cells per ml of the culture media. It was also measured in terms of increase in absorbance at 600 nm.

Growth time optimization

Samples from growing broth were drawn at 4 hours intervals, centrifuged at 10,000 x g for 10 minutes in the cold condition (0 to 4°C). Supernatants were analyzed for xylanase activity.

Harvesting of the bacteria

The cells were harvested by centrifuging broth at 8000 rpm for 30 minutes at 0 to 4°C in a Sorvall RC-5B superspeed cooling centrifuge using GSA rotor. Supernatant contained most of the enzyme activity.

Enzyme assay

Xylanase enzyme was assayed by measuring the release of reducing sugar from birch wood xylan following the dinitrosalicylic acid (DNS) method (8). A 1.8 ml sample of 1% xylan from birch wood in 50 mM glycine- NaOH buffer, pH 9.5 was incubated at 55°C for 5 minutes. To this, 0.2 ml enzyme (supernatant of the growth medium considering xylanase as extracellular enzyme) was added and was incubated at 55°C for 10 minutes. The reaction was stopped by adding 3 ml of dinitrosalicylic acid solution (DNS) and the tubes were incubated in a boiling water bath for 15 minutes. A control was also run simultaneously where enzyme was added after the addition of DNS. 5 ml of water was added in all the tubes and the absorbance was measured at 540 nm. A blank was also prepared where no enzyme was added and against the blank, zero was set in the colorimeter. D-Xylose was used as standard during the colorimetric estimation. One unit of the enzyme activity was taken as the amount of the enzyme required to liberate

reducing power equivalent to one micromole of xylose per minute under the conditions of the enzyme assay. Specific activity was taken as units per mg protein.

Protein estimation

Protein was estimated using the method of Lowry et al. (7) using bovine serum albumin as a standard.

Enzyme purification

Being thermostable enzyme, unless otherwise stated, the entire purification procedure was carried out at the room temperature (nearly 25°C). Supernatant of the broth after harvesting the bacterial cells by centrifugation at 8000 rpm for 30 minutes was taken. To it, powdered ammonium sulfate was slowly added with constant stirring to get 0- 80% saturation and the pH was maintained at 9.5 by the addition of dilute ammonia. After storage for 3 hours, it was centrifuged at 15000 x *g* for 30 minutes. The pellet was dissolved in 50 mM glycine- NaOH buffer, pH 9.5, centrifuged and the supernatant was desalted using Sephadex G-25 column chromatography.

Results and Discussion

Screening of the bacteria

A xylanase producing bacteria was isolated from the dung containing soil. Its xylanase producing activity was confirmed by congo red dye staining method. Xylanase activity was confirmed in the bacteria by the presence of a clear zone around the colony. These zones were also enhanced on treating the plate with 1 mM sodium chloride.

Morphological studies revealed it to be a rod shaped , gram positive, endospore forming bacteria. Spore was sub-centrally placed. This infers the bacteria to be *Bacillus*. Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh identified it as *Bacillus halodurans* and registered it as *Bacilllus halodurans*, MTCC No. 9512. The morphological characteristics of the strain are shown in Table1.

Table 1: Morphological characteristics of *Bacillus halodurans* .

Tests	Result
Colony morphology	
Configuration	Circular
Margin	Entire
Elevation	Slightly raised
Surface	Dry
Pigment	Off white
Opacity	Opaque
Gram's reaction	+ve
Cell shape	Big rods
Size (um)	3-9u
Arrangement	Occurring singly
Spore(s)	+ve
Endospore	+ (in chains)
Position	Terminal
Sporangia Bulging	Slightly bulged
Shape	Oval
Motility	+ve

Effect of physiological factors on the bacterial growth

The *Bacillus halodurans* was grown at various temperatures ranging from 10°C to 42°C. It showed good growth in the range of 25 to 42°C (Table 2). Effect of pH on the growth of it was checked in the pH range of 5.0 to 10.0 and was found to have good growth in the pH range of 8 to 10. The effect of sodium chloride on the growth of the bacteria was also observed in the range of 2 to 10% sodium chloride and was found to have similar growth in the presence of various concentrations of sodium chloride (Table 2). However, it could not be grown under anaerobic conditions confirming it as obligate aerobic organism (Table 2).

Table 2: Effect of physiological factors on the growth of *Bacillus halodurans* .

Tests	Result
Growth at temperatures	
10^0 C	-
25^0 C	+
30^0 C	+
37^0 C	+
42^0 C	+
Growth at pH	
pH 5.0	-
pH6.0	-
pH7.0	+
pH 8.0	+
pH 10.0	+
Growth on NaCI (%)	
2.0	+
4.0	+
6.0	+
8.0	+
10.0	+
Anaerobic Growth	-

+ : Positive, -: Negative

Biochemical characteristics of the *Bacillus halodurans*

Various tests viz. indole test, methyl red test, Voges Poskauer test, hydrogen sulfide production, gas production, casein hydrolysis, Mac Conkey test, citrate test were found negative with the bacteria and were found all to be negative (Table 3). The bacteria showed negative test for esculin and urea hydrolysis whereas it showed positive test for gelatin and starch hydrolysis (Table 3).

Table 3: Various Biochemical characteristics of *Bacillus haloduran*

Tests	Result
Indole test	-
Methyl red test	-
Voges Proskauer test	-
H_2S production	-
Gas production	-
Casein hydrolysis	-
Mac Conkey	-
Citrate	-
Esculin hydrolysis	NG
Gelatin hydrolysis	+
Starch hydrolysis	+
Urea hydrolysis	-
Nitrate reduction	(+)
Arginine dihydrolase	-
Tween 20 hydrolysis	+
Tween 40 hydrolysis	+
Tween 80 hydrolysis	+
Catalase test	+
Oxidase test	+
Acid Production from	
Dextrose	+
Maltose	-
Mannitol	-
Xylose	-
Mannose	+

+ : Positive ; -: Negative ; NG: No Growth

Production of Xylanase

Although many growth media were tested for the xylanase producing bacteria, ultimately on the basis of growth of the bacteria and production of xylanase, Emerson medium was selected for the growth of the bacteria.

Time optimization for xylanase production

The growth and enzyme production were monitored for 52 hours. It was found that the microbe grew logarithmically up to 35 to 40 hours (Fig. 1). Xylanase production was observed to increase up to 42 hours and thereafter, it was constant (Fig. 1).

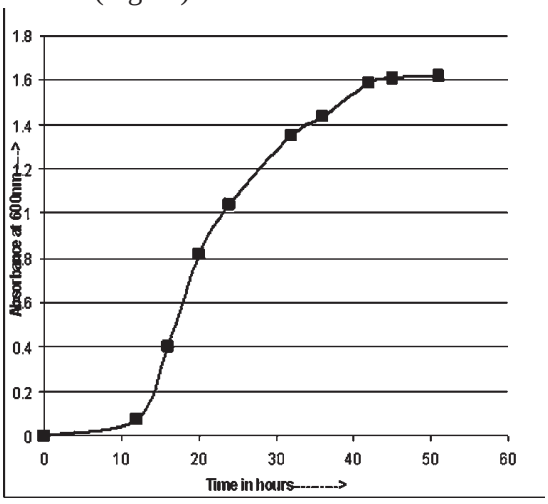

Fig. 1a. Bacterial growth pattern with time

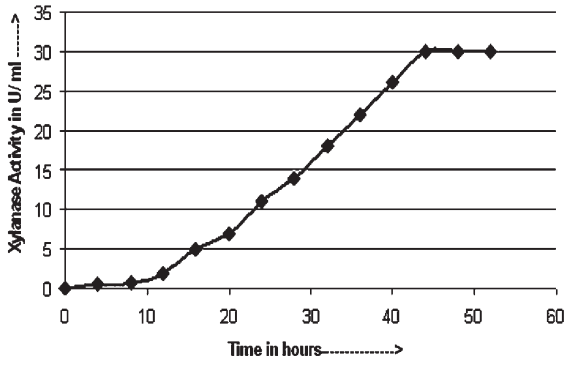

Fig. 1b. Production of xylanase in the growth medium by the microbe as a function of growth time

Effect of different carbon sources on xylanase production

Different carbon sources viz. xylan, sucrose, starch and glucose were used for xylanase production. Emerson medium containing different carbon sources at the concentration of 0.5% were used for growth and enzyme production. Maximum biomass growth was found to be associated with xylan while enzyme production was maximum in case of glucose. Although all of these carbon sources act as inducer for xylanase production, only glucose was found to be strong inducer. The pattern of biomass growth and enzyme production on different carbon sources is shown in Fig. 2.

1=Xylan, 2=Glucose, 3=Sucrose, 4=Lactose

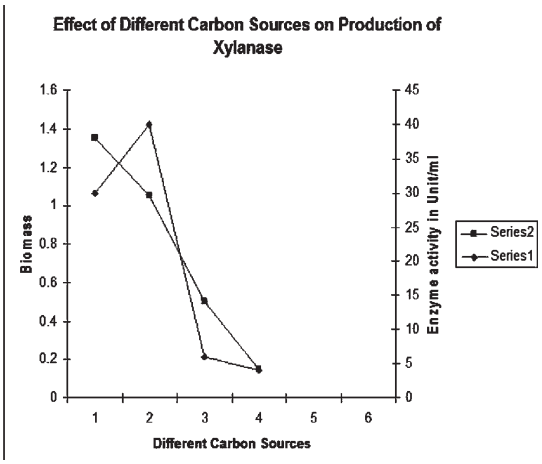

Fig. 2: Effect of different carbon sources on the growth of the microbe and production of xylanase.

Enzyme purification

The culture after 42 hours of growth was centrifuged and the pellet was discarded. The supernatant having xylanase enzyme activity was subjected to protein precipitation using 0 to 80% ammonium sulfate. After ammonium sulfate precipitation, xylanase activity in the sample was only 16.6%. The fraction after centrifugation to get clear supernatant was desalted through

Sephadex G-25 column chromatography. After desalting, recovery increased to nearly 50%. These results showed that ammonium sulfate is inhibitory to xylanase activity.

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Development of a Novel Transdermal Ibuprofen Ointment

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Abstract

The present work was undertaken to develop and evaluate transdermal ointments of ibuprofen containing supersaturated drug and menthol as a penetration enhancer. Trandermal ointments were prepared using white petrolatum, bees wax, propylene glycol, PEG 400 and menthol using a fusion technique. Microscopy was used to determine the supersaturation of the drug in the vehicle. The formulated ointments were subjected to *in vitro* release studies and skin permeation studies. These studies were conducted in the diffusion cells developed in our laboratory, specifically for this purpose. Selected formulations were evaluated for their anti-inflammatory activity using the carrageenan-induced paw edema in rats. The formulation containing menthol demonstrated more transport across the skin. The final formulations selected for topical and systemic investigation had menthol in both the formulations. The results corroborated the fact that the drug was released into the systemic circulation from ibuprofen ointments after topical application with one containing penetration enhancer releasing more. The study clearly indicates that trans-dermal delivery of ibuprofen using a topical petrolatum base ointment is a viable option.

Key words: *transdermal; ointment; ibuprofen; co-solvency; solubility; carrageenan*

Introduction

Ibuprofen, a non-steroidal anti-inflammatory drug (NSAID) is used in several inflammatory conditions (1). Several semi-solid dosage forms for ibuprofen with variable drug content are used for topical inflammatory conditions such as backache and muscular pain (2). Gels and creams with 10% and 15% ibuprofen, respectively, are available. Although popular for topical inflammatory conditions, it is also useful in migraine, dental pain, pain associated with PMS, sore throat, cold/flu and fever, all of which needs systemic delivery of ibuprofen and is more effective than paracetamol in some of these conditions (3,4). For systemic delivery of drugs, dosage forms such as oral tablets, capsules, caplets, intravenous solution, oral suspension, oral solution and suppositories (5) are available. In pharmaceutical market, ibuprofen is also available in all these dosage forms and for systemic delivery of ibuprofen these dosage forms are popularly prescribed by physicians (6). However, on many of these occasions the drug has to reach the systemic circulation via the oral route where it could cause very significant side-effects like peptic ulcers. Thus, oral route is generally not a preferable route for ibuprofen. Other routes to reach systemic circulation could be conveniently attempted. In this regard, a transdermal route which has many advantages could be the choice for ibuprofen. We have conducted some preliminary literature search regarding the transport of ibuprofen across skin and entrance into the systemic circulation. Interestingly, several reports suggested that ibuprofen enters the systemic circulation from topical route at a very high rate and extent (7,8). However, transdermal formulations for ibuprofen are not yet available in the market. Further, literature search suggests

that this route and mode of delivery using semisolids for systemic delivery is promising and is slowly gaining prominence (9). There are several advantages for systemic delivery of drugs with the ointment usage compared to a transdermal patch. Thus, this gave us enough leads to investigate further on this novel transdermal ointment approach for systemic delivery of ibuprofen.

Transdermal patches and its modifications such as electrically based enhancement techniques, photomechanical waves and microneedles are different topical approaches that could lead to drug levels in the systemic circulation (10). The very well known examples present in the market are nitroglycerin, fentanyl, lidocaine, estradiol patches, etc. On the other hand semisolids for transdermal delivery into systemic circulation can also be attempted for drugs with high penetration into the skin. There are some additional advantages to these semi-solids compared to transdermal patches and its modifications and these include ease of application, cosmetic appeal and reduced skin irritation (10). The aim of this investigation was to develop a petrolatum-based ibuprofen ointment that could lead to convenient systemic levels after topical administration. The selection of petrolatum base has several advantages for transdermal delivery of drugs and as well, currently high grade and high purity petrolatum with clear qualification and instructions, which was not previously available is sold in the market and for these reasons it was naturally, the selected ointment base in this study.

Experimental

Materials and Methods

Ibuprofen was obtained from Boots India Ltd., Mumbai. White bees wax, hard paraffin were purchased from Loba Chemic, Mumbai. White soft paraffin was purchased from Burgoyn Urbidges & Co., Mumbai. Polyethylene glycol 400, propylene glycol, sodium carboxymethyl cellulose (CMC) and menthol were obtained from S.D. Fine Chemicals, Bombay. Methanol was obtained from Ranbaxy Chemicals, Delhi. Magnetic stirrers were obtained from Remi Equipments Pvt. Limited. A Double Beam UV-Vis Spectrophotometer (SL 164) used to analyze the samples was obtained from Elico, Mumbai. A diffusion cell used to study drug release from the ointments was designed in our laboratory. Carrageenan sodium salt was obtained from SD fine Chemicals Ltd., Mumbai. Microscope was obtained from Ajay Optics. Centrifuge, vortex mixer and magnetic stirrer were obtained from Remi Industries Ltd. Menthol was obtained from Final Chemicals. Diethylether was obtained from Finar Chemicals. Plethysmograph used to determine the extent of inflammation in a rat was locally made.

Development of the ointments

To develop the appropriate Ibuprofen ointment, petrolatum based ointment excipients which include white beeswax, and hard paraffin were used (Table 1). In its preparation PEG 400 and propylene glycol were used as co-solvents. This could lead to higher solubility of the drug in the base. Ointments containing increased concentration from 3% to 7% of PEG 400 and propylene glycol were prepared and the solubility of the drug was determined using a microscopic method previously published (11). The final ointment formulation is anticipated to have high drug levels in the soluble form. A high drug level especially in the soluble form can lead to therapeutic drug levels in the systemic circulation after ointment administration than compared to the existence of the same drug in the insoluble form. As a reason, a 12.5% drug containing formulation was selected. This concentration is well below 10% of its solubility in the selected formulation with a minimum amounts of the cosolvent used in this study (its solubility in the minimum cosolvent containing formulation is

Table 1. Compositions of Ointments Investigated for Drug Release and Transport across the Skin

Composition/Formulation (% W/W)	Form 1	Form 2	Form 3	Form 4
Drug	12.5	12.5	12.5	12.5
PEG 400	3	3	3	3
Propylene Glycol	7	7	8	8
Menthol	-	3	3	5
Petrolatum and Beeswax	upto 100	upto 100	upto 100	upto 100

13.75%; see results). Although gels and creams with such a high content are available, there could be significant precipitation of the ibuprofen in these formulations of higher aqueous nature because of its poor water solubility. This may not result in systemic therapeutic levels. Thus, these are used only for local applications. On the other hand, petrolatum base can incorporate more drug in soluble form and thereby can lead to higher systemic levels. Paraffin/PEG/Propylene glycol ointment was prepared by melting white bees wax, hard paraffin, to which the drug dissolved in PEG 400 or propylene glycol with or without menthol added while stirring. The entire mixture was stirred while cooling to form Ibuprofen ointment.

Drug release into dissolution medium

In vitro release studies are important for a number of reasons including product optimization and *in vitro- in vivo* correlations. Drug release measurements were carried out in a diffusion cell designed in our laboratory (Figure 1) in optimized dissolution media using all the four formulations prepared in this study. A dialysis membrane (gelatin paper soaked in water at 50ºC for 10 min) was placed between the donor and the receiver. The donor always contained 500 mg of the ointment. Since the ointment was prepared using a fusion technique and contained the drug in the soluble form and was used for the release studies just after it was manufactured, it was assumed that the content of the drug in all the ointments applied on the donor side is the same. As a reason, we did not estimate the drug amount in the ointments prior to its usage in the release studies and thus the drug content, content uniformity, spreadability and viscosity were not determined. The dissolution media was optimized by investigating the drug release from a 5% ibuprofen suspension prepared using CMC as the suspending agent into different compositions of methanol:water (30 : 70) (Media 1), methanol:water (15 : 85) (Media 2) and pure water (Media 3). Pure water offered a better sink compared to PBS that occasional showed interference in the UV assays in the presence of methanol. Addition of methanol in the media can result in a better sink condition. Release drug contents were measured using UV-double beam spectrophotometer at wave length 221nm. The media which supported sink conditions the best was taken as an optimized media. The dissolution studies were conducted as previously described (11) and the diffusion coefficient calculations are based on Higuchi equation (12) which is shown below:

$$Q = 2C_0(Dt/\delta)^{1/2} \ldots\ldots\ldots\ldots \text{ eqn 1}$$

Where 'C_0' is the initial drug concentration in the donor,

'Q' is the cumulative amount of drug,

't' is the release time and

'D' is the diffusion coefficient.

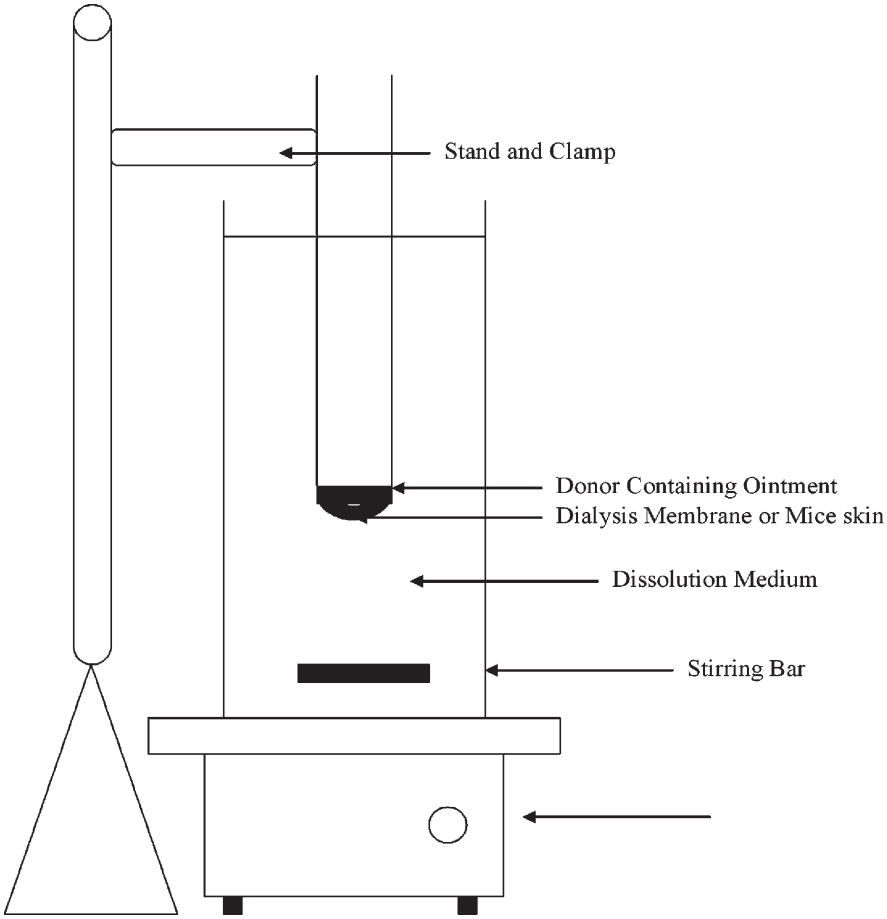

Figure 1. Diffusion Cell Used in this Study

Transport across mice skin

The skin used for transport studies was removed from the mice and the section was prepared as described previously (13). The skin section thus prepared was clamped carefully to one end of the hollow glass tube (dialysis cell) so that the stratum corneum faced up on the receiver compartment. The dissolution medium selected by optimization technique previously mentioned was used as receiver compartment. The donor compartment was immersed into the receiver compartment so that the edge just touched the receiver compartment. For first 30 min skin washing was performed. Then the receiver fluid was replaced with fresh dissolution media. The known quantity (500 mg ointment) was spread uniformly and the experiment was continued as mentioned in the release studies section. Permeation profiles were constructed by plotting the cumulative amount of the drug permeated Vs time.

Antiinflammatory effects (Carrageenan rat paw method)

The anti-inflammatory activities of the formulations under investigation were studied using the carrageenan-induced edema model as previously mentioned (14). Male wistar rats (140-

175 g) were used. Formulations were prepared just before the administration. Drug formulations administered were: two selected ointments and one oral suspension formulations. Carrageenan 1% solution to be injected into a rat was prepared by adding 250 mg of carrageenan in 25 ml of Normal saline. The solution was injected into the hand paw of the rat to cause inflammation. For investigating the systemic effects, the ointments (500 mg) were applied to the shaved surface on the abdomen of the rat and for investigating the local effects the ointments were applied near the paw at the site of inflammation (a fairen electronic shaver with trimmer were used to shave the abdomen of the rat). It is assumed that the drug diffuses from the ointment, reaches the systemic circulation via transdermal route and thereby elicits the action. Inflammation was measured by the equipment called plethysmograph (Narsaiah Enterprises, Warangal, India). The percentage increased in the volume of paw was calculated using the formula:

$$\% \text{ Increase in paw swelling} = \frac{V - Vi}{Vi} \times 100$$

Where V = Volume of the paw 2 hr after the carrageenan injection

Vi= The initial paw volume

Results

Upon dissolving ibuprofen in propylene glycol and PEG 400 at a ratio of 7:3 in the total ointment composition and thereby dispersing into the petrolatum based ointment base, a 12.5% w/w ibuprofen ointment containing drug in the solubilized formed. A 3% menthol could be conveniently incorporated as a penetration enhancer into this. The final formulations have drug with 10% of excess in solubilized form. The compositions are tabulated (Table 1). To investigate the release of the drug from the prepared 12.5% ibuprofen ointment, tailoring (optimizing) of dissolution medium is essential such that sink conditions are maintained during the release. Three different media (Media 1, Media 2 and Media 3) were investigated for this purpose and finally Media 1 was found to be optimum and we used this dissolution medium to investigate drug transport in this study (Figure 2). Drug release studies were investigated to determine the rate and extent of drug release from the ointment. The drug release from all the four formulations was investigated. The release depended on the composition of the medium (Figure 3). From cumulative amount release data, Form 4 was more effective than Form 2, which was more effective than Form 1, and Form 1 was more effective than Form 3. When square root time vs cumulative amount drug release was plotted (Higuchi plot), it yielded a straight line for all the formulations (Figure 4). Thus, using Higuchi equation, we could calculate the diffusion coefficient of the drug from the vehicle. The calculated diffusion coefficients for Form 1, Form 2, Form 3 and Form 4, were 8.86 *10E^7, 11.34 *10E^7, 5.57 *10E^7 and 14.33 *10E^7, respectively. In drug transport across mice skin studies, it was found that ibuprofen transported across the skin from all the formulations. The transport was enhanced in the presence of menthol (Figure 5). Local and systemic effects of the drug after topical application in the form of the ointment were tested in a carrageenan-induced rat paw inflammatory model. Based on the drug release from the ointments and skin permeation, Formulation 2 and Formulation 4 were selected to investigate this. The percentage inhibition of inflammation in the rat paw method in case of Formulation 4 which contained a penetration enhancer and administered at a remote location was 65%, while with Formulation 2 that contained no penetration enhancer had 35% percent inhibition. However, in case of local effects the inflammation reduction with both the ointments was 100% suggesting that this mode of administration better suits for ibuprofen ointment. In either case, a placebo control both for administering at the inflammatory site (local application) as well as at the remote location (systemic application) was used.

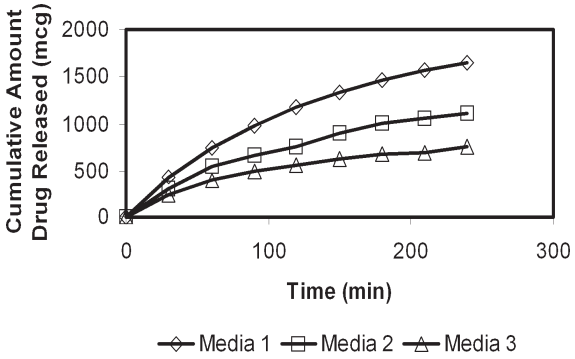

Figure 2. Optimization of the Dissolution Medium

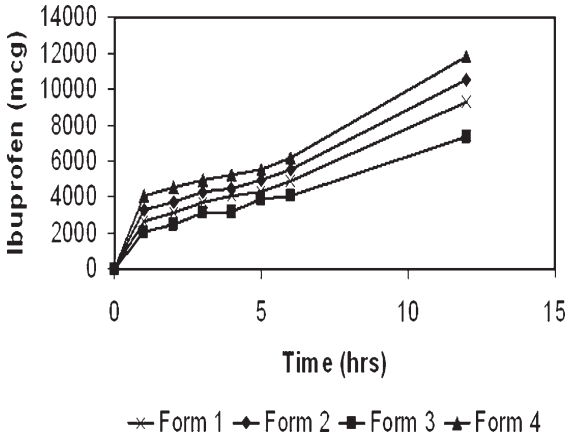

Figure 3. Cumulative Amount of Drug Release From the Selected Formulations

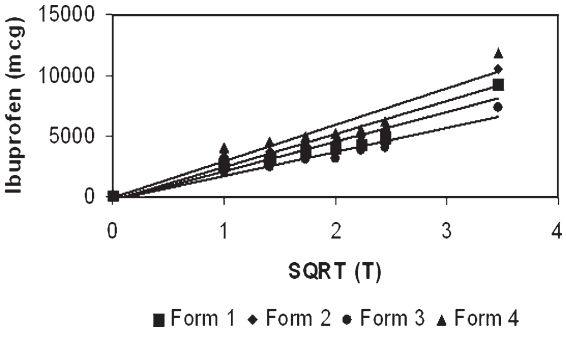

Figure 4. Higuchi Plot for the Release of the Drug From the Selected Formulations

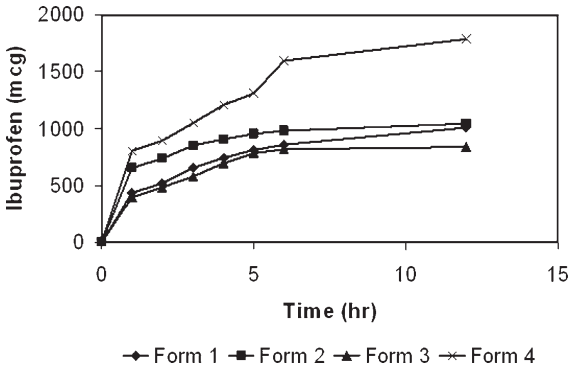

Figure 5. Cumulative Amount of Drug Release From the Formulations Across the Mice Skin

Placebos did not demonstrate any anti-inflammatory effects.

Discussion

Currently, delivery of drugs into systemic circulation via topical route by applying ointments is the state of art in this area of research (9). In this study, we aimed at investigating this issue taking ibuprofen as the drug of choice as it has several systemic applications as well it has been previously shown that it is taken at a very high level into systemic circulation after topical application, although this issue regarding its systemic delivery was not the focus (7, 8). To facilitate drug transport into the skin and thereby into the systemic circulation, methods like hydrating the skin, saturating the vehicle with the drug or adding chemical penetration enhancers (15) have been researched. All these three factors were incorporated in the formulation development of a transdermal ibuprofen ointment. A petrolatum base hydrates the skin very well. Thermodynamic activity of the drug in the formulation, which is one of the deciding factors for enhanced absorption into deeper layers of the skin and also into the systemic circulation, can be enhanced by increasing the solubility of the drug in the vehicle (16). On these lines supersaturation of the drug in the vehicles is the need of the hour. Additionally,

we have incorporated a penetration enhancer. We have opted to prepare a 12.5% ibuprofen ointment containing the active in a soluble form so that super-saturation of the drug occurs in the presence of co-solvents and also optimum viscosity or thixotrophic properties are the characteristics of the formulae investigated. Propylene glycol and PEG was used as co-solvents to enhance the solubility of the drug in the ointment base. Methods of increasing the solubility of the drug in the petrolatum based ointment by cosolvency techniques have been previously described (17). As the concentration of propylene glycol was increased to 7% the solubility reached to more than 12.5%. The solubility was determined using a microscopic method. The final topical ointment base incorporated PEG 400: 3%, propylene glycol: 7%, drug: 12.5% and rest petrolatum base. Further, another set of ointment base for systemic delivery of the drug was prepared. This additionally incorporated 3% and 5% of menthol in it.

Subsequently, the ointments were characterized for *in vitro* drug release into the dissolution medium and drug transport across mice skin and diffusion coefficients from the release data and drug transport across the skin determined. The calculations and interpretation of drug release studies followed a modification of protocols published by Ozsoy et al., 2004 (11) and Zhang et al., (2002) (18). In the *in vitro* drug transport across the mice skin, the amount of drug transported through unit area of skin was more in case of formulation containing penetration enhancer, suggesting that for systemic delivery second formulation is better. The results with the two formulations in carrageenan-induced inflammation model corroborated the fact that the drug was released into the systemic circulation from ibuprofen ointments after topical application with the one containing penetration enhancer releasing more. Results also indicated that local use is better, however, systemic administration was also able to subside the inflammation. Thus, the study clearly indicated that transdermal delivery system for ibuprofen is a viable option. However, more studies are to be conducted to further develop an effective and clinically viable ointment for ibuprofen for systemic delivery.

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Detection of telomerase activity in different cancer tissues: a diagnostic marker

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Abstract

Telomerase is a ribonucleoprotein enzyme that plays an important role in cell immortalization and carcinogenesis. It is not detectable in normal somatic cells. In this report, we examined the usefulness of telomerase activity for diagnosing cancer by utilizing telomeric repeat amplification protocol (TRAP) assay. Telomerase activity was analyzed in cancer tissue samples (breast, prostate, lung) using highly sensitive non-isotopic PCR-based TRAP assay. In total, 45 histologically diagnosed specimens were analyzed including breast cancer (10), prostate cancer (10), lung cancer (10) and normal tissue samples (breast, prostate and lung) (15). In nine out of ten breast cancer, eight out of ten prostate cancer and ten out of ten lung cancer samples showed telomerase activity. Telomerase activity was detected in positive control and in all most all tumor samples but not detected in normal, heat treated and negative control samples. Detection of telomerase is important for the clinical diagnosis and treatment of cancer.

Key words: Telomerase, diagnostic marker, ribonucleoprotein, TRAP

Introduction

Telomerase is a cellular reverse transcriptase enzyme which catalyzes the synthesis and extension of telomeric DNA (1), helps to stabilize telomere length in human stem cells by adding TTAGGG repeats on to the telomeres using its intrinsic RNA as a template for reverse transcription (2). Telomerase activity is expressed in approximately 90% of tumors and is absent in nonneoplastic tissues and normal somatic cells (3). Therefore, telomerase activity or telomerase components could be potentially useful as novel diagnostic marker for a wide range of cancers (4) and its potential to predict clinical out come in a range of different neoplasias has been largely documented (5,6, 7). The most prominent hypothesis is that maintenance of telomere stability is required for the long-term proliferation of tumors (8). Thus, escape from cellular senescence and becoming immortal by activating telomerase, or an alternative mechanism to maintain telomeres (9), constitutes an additional step in oncogenesis that most tumors require for their ongoing proliferation. This makes telomerase a target not only for cancer diagnosis but also for the development of novel anticancer therapeutics agents.

There is much evidence that, in human cells, cell division in the absence of telomerase activity leads to telomeric shortening, providing a mechanism to limit the proliferative capacity of normal cells, which senesce and stop dividing after undergoing a given number of cell divisions (10). Telomerase may need to be activated to allow cells to escape from senescence and thus proliferate indefinitely, a process referred to as

immortalization. This supported by the findings that there is no telomerase activity in most somatic cells, whose telomeres shorten with replicative age (11).

Among more than 100 proposed cancer markers, telomerase is unique as it is detected in almost all kinds of cancer tissues with a very high positive rate. In some instances, when telomerase activity appears in most tumor cells at the preneoplastic or in situ stage, telomerase activity may be useful for early detection of cancer, especially in cytology samples. In other instances, in which the level of telomerase activity is not high but increase with cancer progression (9), telomerase activity levels in tumor tissue may be prognostic indicator of patient outcome. Thus in the present investigation telomerase is being studied in anticipation of clinical usage. In fact several clinical trails of telomerase assay for cancer diagnosis are now in progress (12).

The aim of this study was to use telomerase activity as a molecular marker for the detection of cancer cells with replicative potential in the tissues of patients with breast, prostate, lung cancer. The analysis of telomerase activity status has potential clinical utility for diagnosis, screening and monitoring treatment.

Materials and Methods

Tissue procurement

Tissue samples were obtained from surgical specimens resected in the local Hospitals (Warangal, Andhra Pradesh, India).Tissues were frozen immediately after resection and stored at -80^{o}C until analysis. Samples were obtained from patients aged between 35-60 years.

Preparation of tissue extracts

Tissue extracts were prepared using the protocol developed by Kim and colleagues (13) with modification. A positive control for telomerase activity was prepared using lysates of the cell pellet (10^6 cells) provided in the kit and stored at -80^{o}C. Each tissue sample (40-100mg) was homogenized in 200 µL of (3-[(3-chloramidoproply) dimethylamino]-1-propane-sulphonate) (CHAPS) lysis buffer and RNase inhibitor (100-200 units/ml). After 30 min. on ice, the lysate was centrifuged at 12,000g for 20 min. at 4^{o}C.the supernatant was quickly frozen on dry ice and stored at -80^{o}C. The protein concentration of the extract was measured using Lowry's method. For heat-inactivated control incubate 10µL of each tissue sample extract at 85^{o}C for 10 min. When performing the TRAP assay, 2 µL of protein was analyzed according to the manufacturer's instruction.

TRAP assay

Telomerase activity was assayed by the modified TRAP assay using the TRAP^{EZE} telomerase detection kit (CHEMICON, USA). The assay is a one buffer, two enzyme system using the PCR. In the first step of the assay, telomerase adds 6 bp telomeric sequence (TTAGGG) on to the 3'end of a substrate oligonucleotide (5'-AATCCGTCGA GCAGA GTT-3'). In the second step, the extended products are amplified by PCR. Aliquots of different protein concentrations were tested by TRAP assay. Because the best differentiation of the samples was seen with extracts containing 500ng protein we used this concentration as the standard concentration. The reactions were carried out on a total volume of 50µl which contain TRAP reaction buffer (200mM Tris HCL ,pH 8.3, 15 mM $Mgcl_2$, 630 mM KCL, 0.5% Tween 20, 10 mM EGTA), 50X dNTP mix, TS primier, Primer mix(RP primier,K1 primier, TSK1template) Taq polymerase (5Units/µL),PCR grade water and samples. After 30min. incubation at 30^{o}C, the samples were subjected to 33 PCR cycles of 94^{o}C for 30sec., 59^{o}C for 30sec and 72^{o}C for 1min. once the reactions were completed, a non denaturant electrophoresis was done on 10% polyacrylamide gels in 0.5X TBE buffer.

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Subsequent to electrophoresis, the gels were immersed for 30min. in a solution of Ethidium Bromide strain, which was prepared in deionized water according to the manufacturer's recommendations, then visualized at UV (302nm) under transilluminator and photographs were taken by using CCD imaging system.

Result

Telomerase is a ribonucleoprotein. Kim and co-workers have developed an extremely sensitive assay for the detection of telomerase activity, however, it requires radioisotopes for the reaction. In this study, telomerase activity was detected by a non isotopic TRAP assay combined with ethidium bromide staining. A representative TRAP assay is depicted in (Fig. 1and 2). The presence of active telomerase in a sample is revealed by a characteristic ladder of products, created by PCR amplification of the DNA synthesized by the enzyme that is entirely composed of 6-bp TTAGGG tandem repeats. All samples were evaluated using 500ng of total protein to confirm telomerase status. Telomerase activity was detected in nine out of ten breast cancer (90%), eight out of ten prostate cancer (80%) and ten out of ten lung cancer (100%) cases. This activity was sensitive to heat (80^{o}C) in each case, heat inactivation of tissue extracts completely eliminated the signals demonstrating the specificity of the enzymatic detection (fig.1, lanes 4 fig. 2, lanes 3, 7, 8) and no telomerase activity was found in patients with no evidence of breast, prostate, lung cancer (normal samples) (fig.1, lanes 1 fig. 2, lanes2, 5, 6) using TRAP assay. The inability to detect telomerase activity in these extracts may have been a true indicator of the absence of telomerase activity. Furthermore, telomerase positive samples showed the characteristic processive 6-bp ladder up on PAGE (fig.1and 2).

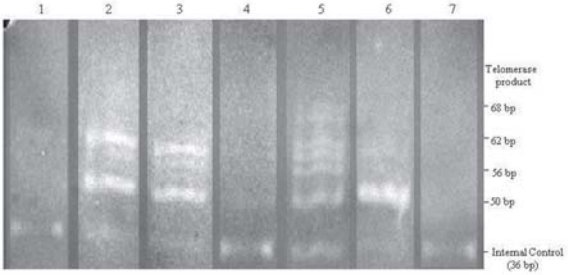

Figure 1
TRAP gel assay of the tumor samples. The modified TRAP assay was used to analyze the telomerase activity of the normal and cancer samples. Lane: 1, normal breast; Lanes: 2, 3 show telomerase activity in breast cancer samples; Lane: 4, heat treated control; Lane: 5, positive control; Lane: 6, shows telomerase activity in prostate cancer sample and Lane: 7, negative control (CHAPS lysis buffer only).

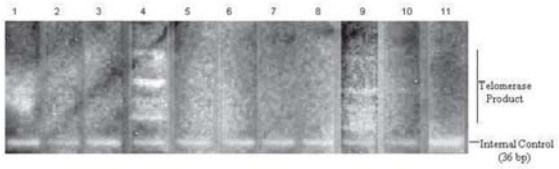

Figure 2

TRAP gel assay of tumor and normal samples. The modified TRAP assay was used to analyze the telomerase activity of the tumor samples. lane: 1, prostate cancer ; Lanes: 2, 3 normal prostate, heat treated; Lane: 4, lung cancer; Lanes: 5 and 6 normal lung; Lanes: 7 and 8 heat treated; Lane: 9,lung cancer; and Lane: 10, 11 negative controls (CHAPS lysis buffer only)

Discussion

There is increasing interest in identifying molecular markers which could ultimately replace the older anatomically or cytologically oriented methods for the early detection of cancer. The diagnosis based on cytology alone is often difficult. It is noteworthy that, in the present study all most

all of the patients with stage-I cytology had telomerase activity detected using sensitive PCR-based TRAP assay. These observations illustrate one of the limitations of morphologic cytology. Since cytology specimens may contain degenerated cells and many contain only a few cancerous cells such specimens may hamper the proper cytologic diagnosis. Thus using new diagnostic markers such as telomerase in combination with cytology may prove more reliable in diagnosis of cancer. Measurement of telomerase activity may be most helpful when cytologic examination fails to detect cancer cells. The usefulness of validating whether telomerase is such a marker is that it may affect the duration of clinical trials, require a smaller sample size, and reduce costs.

The development of the sensitive TRAP assay (13) has enabled the evaluation of telomerase activity in many types of human cancers. The reported activity of positive TRAP assays of solid tumor samples are ~90% (14), and it is now widely accepted that, except for a new specialized cell type telomerase activity in cells of somatic origin is indicative of immortal transformation of one of the major advantages of using this analyte is the ability to use minimal amounts of clinical sample material.

The present study detects telomerase activity in cancer tissues by using sensitive method that is available commercially in the market. The methodology utilized in this kit method is based on an improved version of the original method described by (13). The assay is a one buffer, two-enzyme system utilizing the Polymerase Chain Reaction (PCR). Using a PCR-based telomerase assay, telomerase activity was found nearly all of the human cancer tissue samples investigated in the present study which was not seen in normal tissues.

Breast cancer is the most common cause of mortality due to malignant diseases in women, and despite major advances in adjuvant therapy, improvement in survival has been disappointingly small (15). In the present study telomerase activity was detected in 90% of breast cancer samples. The current results confirm previous reports (16, 17,) and a recent report has shown that telomerase activity is nearly ubiquitous in invasive breast tumors (18). In a retrospective study of a large number of breast cancers the levels of telomerase activity significantly correlated with clinical outcomes and several prognostic indicators (19).

Telomerase activity is detectable with a high frequency in lung cancer tissues. In the present study we demonstrated that 100% of lung cancer samples from patients with stage-I had telomerase activity by using PCR based TRAP assay. The current results confirm and extended previous reports (20, 21) that high proportions of lung cancers have telomerase activity. More importantly our results strongly suggest that the level of telomerase activity correlated with the prognosis of the patients.

Telomerase activity of the prostate cancers investigated in the present study 80% of the samples showed telomerase activity**.** In accordance with previous results (22) a lack of telomerase activity has been reported by others (23). Telomerase is generally expressed at low levels in tumors with a favorable prognosis, at very high levels in tumors with an unfavorable prognosis and is not detected in tumors which subsequently regressed (24). Moreover, in a study of bladder cancer, the tumors with high telomerase activity were mostly those of an advanced grade, where as tumors with low telomerase activity was of low grade (25).

There is evidence in breast (26) and gastrointestinal cancers (24) that the presence of high levels of telomerase correlates with poor prognosis. In the present investigation we observed same results in prostate cancer samples.

Conclusion

Detecting telomerase activity is an important and necessary step in studies associated with this enzyme and its implications on cell proliferation. As telomerase appears to be involved in carcinogenesis, this ribonucleoprotein has gained great attention in cancer research, especially as it might serve as a diagnostic and also prognostic marker.

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Gliomastix indicus sp. nov.

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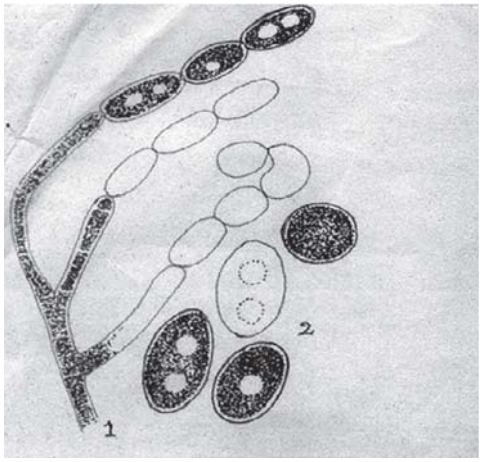
Abstract

Gliomastix indicus S. Nagalakshmi & A. Subrahm sp.nov. was isolated from a wasteland soil sample collected at Tiruchengode, Tamilnadu. It rapidly grows on all common mycological media like malt extract, potato-dextrose agar etc. Due to the presence of catenulate amerospores, it is assigned to the genus *Gliomastix.* It stands distinct from all the known species in having rapid growth rate and larger conidia. Its ability to reduce paper mill effluent and colour is an additional feature of distinction.

Keywords : *Gliomastix indicus,* soil

During the course of study on fungal flora of Salem district, Tamilnadu, an interesting fast growing isolate assignable to G*liomastix* Guegen was isolated on potato-dextrose agar at room temperature (30±2ºC). On detailed investigation and comparison with known species of the genus, it was found to be very distinct from all of them in conidial characters and hence is described here as new species under the name *Gliomastix indicus* S.Nagalaxmi & A. Subrahm (Figs. 1-2).

On 2% malt extract agar at room temperature, culture is fast growing covering 80 mm plate in 72 h. It is hyaline in the beginning but soon become ropy with strands of mycelium radiating from center, turn to sooty black with abundant sporulation. Its margin is off white; reverse side of colony is black. Colony is dark in the middle and fades gradually towards periphery with no diffusible pigment production.



Gliomastix indicus S. Nagalakshmi & A. Subrahm sp.nov.

Fig.1: Showing comidiophores with catenulate conidia 100x Mature conida 1000x

Mycelium is pale brown, branched, septate occasionally partly joined to form mycelial strands radiating from the centre; conidiophores undifferentiated, pale brown, septate and smooth: conidia brown in reflected light, one celled, oval to spherical or oblong occasionally reniform, catenulate, generally vacuolated in fresh cultures, one or two vacuoles per conidium which may not be seen in old cultures. Spherical conidia are 11.0 µm in diam., oval conidia measure 13.8-18.5 x 7.0-9.0 µm, oblong conidia 14.0-17.5 x 6.5 -11.0 µm in size.

Habitat: Waste land soil, Tiruchengod, Tamilnadu
MTCC Chandigarh No. 3869

Identity: Due to the presence of amerospores on an undifferentiated nearly hyaline sporogenous cell, it is assigned to *Gliomastix*. But it stands distinct from all the known species in bigger size of conidia (with vacuoles) and its rapid growth rate. Its unique ability to reduce paper mill effluent and color is an additional feature of distinction.

Games (1) in his treatment of *Acremonium* included monophialidic species of *Paecilomyces* and all species of *Gliomastix* under *Acremonium.* Most of the workers accepted the transfer of monophialdic *Paecilomyces* under *Acremonium* and continued to recognize *Gliomastix* as distinct genus. Barrown (2) also maintained *Gliomastix* as a separate and distinct genus and according to him "Chains or balls" or dark amerospores arising from almost hyaline sporogenous cells give the common *Gliomastix* species a distinct appearance.

Therefore it is preferred to maintain *Gliomastix* as a distinct genus from *Acremonium* at least until such time a comprehensive investigation dealing with several isolates become available indicating a clear distinction between *Gliomastix* and related genera on sound taxonomic characters.

Latin diagnosis

Gliomastic indicus S. Nagalaxmi & A. Subrahm. sp. nov. colonies agaro malt extract 80 mm diametro biebus: reverse melano pigmento : sinopigmente diffusible: conidia glabra, sphericae 11.0 µm vel vate 13.8-18.4 x 7.0-9.0 µm. vel oblongatus 14.0 - 17.5 x 6.5-11.0 µm contines mono vel bigillatus.

This is probably the first report of an imperfect fungus far exceeding the classical *Phanerochaete* in its degradative activity of paper mill effluent and color. By virtue of its rapid growth rate, ability to grow on simple media, it stands as choice organism for bioremediation process. These aspects will be published in due course.

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