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Editors

Prof.K.R.S. Sambasiva Rao, India
krssrao@abap.co.in

Prof. Karnam S. Murthy, USA
skarnam@vcu.edu

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Research Papers

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Performance assessment of biodiesel transesterified from *Labeo rohita* visceral oil using IC engine

N.S. Sampath Kumar[1*], R. Sruthi Geetha[1], A. Thirumala Vasu[2], D. Vijaya Ramu[1]
[1]Department of Biotechnology, Vignan's Foundation for Science Technology and Research Vadlamudi, Guntur, Andhra Pradesh, India- 522213, India.
[2]Department of Mechanical Engineering, National Chung Cheng University, Min-Hsiung Township, Chiayi County 62102 Taiwan
*Corresponding Author : nssk84@gmail.com

Abstract:

During recent years there is a growing interest in producing biodiesel from fish wastes as alternative renewable source for declining fossil fuel resources. Present study aims at producing and transesterification of biodiesel from *Labeo rohita* visceral mass. Resulted fatty acids methyl esters was mixed with pure diesel in different ratios to investigate its efficiency as fuel in IC engine and the results were compared with pure diesel. Ratios of fish-oil to diesel used in experiments were 0:100, 10:90 and 20:80 respectively. Results indicated that the performance parameters of ignition power, IMEP and airflow ratios were higher with 10:90% and IP, BP, ITH, BTH, IMEP, BMEP and air flow ratios were higher with 20:80 respectively.

Keywords: *Fish oil, diesel, IC engine, Combustion, transesterification*, *biodiesel, Labeo rohita*

Introduction

India being one of largest freshwater fish producer in the world, with an annual contribution of 1.05 million tonnes of fish annually (Laxmappa, 2015), Rohu being most preferred species in country comprises of 11,33,233 tonnes (FAO, 2013). The large production of fish has contributed to the generation of underutilised body parts viz., visceral mass, hard skin, trimmings, bones and roe, which is considered as waste by fish traders. Chalamaiah et al., (2012) reported that, these body parts would account for more than 60% of biomass and were disposed without making any recovery attempts. On the other hand, researchers have made attempts to extract and explore applications of fish waste into commercially important products such as therapeutic proteins and their enzymatic hydrolysates, peptides with various bioactivities, oils, etc (Sampathkumar et al., 2013; Ghaly et al., 2013; Nasri et al., 2013).

Despite the fact that presence of omega-3 and omega-6 fatty acids in fish oil has the ability to treat many diseases viz., heart diseases, type-2 diabetes, cancer, attention deficit hyperactivity disorder (ADHD), anxiety and depression, Alzheimer's/ Parkinson's and age related macular degeneration; transesterified fish oil can also be utilized as an alternative fuel in CI/diesel engines which serves as an renewable energy source. Because petroleum distillates and fish oil has the same calorific value (Blythe, 1996) and previous studies have strengthened this view with slight differences (Ramesha et al., 2012; Savariraj et al., 2013). Biodiesel is identified as a renewable, biologically degradable with no toxicity issues and maintains carbon neutrality for CI engines has increased substantial attention all over the world because the existing fuel reserves are depleting at a fast pace and a number of vehicles are on the exponential rise Gupta et al., 2013; Hari Prasad et al., 2015).

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In consideration of above mentioned facts and to reduce environmental pollution caused by disposing of waste from processing units, transesterifying fish oil would be a better move toward converting waste into wealth. This paper highlights the usage of fish viscera as a feedstock for production of biodiesel via two step process, transesterification which includes pretreatment of samples with acid catalyst to reduce the value from 12 to 3 mg [KOH]g[oil]$^{-1}$ and followed by KOH-catalyzed transesterification. During this process, esters are removed due to the formation of the emulsion and leads to the separation of glycerol as shown in the figure (1).

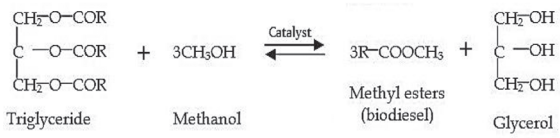

Fig. 1: Transesterification of oils

Mofijur et al., (2013) reported that biodiesel from fish source will be a substantial prospect as a long-term replacement for diesel, hence considered as one of the alternative fuel options for petroleum distillates. Moreover, fish oil has low carbon content and slightly higher hydrogen content which provides characteristics viz., flash point with a much lower kinematic viscosity (Yahyaee et al., 2013). Lower viscosity could decrease the necessity for preheating the fuel and progress the atomization of burner which directly reduces emissions of CO, SO_2 (Preto et al., 2007; 2008). Even though it is eco-friendly fuel, if directly used for engines either 100% or by diluting it with solvents, it has certain disadvantages viz., carbon deposits in the engine, reduce engine durability and contamination of the lubricating oil. But considering the benefits caused to the environment in long run, fish oil is considered as a potential renewable biological source of liquid fuels. In this paper, we present the usage of biodiesel extracted from *Labeo rohita* viscera in IC engines and various studies were carried out on a single cylinder diesel engine to assess the performance and combustion levels.

Material and methods

Sample collection

Rohu (*Labeo rohita*) was collected from Guntur fish market (16^0 30'67" N 80^0 43' 65" E), Andhra Pradesh, India. Fish viscera was separated, blotted, weighed and kept at – 20 °C until further usage.

Biochemical analysis : Moisture content, ash content, lipid and protein composition was analyzed on wet weight basis. Sample (2 g) was taken in a pre-weighted aluminium dish and moisture content was determined using the standard protocol of AOAC (1991). Pre-dried sample was placed in a crucible at 600 °C until a white ash was formed to estimate ash content (AOAC 1991). Kjeldahl method (AOAC 1991) was used to determine the total crude protein (N x 6.25) in raw material. Bligh and Dyer (1959) method was used to determine gravimetrically the lipid content.

Extraction of oil from fish viscera : The crude fish oil was extracted from the visceral mass collected from *L. rohita*. Visceral mass was cooked for 30 min at 105 °C, followed by squeezing and centrifuging at 3000 rpm. Collected fish oil was brown in colour and the same was used to produce biodiesel (Cherng and Rong, 2009).

Transesterification of fish oil : Fish oil was transesterified with methanol and NaOH (1% wt) at 1:6 ratio to produce biodiesel. This solution was transferred into a reaction tank along with refined fish oil supported by a homogenizer at 60 °C for 50 min. The mixture was then centrifuged to separate crude biodiesel from glycerol based on the difference in their density. The supernatant containing biodiesel was distillated at 70 °C to separate the un-reacted methanol followed by washing with petroleum ether (50% wt) to remove other contaminants. The same process was repeated at 105 °C to remove water and unwanted molecules to collect biodiesel (Zhang et al., 2003).

Preliminary analysis of biodiesel blends : Before running the engine, a preliminary analysis was done for oil blends (80% & 90% of diesel with fish oil) to obtain the important fuel parameters

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(viscosity, flash and fire point) to assess its appropriateness as fuel for IC engine.

Experimental system and process : To analyze the performance and combustion features of unified oil IC engine experiments was performed on a Kirloskar TV-1 made a single cylinder, 4-stroke engine, aspirated direct injection, 7HP capacity water cooled diesel engine works at 1500 rpm directly coupled to an eddy current dynamometer (Table 1). As shown in the schematic diagram (Figure 2) an orifice meter coupled with a big tank was fixed to the engine which permits the calculation process of air flow. The glass burette and stopwatch helps in measuring the rate of fuel consumption. Digitalized tachometer was coupled for analyzing the speed of engine. Encoder such as AVL shaft position helps to provide signals at TDC and AVL GM12D miniature pressure transducer was used to enhance the pressure of cylinder.

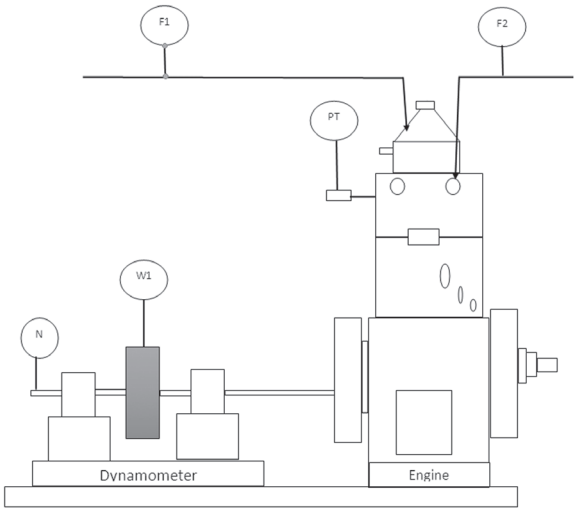

Fig. 2 Graphical representation diagram of IC engine

Results and discussion

Proximate analysis

Freshwater fish rohu was selected for the present work due to its abundance in the state of Andhra Pradesh and the biochemical composition of the crude visceral mass was performed as shown in table (2). Current results have proven that rohu viscera have shown good protein (11.4%) and lipid (6.3%) contents. Suriah et al., (1995) has classified fishes into three categories as lean (<5%), medium (5-10%) and fatty fishes (>5%) based on their lipid content. So, based on this classification rohu can be considered as medium fish.

Table1: Test engine specifications

Parameter	Specifications
Engine model	Kirloskar TV-1
Type of engine	IC natural aspirated water cooled
No. of cylinders	One
Cylinder diameter	87.5 mm
Number of Stokes	4
Stoke length (mm)	110
Dynamometer length (mm)	185
Connecting rod length(mm)	234
Maximum power (kw)	3.5 kw @ 1500 rpm
Orifice diameter (mm)	20

Table 2 Biochemical analysis of rohu visceral mass

Biochemical Composition	Percentage (%)
Moisture content	80.1±1.7
Ash content	2.1±0.3
Protein content	11.4±1.6
Lipid content	6.1±0.19
Mean ± SD where n=3	

Biodiesel

Bio-fuels has been extensively under consideration in recent times as an alternative fuel because of their nature in terms of diversifying energy sources, declining Green-house gases (GHG) and particulate matter emission (PME); above all transesterified fish oils have similar physical and chemical properties to the refined petroleum distillates. The emission characteristics tested on IC engine at constant speed with different loads for the different fuel combinations is B0 (100% neat diesel), B10 (10% biodiesel +

90% diesel) and B20 (20% biodiesel + 80% diesel) tested has shown impressive results.

Kinematic viscosity for pure diesel (B0), 10% (B10) and 20% (B20) fish oil blends with diesel were 1.4, 1.6 and 1.67 centistoke respectively. Another important feature is a flash point which is associated to the vapour pressure of a flammable fuel at the lowest possible temperature at which it can mix with air (Crowl and Louvar, 2002). Flash point for B0, B10 and B20 were 60, 62 & 65°C respectively as shown in the table (3); which confirms that below 80 °C is sufficient to ignite and combust the mixture. Increasing temperature increases the pressure of vapour and the amount of evaporated flammable fuel in equilibrium with the air also increases (Natália et al., 2012) and usually this value changes for blends because of various mixtures based on the combination than compared with pure fuels (Guo et al., 2009; Boog et al., 2011). Similarly, fire point for aforementioned sample showed 80°C, 82 °C and 86°C respectively.

Performance studies

Blending of biodiesel along with diesel as an oxygenated fuel was efficient in ignition at high engine loads than low engine loads. Therefore, biodiesel blends resulted in different effects on the performance and the emissions at different engine loads (*ie.* 0 and 4). The engine performance characteristics viz., ignition power, brake power, mechanical competence, indicated thermal efficiency, break thermal efficiency (BTE), indicated mean efficiency pressure, break mean efficiency pressure (BMEP), consumption of specific fuel, air flow and air fuel ratio were analyzed on an I.C engine equipped with combustion analyses software for B0, B10 & B20 unified fuels. Sufficient time was given for engine to consume the remaining fuel from the previous experiments before running the engine with new fuel for the current experiment. As tabulated, IC engine performance parameters for B0, B10 and B20 at different loads (0 and 4 loads) had shown certain interesting patterns like the value increased with the increase in the concentration of blending with regards to the IP, IMEP and air flow. On the contrary, values decreased for BP, Mechanical efficiency and BMEP; despite these variations, brake thermal efficiency is directly proportional to applied load *ie*., it increases with increasing applied load.

Because of the reduction in heat loss and increase in power developed with an increase in load, the maximum BTE obtained is about 21.6% for B20, which is actually more than the regular diesel (20.24%). Blending of biodiesel in diesel has yielded good thermal efficiency curves because of the additional lubricity (Ramadhas et al., 2005). Brake specific fuel consumption (BSFC) is another important parameter to compare the performance of fuels. As seen in the table (4), BSFC values of biodiesel blends (B10 & B20) were lower than those of diesel fuel (B0). As seen in the table (3) biodiesel blends have higher flash and fire point than that of diesel fuel, this might have caused less fuel consumption. A similar correlation between results was reported by Lei et al., (2016) and Ertan et al., (2017).

Table 3 - Properties of unified oils

Samples	Kinematic viscosity (Centistokes)	Flashpoint (°C)	Fire point (°C)
B0	1.4	60	80
B10	1.6	62	82
B20	1.67	65	86

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Table 4: Performance studies of various samples at different loads

Sample	B0		B10		B20	
Load	**0.00**	**4.00**	**0.00**	**4.00**	**0.00**	**4.00**
Indicated Power (KW)	2.03	2.48	3.30	3.49	5.70	4.50
Brake power (KW)	1.10	1.20	0.10	1.25	0.00	1.30
Mechanical efficiency (%)	2.50	47.3	1.60	37.65	1.20	28.00
Indicated thermal efficiency (%)	0.00	42.76	0.00	60.11	0.00	77.47
Brake thermal efficiency (%)	0.00	20.24	0.00	20.95	0.00	21.69
Indicated mean effective pressure (Bar)	2.50	3.10	3.80	4.20	5.20	5.30
Brake mean effective pressure (Bar)	1.40	1.50	1.00	1.50	0.70	1.50
Consumption of specific fuel (kg/kWh)	0.04	0.64	0.00	0.34	0.00	0.04
Air flow (kg-hr)	27.9	27.8	31.0	30.65	35.5	30.30
Air fuel Ratio	0.00	558.1	0.00	583.45	0.00	608.8

Cylinder gas pressure

The result of the pressure curve shown in figure (2& 3) reveals that the peak pressure in the cylinder increases and the delay is reduced proportionally with the amount of fuel injected. This peak rises from the 38 bar for B10 to 46 bar at the crank angle of 10 deg with SOC at 23 bars cylindrical pressure and 15 deg crank angle, EOC at 16 bar cylindrical pressure and 40 deg crank angle. Further increase of biodiesel concentration in diseal ie for B20 the maximum cylindrical pressureraised till 54 bar at the same crank angle, SOC and EOC parameters.This increase is a result of flaming properties of fuels, which provides a rapid combustion of the blended fuel which in turn improves the combustion process (Deb et al., 2015; Hendrick et al., 2017).

Net and cumulative heat release Figure (4) and (5) represents the crank angle vs. net heat release for the experimental blends B10 & B20. The maximum net heat release was found at *30 J/deg at 5 deg* of *crank angle* for B10, and *40 J/ deg at 3 deg* of *crank angle* for B20. Similarly, figures (6) and (7) show the cumulative heat release peek at 0.68 KJ at 40 deg crank angle for B10 and 0.88 KJ at 40 deg crank angle for B20. Fuel blends B20 has shown higher heat release per crank angle than B10. Upon comparing two blends the shift in the angle of crank for heat release was found to be moving rightward in direction, which may be found common in oxygenated fractions. Because the blend enhances the release of heat during combustion and it inn turn may be the reason for changes in net in cumulative heat release (Eliezer et al., 2017).

Mass fraction burned : Conversion of energy during combustion cycle can be expressed by the MFB (mass fraction burned) at a specific degree of crank angle (CAD) which is used as a feedback control systems (Krzysztof et al., 2002). Figure (8) and (9) shows 100% mass fraction burned at 36 deg and 40 deg crank angle for B10 and B20 respectively. Reaching 100% of combustion in a cylinder connected to IC engine is very essential because it affects the efficiency related to thermal properties of engine, pressure and temperature of peak cycles & exhaust emissions (Mittal et al., 2009).

Mean gas temperature : From the above figures (10) and (11) Crank Angle Vs Mean Gas Temperature, B10 showed 1020 ^{0}c temperature at 23 deg crank angle and B20 showed 1040 ^{0}c temperature at 25 deg crank angle respectively. Saseet al., (2016) reported blends of thumb biodiesel had 180 & 540 ^{0}c, which was

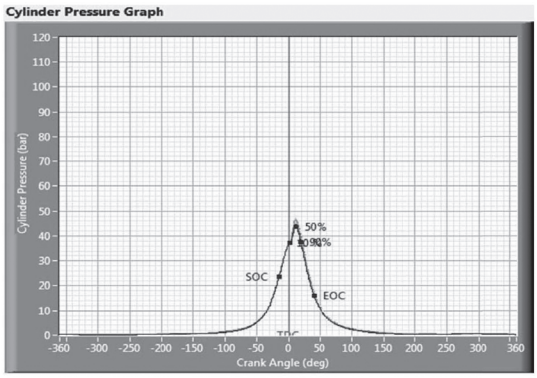

Fig. 2: Cylinder pressure graph for B10

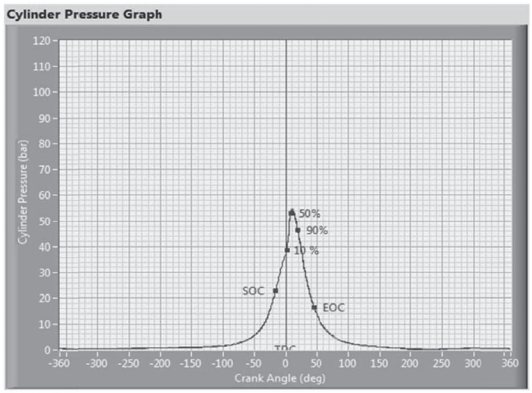

Fig. 3: cylinder pressure graph for B20

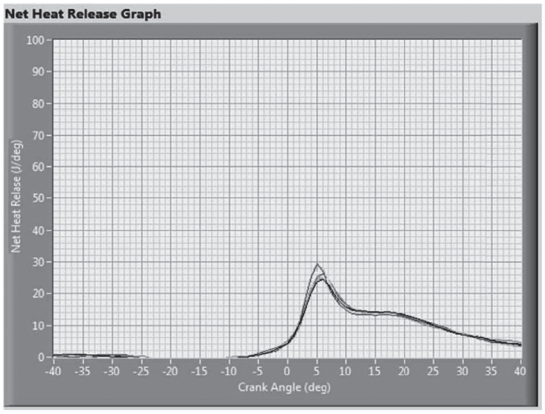

Fig. 4: Net heat released for B10

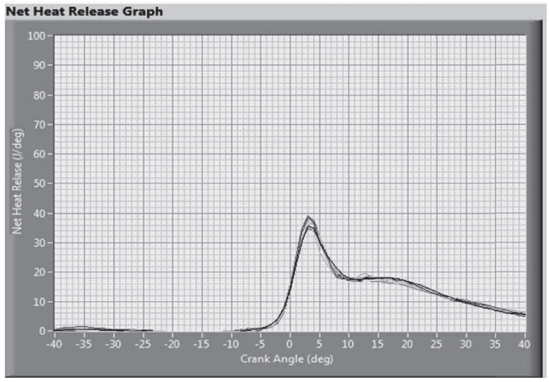

Fig. 5: Net heat released for B20

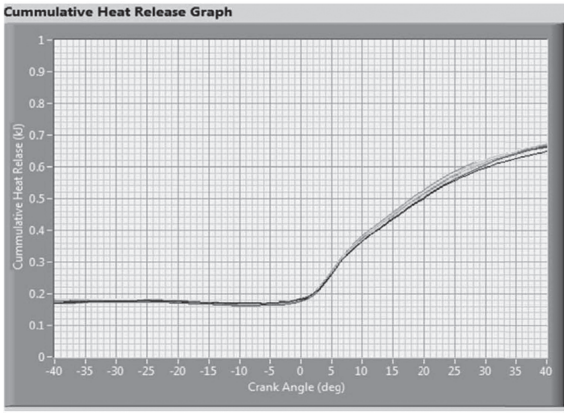

Fig. 6: Cumulative heat release for B10

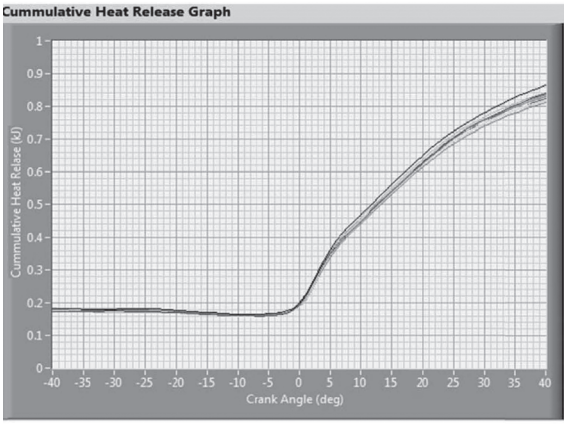

Fig. 7: Cumulative heat release for B20

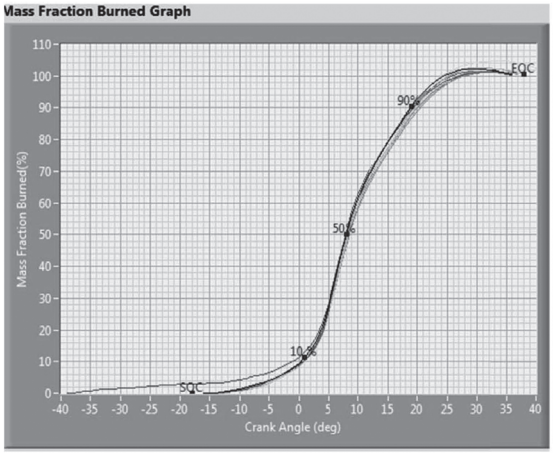

Fig. 8: Mass fraction burned graph for B10

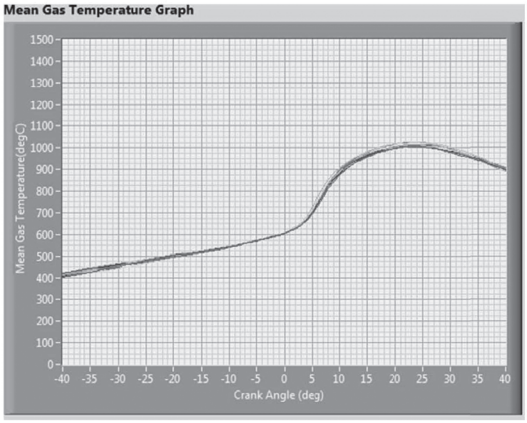

Fig. 10: Mean gas temperature graph for B10

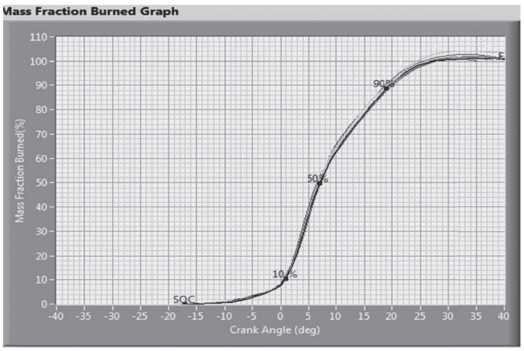

Fig. 9: Mass fraction burned graph for B20

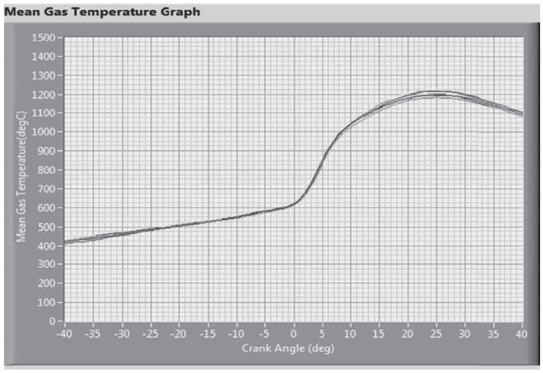

Fig. 11: Mean gas temperature graph for B20

comparatively very less than the fish oil blends.

Conclusion:

This project was carried out to characterise the biodiesel transesterified from the visceral oil of *Labeo rohita* and test its performance on a IC engine. Analyzing the B10 & B20 blends has proven to possess good fire and flash points with moderate viscosity. Both the blends have considerably good values of BP, Mechanical efficiencey, ITH efficiency, BTH efficiency, IMEP, BMEP and SFC at 0 and 4 load conditions. Results obtained from this work are confined only for the engine and engine parameters applied in this work. Hence, other percentage of biodiesel blends and their parameters should be assessed in other types of diesel engines at different loads before the fuel is commercialized.

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Biochemical characterization and dye decolorization of selective microbial isolates from natural sources

Saloni Soni, Shellina Khan and Navneet Joshi*
Department of Biosciences, Mody University of Science and Technology,
Lakshmangarh, 332311 Sikar, Rajasthan (INDIA)
*corresponding author Email: navybiotech@gmail.com

Abstract

The estimation of dye decolorizing efficiency of microorganisms was initially carried out by the primary screening of isolates from the soil samples of Ambabari region Jaipur. Two microbial Strains *Bacillus subtilis* and *Psedomonas stutzeri* were isolated from the soil samples. Dye decolorizing efficiency was checked by plating and shake culture flask experiments, provided with the optimized conditions of temperature / time period. Selective isolated microbial strains were further analyzed spectrophotometrically for decolorization percentage, morphological analysis carried out by visualizing the colony parameters with respect to their size/ shape or color. Selective microbial isolate *Bacillus subtilis* was found with the capability of 73.52% to decolorizie1 ppm methyl red in 96 h and 79.71% capability to decolorize 100 ppm methyl red in 96 h. The same isolate *Bacillus subtilis* was found with the capability of 74.64% to decolorize the 1 ppm orange G in 96 h and 80.80% capability to decolorize the 100 ppm orange G in 96 hrs. *Pseudomonas stutzeri* was found with the capability of 70.16% to decolorize 1 ppm methyl red in 96 h and 77.17% capability to decolorize 100 ppm methyl red in 96 h. Microbial isolate pseudomonas stutzeri was having capability of 72.80% to decolorize the 1 ppm orange G in 96 h and 75.81% capability to decolorize 100 ppm orange G.

Keywords: dye, microorganisms, soil, decolorization

Introduction

Dye is synthetic organic compound that is used for the coloration of textiles. These organic chemicals are usually classified as azo, anthraquinone, vat, phtalocyanine, indigo, polyethylene, carbonium and nitro dyes [Abrahart 1977, Sponza 2006]. There are two components in the dyes by which they attach to the fibers and make resistance towards the other chemicals like soap and detergents. The first componant is chromophore which is capable to absorb the light in UV region, like;-N=N-, -C=N-,-N=O-,CH=CH and the components with the properties of chromophore are known as chromogen. The second componant is auxochrome, these are the stable chemical bonds by which the fibes make bonding with dyes, like; NH, NHR, NR these are basic in nature and OH, -SOH, -COOH these are acidic in nature[Wang *et al.,* 2009]. If the compound do not have the properties of auxochrome than it is impossible to colorize the textiles because of the lack of stable chemical bonds between the fibres and dyes.Azo dye contains azo bonds (-N=N-). It is highly persistent in aquatic environment and highly toxic and carcinogenic and explosive in nature because of the presence of aromatic rings, azoic linkages,and amino groups. There are approximately 10,000 different synthetic dyes available in the market with a global annual production of almost 800,000 tons [Bazin *et al.,* 2012]. These synthetic dyes are widely used in the textile, photograpgy, paper, cosmetic and food industries. It is estimated that 10-15% of the total production of colorants in textile industriesis lost during the dyeing process

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[Maguire,1992] and that lost dye effluent is dischared openenly in near by area including water ponds and rivers, that causes the severe environmental problems. Those effluents are characterized by strong colors, high pH variations, high chemical oxygen demand (COD) and increased bio toxicity against bacteria [Walker *et al.*,2000].Microorganism plays a very important role in the biodegradation and mineralization of these dyes which is of great significance [Lie *et al.*, 1996 and Khalid *et al*, 2008].Soil can become a effective source for the purpose of decolorization of industrial dyes, because soil contains numerous microbial species that have their unique role in the environment [Gahlout *et al.,* 2013] Biological process including different taxonomic group of microorganisms such as bacteria, fungi, yeast, and algae received increasing interest due to their cost effectiveness, ability to produce less sludge, and eco-friendly nature. [Kalyani *et al.,* 2008] Different trophic groups of bacteria can achieve a higher degree of degradation and even complete mineralization of dyes under optimum growth conditions [Asad *et al.*, 2007].

The contaminated soil sample was collected from the Ambabari region Jaipur, which is connected to the Amanishah nala that included with the textile industry effluents discharge. By applying the technique of microbial isolation, biochemical characterization and identification of the microorganisms, can know that which microorganisms are very effective for biologically decolorization of these toxic dyes (Methyl red and Orange G), used in the textile print industries. These microorganisms can be made effective solution of these dye toxicity problems.

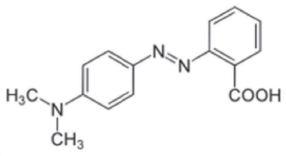
Materials and Methods

Sample collection

The contaminated soil (that consist of textile industry's effluent) was collected from the Ambabari region,jaipur. This contaminated soil was the source of isolation of different microorganisms that have ability to decolorize the toxic dyes.

Dyes and chemicals : The azo dyes Methyl red, Orange G used in the experiment. 1ppm and 100ppm of dye solutions were used.The chemical structure of both the dyes :

Methyl red:

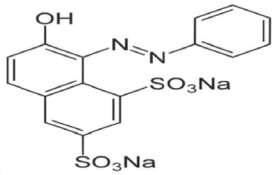


$C_{15}H_{15}N_3O_2$

Molar mass -269.30g/mol

λ_{max} – 410nm

Orange G :-



$C_{16}H_{10}N_2Na_2O_7S$

Molar mass- 452.38g/mol

λ_{max} – 480nm

Growth medium

(1) Nutrient agar: it is a general growth medium used for primary screening of microor ganisms,which consist of following composition (g/L^{-1}); peptone (5), sodium chloride (5), yeast extract (1.5), agar (15). The final pH adjusted to neutral or 6.8 at 25^0c.

(2) Nutrient broth: A liquied growth medium,used for primary screening and for spectrophotometry analysis of microorganisms.

(3) Mineral salt medium:It is an enrichment culture medium used for the isolation and characterization of microorganisms, which consist of composition (g/L^{-1}) ; $(NH_4)_2HPO_4.4H_2O$ (3.5), $K_2HPO_4.3H_2O$ (7.5), KH_2PO_4(3.7), $MgSO_4$(0.17), Yeast extract(0.04), glucose(2), microelement solution(10 ml) which contains; $FeSO_4.7H_2O$(2.7mg), $MnCl_4.4H_20$ (1.98mg), $CuSO_4.7H_2O$(2.8mg), $CoCl_2.2H_2O$ (0.17mg), $ZnSO_4.7H_2O$(0.29mg).

Preparation of dye solutions : 0.001g of solid dye (methyl red and orange G) was dissolved in 1000ml of distilled water. The prepared dye stock solutions were referred as 1ppm solution.

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0.1 g of solid (methyl red and orange G) was dissolved in 1000ml of distilled water. The prepared dye stock solution was referred as 100ppm.

Isolation and screening of dye decolorizing microorganisms

For the primary screening, serial dilution was performed with contaminated soil sample and the dilutions were streaked over the nutrient agar medium.11 morphologically distinct colonies were obtained that were subcultured into MSM media and flasks were incubated at 35°C under shaking conditions for 24-48 h.

For the secondary screening, 1ml from the grown culture was inoculated into the prepared nutrient broth media including 1ppm and 100ppm of dye solutions (methyl red and orange G) and incubated for 48-96 h under shaking condition at 120rpm and at 35°C temperature. Out of 11microbial isolates, 2 isolates (A & F1) degraded the azo dyes. Due to the decolorization of dyes, these two microbial isolates were selected for the further characterization and identification studies.

Slants were prepared of those microbial isolates that decolorized the dyes, for the preparation of stock samples.

Decolorization assay

The decolorized samples were spectro photometrically analyzed, by measuring the absorbance maximum ($ë_{max}$) of the dyes i.e. 410nm for methyl red and 480nm for orange G. The decolorization % of microbial isolates was determined by the equation:

Decolorization %= [(Ai-At)/Ai] × 100

Where, Ai is initial absorbance and F is final absorbance of decolorized medium.

Gram character analysis : The Gram characters of selected microbial isolates were identified by Gram staining.

Biochemical characterization of microbial isolates : Isolated microorganisms were further analysed by the biochemical methods.

Molecular characterization of microbial isolates : Selected microbial isolates A and F1 were cross verifiedby the Yaazh xenomics, Chennai.

Results and Discussion

In this study soil sample were collected from the ambabari region Jaipur. Contaminated soil sample were subjected to the serial dilution and subsequently plated on a solid media. 11 distinct colonies were observed. Each colony was named as A,B,C1,C2,C3,D1,D2,E,F1,F2,F3.

All the strains were cultivated firstly in MSM media and further the grown culture in MSM was again cultivated in nutrient broth media with dyes orange G and methyl red [Graph: 1-4, Table: 1-10].

Among the isolated colonies, colony A and colony F1 showed the decolorization and these two strains were selected for identification [Fig1-18].

Molecular identification results

Microbial isolates A and F1 were further confirmed as *Bacilluc subtilis* and *Pseudomonas stutzeri.*

Microbial Sample A Sequences identified

>contig sample A

CCCCCGGCTC AACCGGGGAGG GTCAT
TGGAAACTGG GGAACTTGAGTGCAGAAGAG
GAGAGTGGAATTCCACGTGTAGCG
GTGAAATGCGTAGAGATGTG
GAGGAACACCAGT GGCGAAGGCGACTCTC
TGGTCTGTAACTGACGCT GAGG AGCGA
AAGCGTGGGGAGCGAACAG
GATTAGATACCCTGGTAGTCCACG
CCGTAAACGATGAGTGCTA
AGTGTTAGGGGGTTTCCGCCC CTTAGT
GCTGCAGCTAACGCATTAAGCACTCCGCC
TGGGGAGTACGGTCGCAA GACTG AAACTC
AAAGG AATTGA CGGGGGCCCGC ACAAGC
GGTGGAG CATGTG GTTTAAT TCGAAGC
AACGCG AAGAACCTTA CCAGGT CTTG
ACATCCT CTGACA ATCCTAGA GATA GGAC
GTCCCCTTCGGGG GCAGAGT GACAGG
TGGTGCATGGTTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGC
AACGAGCGCAACCCTTGATCTTAGTTGCC

A G C A T T C A G T T G G G C A C T C T A A G G
TGACTGCCGGTGACAAACCG GAGGAAGGTG
GGGATGACGTCAAATCATCATGCCCCTTATGA
Bacillus subtilis

Microbial Sample F1 Sequences identified
>ContigSample F1
C T T C G T G C C A G C A G C C G C G G
TAATACGAAGGGTGCAAGCGTTAATCGGAAT
TACTGGGCGTAAAGCGCG CGTAG GTGGTTCG
TTAA GTTGGAT GTGAAAGCCCCG GGCTCAA
CCTGG GAACTGCATCCAAAACT GGCGA
GCTAGAGTATGGCAGA GGGTGGTGGAATTTC
CTGTGTAG CGGTGAAATGCGTAGATATAGGAA
GGGAACACCAG TGGCGAAG GCGAC
CACCTGGGGCT AATACTGACA CTGAGGTGC
GAAAGCGTGGG GAGCAAA CAGGATTAGA
TACCC TGGGTAG TCCACGCCCGTAA
ACGATGTTC GACTATGCCGTTGG GATCCTTG
AGATCTTAGTG GCGCAGCTAACGCATTAAGTC
G A C C G C C T G G G G A G T A C G G
C C G C A A G G T T A A A A C T C A A A T G A A T T
G A C G G G G G C C C G C A C A A G C G G T G G
AGCATGTGGTTTAATTCGAAG CAACGCG
AAGAACCTT ACCAGGC CTTGACATGCA
GAGAACTT TCCAGAGAT GGATTGGTGCCTT
CGGGAACTCTGACACA GGTGCTGCATGGCT
G T C G T C A G C T C G T G T C G T G A G A T G T
T G G G T T A A G T C C C G T A A C G A G C
GCAACCCTTGTC CTTAGTTACCA GCATGTTA
AGGTGGGC ACTCTAAGGA GACTGCCG
GTGACAAACCGGA GGAAGGTGGGGAT
G A C G T C A A G T C A T C A T G G C C C T T A
C G G C C T G G G G C T A C A C A C G T G
CTACAATGGTCGGT ACAAA GGGTT
GCCAAGCCGCG AGGTGGAGCTAATCCCA
TAAAACC GATCGTAGTCCGGATCGCAG
T C T G C A A C T C G A C T G C G T G A A G
T C G G A A T C G C T A G T A A T C G T G A A
TCAGAATGTCACGG TGAATACG TTCCCGGG
CCTTGTACACACCGC CCGTCACACC
ATGGGAGTGGGTTGCTCC

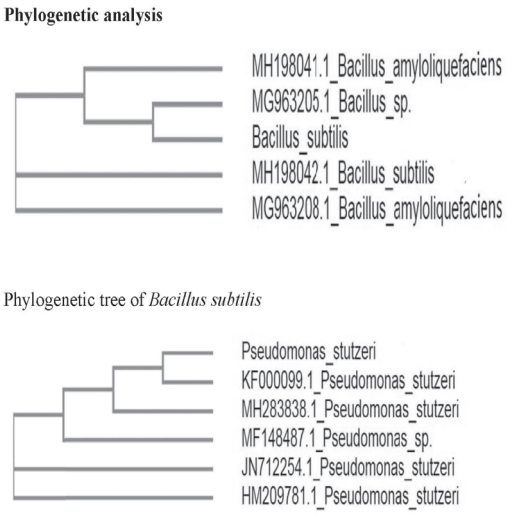
*Pseudomonas stutzer*i

Phylogenetic analysis

Phylogenetic tree of *Bacillus subtilis*

Phylogenetic tree of *pseudomonas stutzeri*

Dye decolorization



Phylogenetic tree of pseudomonas stutzeri

Discussion

From the present study, it becomes clear that the selected microbial isolates are able to decolorize the dyes. The 16S-rRNA sequencing reveals the micro-organisms of bacterial class. The soil samples are highly rich in microbial loads and thus soils from natural sources are being selected**(Ponraj*et al.,* 2011).**Selective microbial isolate *Bacillus subtilis* was found with the capability of 73.52% to decolorize1 ppm methyl red in 96 h and 79.71% capability to decolorize 100 ppm methyl red in 96 h. The same isolate *Bacillus subtilis* was found with the capability of 74.64% to decolorize the 1 ppm orange G in 96 h and 80.80% capability to decolorize the 100 ppm orange G in 96 h. *Pseudomonas stutzeri* was found with the capability of 70.16% to decolorize 1 ppm methyl red in 96 h and 77.17% capability to decolorize 100 ppm methyl red in 96 h. Microbial isolate *Pseudomonas stutzeri*was having capability of 72.80% to decolorize the 1 ppm orange G in 96 h and 75.81% capability to decolorize 100 ppm orange G [Graph:1-4,Table:1-10].

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Fig 14: Methyl red Control decolorized by Isolate A

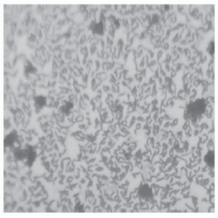
Fig 15: Test flask methyl red decolorized by Isolate F1

Fig16: Test flask methyl red

50ml of 100 ppm solution of Orange G was suspended with 50ml of nutrient broth and added 1 ml of inoculum A and F1, separately.

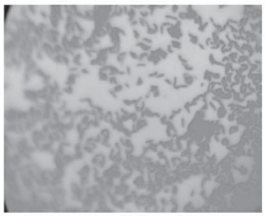
Fig 17: Left-CONTROL Orange G
Right-TEST flask Orange G decolorized by isolate A

Fig 18: Left-Control Orange G
Right-TEST flask Orange G decolorized by isolate F1



Isolate F1

Fig 6: Gram –ve rods



Isolate F2

Fig7: Gram +ve rods

Sample A Sample F1

Isolate A: Starch hydrolysis test

Indication of Test: Positive

Fig 8: Control

Fig 9: Test

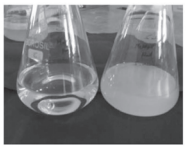


Fig 10:Left- control methyl red
Right-test flask 1ppm methyl red red decolorized by isolate F1

Fig11: Left-control methyl red
Right- test flask of 1ppm methyl decolorized by isolate A.

50ml of 1 ppm solution of Orange G was suspended with 50ml of nutrient broth and added 1 ml of inoculum A and F1, separately.

Fig 12: Left- control orange G
Right-test flask orange G decolorized by the isolate A.

Fig13: Left-control orange G
Right- test flask orange G decolorized by the isolate F1.

Fig 14: Methyl Red Control decolorized by Isolate A

Fig 15: Test flak: methyl red decolorized by Isolate F1

Fig16: Test flask methyl red

50ml of 100 ppm solution of Orange G was suspended with 50ml of nutrient broth and added 1 ml of inoculum A and F1, separately.

Fig 17: Left-CONTROL Orange G

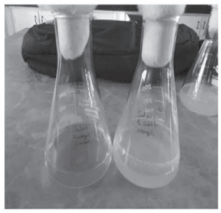


Fig 18: Left-Control Orange G

Right-TEST flask Orange G decolorized by isolate A
Right-TEST flask Orange G decolorized by isolate F1

Biochemical characterization and dye decolorization

Table 1: Test characteristics

Sample	Colony morphology	Cell morphology	Gram character
A	Gummy, yellowish	Cocci+rod shaped	positive
F1	Circular, pinhead	Rod shaped	positive

Table: 2 Biochemical characteristics

Test	**Sample A**	**Sample F1**
Starch hydrolysis	Negative	Positive
Catalase	Negative	Negative
Mannitol salt agar test	Positive	Negative
MRVP test	Negative	Negative
Carbohydrate fermentation test	Negative	Negative
SIM agar test	Negative	Negative

Table 3: Spectro-analysis of 1ppm methyl red at 410nm

Sample	**24 hrs**	**48hrs**	**72hrs**	**96hrs**
Blank	0.000	0.000	0.000	0.000
Uninoculated methyl red	0.476	0.476	0.476	0.476
Sample A	0.302	0.283	0.138	0.126
Sample F1	0.390	0.289	0.150	0.142

Table 4: Spectro-analysis of 100ppm methyl red at 410nm

Sample	**24hrs**	**48hrs**	**72hrs**	**96hrs**
Blank	0.000	0.000	0.000	0.000
Uninoculated methyl red	0.552	0.552	0.552	0.552
Sample A	0.319	0.251	0.159	0.112
Sample F1	0.329	0.292	0.168	0.126

Table 5: Spectro-analysis of 1ppm orange G at 480nm

Sample	**24hrs**	**48hrs**	**72hrs**	**96hrs**
Blank	0.000	0.000	0.000	0.000
Uninoculated Orange G	0.489	0.489	0.489	0.489
Sample A	0.326	0.218	0.139	0.124
Sample F1	0.381	0.252	0.171	0.133

Table 6: Spectro-analysis of 100ppm orange G at 480nm

Sample	24hrs	48hrs	72hrs	96hrs
Blank	0.000	0.000	0.000	0.000
UninoculatedOrange G	0.521	0.521	0.521	0.521
Sample A	0.272	0.210	0.121	0.10
Sample F1	0.312	0.241	0.129	0.126

Table 7: % decolorization of methyl red by isolates A

Sample	24hrs % decolorization	48hrs % decolorization	72hrs % decolorization	96hrs % decolorization
Methyl red 1 ppm	36.55%	40.54%	71.00%	73.52%

Table 8: % decolorization of methyl red by isolates F1

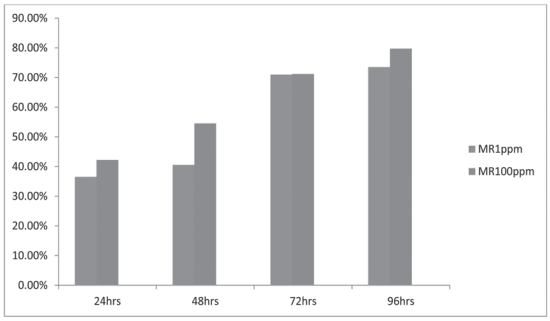
Sample	24hrs % decolorization	48hrs % decolorization	72hrs % decolorization	96hrs % decolorization
Methyl red 1 ppm	18.06%	39.28%	68.48%	70.16%
Methyl red 100ppm	40.39%	47.10%	69.56%	77.17%

Table 9: % decolorization of Orange G by isolates A

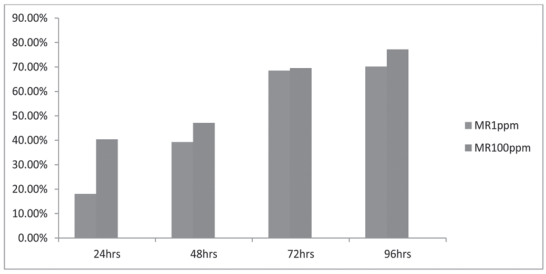
Sample	24hrs % decolorization	48hrs % decolorization	72hrs % decolorization	96hrs % decolorization
Orange G 1 ppm	33.33%	55.41%	71.54%	74.64%

Table 10: % decolorization of Orange G by isolates F1

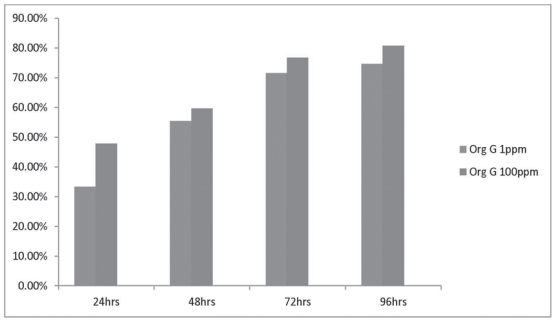
Sample	24hrs % decolorization	48hrs % decolorization	72hrs % decolorization	96hrs % decolorization
Orange G 1 ppm	22.08%	48.46%	65.03%	72.80%
Orange G 100ppm	40.11%	53.74%	75.23%	75.81%



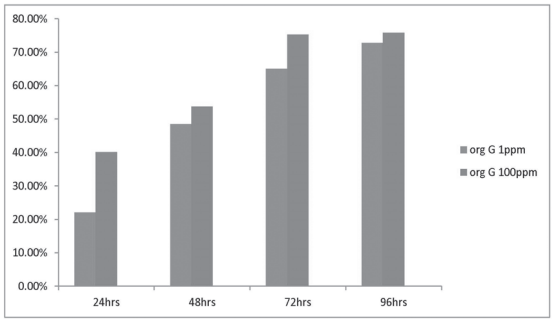
Graph 1: % decolorization of methyl red 1ppm and 100ppm by isolate A



Graph 2: % decolorization of methyl red 1ppm and 100ppm by isolate F1



Graph 3: % decolorization of orange G 1ppm and 100ppm by isolate A



Graph 4: % decolorization of orange G 1ppm and 100ppm by isolate F1

Conclusion

1. Two microbial isolates A and F1 from soils showed maximum decolorization of methyl red (100 ppm) and orange-G (100 ppm) within 24 hours to 96 hours respectively.
2. Orange G (100 ppm) was the compound most remarkably decolorized than Methyl red.
3. The microbial isolates identified were *Bacillus subtilis* and *Pseudomonas stutzeri* respectively.
4. Microbial strain F1 may be a promising bacterium to depollute the effluent containing azo dyes which also could decolorize synthetic mixture effluentcon taining methyl red and Orange G.
5. Both the microorganisms are non-pathogenic and selective strains for environmental cleanup.

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Conflict of Interest:

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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Quantification of phytochemicals in the pseudobulbs of *Crepidium acuminatum* (D. Don) Szlach-a critically endangered medicinal plant

Mamta Arora[1], Gurjinder Kaur[2], Satnam Singh[3], Anupama Mahajan[4], Jaspreet K. Sembi[5]
[1] Faculty Biotechnology, ASBASJSM College, Bela Ropar Punjab
[2] Administrator supervisor, Canada
3 HOD Pharmacognosy, ASBASJSM College of Pharmacy, Bela Ropar Punjab
[4] Assistant Professor, Biotechnology, SUSCET Tangori
[5] Assistant Professor, botany, Panjab University, Chandigarh
mamtaarora.2007@rediffmail.com, 9463027367

Abstract

The present investigation is aimed to quantify the phytochemicals present in the pseudobulbs of *Crepidium acuminatum* (D. Don) Szlach (Family-Orchidaceae). Total fat content was found to be 1.45 ± 0.13%, whereas Alkaloid content as 5±0.52%, Resin content was observed as 0.9±0.1 %, tannin content was 9.25±0.21%, Crude fiber content was found to be 5.1±0.69%. Saponin content was 2±0.48%. Phenolic content was 70.9± 0.53 (µg Gallic Acid equivalent/ml of extract). Total flavonoid content was 34.27 ±0.61 (µg Quercetin equivalents/ml of extract), whereas carbohydrate content was observed as 112±1.7 (µgGlucose equivalents/ml of extract) in 80% ethanol extract, Total saponin content was 184±1.2 (µg saponin equivalents/ml of extract).

Keywords: phytochemicals, Quantification, *Crepidium acuminatum* (D. Don) Szlach, *Malaxis acuminata*

Introduction

Natural products are utilized, as *remedies even* before the dawn of *human civilization.*They have also formed the basis of traditional medicine systems and have been documented in various Pharmacopoeias. Plants and their secondary metabolite constituents have an extensive record of usage in modern western medicines. Recently, interest has surged in herbal products as they are not only in expensive but have better cultural acceptability, better compatibility with the human body and minimal side effects (Pal &Shukla, 2003), therefore trend is shifting from synthetic to herbal medicines. Lead compounds arising from natural product based drug discovery approach, are far superior to synthetic drug discovery approach, due to co-evolution of target sites with biological activities in natural systems (Dias, *et al.,* 2012). A huge proportion of the world's population also does not have access to synthetic medicines (Rates, 2001). Consequently, herbal medicines are now in great demand in the developing and developed countries.

Market for herbal medicines is growing at an amazing speed. In 2003, WHO estimated the annual global herbal market value of approximately US$ 60 billion, whereas in 2012 the global market in Traditional Chinese Medicine only, was reported to be worth US$ 83 billion (Allkin, 2017). Traditional Medical System of AYUSH (Ayurveda, Yoga, Unani, Siddha and Homeopathy) of India is becoming popular in health care sector all over the world. As India is a developing country, it has to adopt pluralistic health care system to meet the requirements of large number of people. National Medicinal Plant Board (NMPB) predicted that the Indian herbal industry would increase from Rs. 80 to 90 billion by 2020 (Shakya, 2016). In India, the herbal drug market is about US$ One

billion, whereas export of herbal drugs is approximately US$ 80 million. Herbal products have market not only as drugs but also as nutraceuticals. Although, India has well practiced traditional medical system, but this has not been able to contribute much to the national economy as compared to China (Kamboj, 2000). Therefore, there is dire need for conservation of herbs, development of appropriate research strategies based on traditional practices, optimum quality control of herbal products, apt documentation to approve the therapeutic efficacy of herbal medicines (Sen *et al.*, 2011).

A large number of herbs used in Ayurveda have been investigated as they hold promise to transform traditional knowledge to modern allopathic medicines. The potential of these herbs as therapeutic and neutraceutical agents in herbal formulations is being increasingly realised in the current times, however, not commensurate with the extent of research being done on them (Senand Chakraborty, 2016). Medicinal plants research, have a promising future as there are about ½ million plants around the globe, whose medicinal activities have not been investigated yet (Singh, 2015), and research in this field not only assist in the treatment of current and future problems but will have preventive role in health care.

Orchids belong to the family Orchidaceae, one of the largest families of flowering plants. Plants of this family are highly evolved and are still in the process of active speciation. The word 'orchid' is derived from the Greek 'orchis' meaning 'testes' describing testicular shaped bulbs of certain orchids (Teoh, 2016). Orchids are acclaimed as ornamentals, therapeutics and ecological indicators. Even though they have been extensivelyexploited commercially for their floricultural value, their therapeutic potential has also been realised since ages. Their reference as medicinal plants has been found in old Chinese and Indian scriptures. Chinese were the first to cultivate orchids and document their medicinal usage (Singh, *et al.,* 2012; Pant, 2013). In 1233, Chao Shih-Keng described 20 orchids in book '*Chin Chan Lan P'u* (Gutiérrez, 2010).

Many orchidaceous preparations have miraculous remedial potential. Several potential compounds have been isolated from this family. Literature survey has shown the occurrence ofalkaloids, flavanoids, phenanthrenes, terpenoids, bibenzyl derivatives and other biologically active compounds in orchids (Singh, *et al.,* 2012). They have been reported as anti-carcinogenic, anti-inflammatory, anti-rheumatic, hypoglycemic, antimicrobial, anticonvulsive, relaxant, neuroprotective, and antiviral (Gutireez, 2010, Arora, et al., 2017[a]). They are used in acidity, arthritis, asthma, blood dysentery, boils, bone fractures, chest pain, cholera, diahorrhea, dyspepsia, earache, eczema, hepatitis, inflammation, jaundice, leucoderma, malaria, menstrual disorder, muscular pain, paralysis, piles, rheumatism, sexually transmitted diseases, sores, spermatorrhea, stomach disorders, syphilis, tuberculosis, tumour, wounds etc. Orchidaceous preparations and formulations are also used as aphrodisiac, bronchodilator, cooling agent, contraceptive, emetic, purgative, in scorpion sting, sex stimulator, in snake bite, vermifuge etc. (Hossain, 2010). This information is limited about their therapeutic potential, and scientific data required is scanty. Hence, these can serve as potent organisms for source of chemical entities for the development of new drugs. Hence, linking of ethnomedicinal usage of orchids to the identification of lead molecules using modern hyphenated analytical techniques is a boon to produce effective entities for drug discovery programmes.

Natural populations of orchids are on a decline due to overexploitation for commercial purposes. Their inherent slow growing character, very low germination in nature, habitat specificity, dependency on symbiotic mycorrhizal association and intricate pollination mechanisms, add to their duress and have thrown the whole family to the verge of extinction. Their numbers are fast dwindling, and are included in Appendix II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora), (Pant, 2013). Therefore, conservation strategies involving large

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scale multiplication of plants are required. *In vitro* propagation is an important strategy for the conservation of orchids, as it offers potential for maintaining the genetic fidelity of plants, and offers possibilities for their commercialisation and conservation (Pathak, *et al.,* 2011).

In Ayurveda, out of the eight herbs of Ashtavarga, four are orchids namely, *Crepidium acuminatum*, *Malaxis muscifera*, *Habenaria intermedia* and *Habenaria edgeworthii* (Singh, 2006). These are known to impart jeevaniya (Vitality), vayashapan (revival of youthful conditions), rejuvenation and antioxidant effects (Balkrishna *et al*, 2012). Ashtavarga plants are suffering from a triple standardization syndrome i.e. three common standardization quality parameters. These are confusion in vernacular names; non-availability of authentic plant; lack of chemical markers (Sharma *et al.,* 2014). It leads to substitution and adulteration of Astavarga plants. Consequently, there is immediate need to establish pharmacognostic standardisation of the Ashtavarga plants along with phytochemical analysis and *in vitro* propagation.

Among various plants of Ashtavarga, *Crepidium acuminatum* (D. Don) Szlach (Syn. *Malaxis acuminata*), known as 'jeevak', is an important ingredient of various formulations like Astavarga churna, Chyavanprash Rasayan, Ghrita, Taila, Gutika, Agada etc. and a polyherbal immune-booster nutraceutical 'Chyavanprash' (Anonymous, 2008). *C. acuminatum* is used in breathing disorders, burning sensation, cough, decrease in bone tissue, bleeding disorders, blood disorders, tuberculosis, insect bites, rheumatism etc. It is reported to be refrigerant therefore used to reduce fever (Febrifuge) (Arora, et al., 2017[b] It has been described as aphrodisiac and used in emaciation and seminal weakness. It is utilized as tonic and in general debility (Warrier & Nambiar, 1993; Teoh, 2016; Balkrishna, 2012).In spite of tremendous miraculous curative nature of the pseudobulbs of this plant, scanty work has been done on this herb (Sharma *et al.,* 2014). Therefore, this is the right time to explore bioactive constituents of *C. acuminatum.*

Materials and Methods

Quantitative estimation of phytoconsti tuents have been for total content of fats, alkaloids, resins, crude fibres, saponins, phenols, flavonoids, carbohydrates. These methods are explained sequentially.

Procurement of Material : Pseudobulbs were procured from market of Chandigarh and were authenticated from NISCAIR, New Delhi and deposited in herbarium of Botany department, Chandigarh. Pseudobulbs were shade dried and grounded to fine powder and preserved as sample.

Total Fat Content : Accurately weighed quantity of sample(3 gm) was extracted with anhydrous ether in a Soxhlet apparatus for 6 h. The extract was filtered into a clean dry previously weighed china dish. The extraction flask was rinsed with small quantity of ether, filtered and added to the weighed china dish. The solvent was evaporated and dried to constant weight at 105°C (Ansari, 2004).

Total Alkaloid Content : Accurately weighed 5 gm of the chloroform extract was mixed with 200 ml of 10% acetic acid in ethanol in 250 ml of a beaker and covered and allowed to stand for 4 h. This was filtered and extract was concentrated on a water bath to one quarter of original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitates were collected and washed with dilute ammonium hydroxide and then filtered. The residue was the alkaloids, which was dried and weighed (Harborne, 1998).

Total Resin Content : A weighed quantity of sample (5 gm) is refluxed in acetone (3×200 ml) for six h. The extract was filtered and evaporated to dryness. The residue was dissolved in distilled water, warmed and filtered; the residue left was dissolved in solvent ether (2×200 ml). The solvent is evaporated and dried to constant weight at 105°C (Ansari, 2010).

Total Tannin Content : The powdered sample (2 gm) was extracted with 100 ml distilled water for

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about 24 h. at room temperature. After 24 h. the mixture was filtered, followed by addition of 5 ml of saturated lead acetate. The addition of lead acetate to the mixture precipitates the tannins as lead tannate. The precipitate was washed withwater and dried. Lead tannate obtained was suspended in ethanol, warmed and decomposed by bubbling in H_2S gas. Black precipitate of PbS was removed by filtration and the filtrate concentrated under reduced pressure. The residue obtained was then dissolved in 50 ml water followed by addition of 25 ml of 1% cupric acetate solution. The precipitate thus obtained was washed, dried and incinerated in a muffle furnace keeping all the material in silica crucible and weighed as cupric oxide. The tannin content was calculated by following formula(Ansari, 2010

$$\% \text{ Tannin} = \frac{(B-A) \times 305 \times 100}{\text{Weight of drug}}$$

Where, A= Weight of silica crucible
B= Weight of silica crucible and material

Crude fibre Content

2 gm of the powdered pseudobulbs powder was accurately weighed and extracted with petroleum ether. Then 200 ml of 1.25% of sulphuric acid was added to the marc and the whole mixture was boiled for 30 minutes under reflux. Then, the mixture was filtered and residue was washed with boiling water until free from acid. The residue was rinsed back with 200 ml of 1.25% of sodium hydroxide solution. The mixture was boiled for 30 minutes under reflux. The liquid was filtered and residue washed with boiling water until neutral, dried at 110°C to constant weight and then, incinerated to constant weight. The difference between the weight of the dried residue and incinerated residue was calculated with reference to the dried sample (Mukherjee, 2002).

Saponin Content

Saponin content of the sample was determined by double solvent extraction gravimetric method (Kassem *et al.,* 2014). 2 gm of the powdered sample was mixed with 50 ml of 20% aqueous ethanol solution. The mixture was heated with periodic agitation in water bath for 90 minutes at 55°C. It was filtered through Whatman filter paper. The residue was extracted with 50 ml of the 20% aqueous ethanol and both extracts were pooled together. The combined extract was reduced to about 40 ml at 90°C and transferred to a separating funnel where 40 ml of diethyl ether was added and shaken vigorously. Separation was done by partition during which the ether layer was discarded and the aqueous layer reserved. Re-extraction by partition was done repeatedly until the aqueous layer become clear in colour. The saponins were extracted with 60 ml of normal butanol. The combined extract were washed twice with 10 ml of 5% aqueous NaCl solution and evaporated to dryness in a pre-weighed evaporating dish. It was dried at 60°C in the oven and reweighed. Percentage saponins were calculated.

$$\% \text{ Saponins} = \frac{(W_2 - W_1) \times 100}{\text{Weight of sample}}$$

Where, W_1 = Weight of evaporating dish;
W_2 = Weight of dish + sample.

Total Carbohydrates Content

The total Carbohydrates contents of the plant extract were determined according to the DNS procedure method (Miller, 1959) by some modification that is addition of 15 ml distilled water.

Reagents:Potassium sodium tartrate (40% *w/v*) and Dinitrosalicylic acid (DNS) reagent were prepared, Prepared dilutions for glucose (standard) of 50, 100, 150….., 500 µg/ml.

Sample Preparation: 100 mg. of powder of Pseudobulbs of *C. acuminatum* were prepared with 80% ethanol twice(5 ml each time). Collected supernatant and evaporated it.Then added 10 ml of distilled water to dissolve the sugars. 3 ml of solution was taken .To this added 3 ml of 3,5-Dinitrosalicylic acid (DNS reagent) and heated in boiling water bath for 5 min.

Preparation of standard:0-500µg/ml of standard dilutions of glucose was taken and added water to make final volume 3 ml. To this added 3 ml of DNS reagent and heated in boiling water bath for 5 minutes. Added 1 ml of sodium potassium tartrate followed by 15 ml distilled water.

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Kept for cooling and absorbance was taken at 510 nm.

Blank solutions: 3 ml of distilled water and 3 ml of DNS reagent. Heated in boiling water not Heat bath for 5 minutes. Added 1 ml of sodium potassium tartrate followed by 15 ml distilled water. Kept for cooling and absorbance was taken at 510 nm.

After taking the absorbance of standard dilutions, calibration curve was plotted. Reducing sugar content in drug was calculated by using standard calibration curve.

Total saponin content : Total saponin determination was done using anisaldehyde reagent. Sample solution was prepared in water. For total saponins estimation 500 µl of sample, 500 µl of 0.5% anisealdehyde reagent, were mixed and kept aside for 10 min. Later, 2 ml of 50% sulphuric acid reagent was added and tubes were mixed. Tubes were then kept in water bath with constant temperature of 60°. After 10 min tubes were cooled and absorbance was taken at 435 nm. The amount of saponins was calculated as saponin equivalent from the calibration curve of standard saponin (100-1000 µg/ml) (Vador *et al.,* 2012).

Results

Total Carbohydrate and Saponin contents are calculated by standard curve methods by using 80 % ethanol and aqueous extract correspondingly. Curves are shown in Figure 1 and Figure 2 for Glucose and Saponins respectively. Results are shown in Table 1. Quantities are found to 112±1.7 (µg Glucose equivalents/ml of extract) in 80% ethanol extract and 184±1.2 (µg saponin equivalents/ml of extract) in aqueous extract. Saponin content has also been measured by double solvent gravimetric method and it is found to be 2±0.48%. Quantity of Total Fat Content, Total Alkaloid Content, Total Resin Content, Total Tannin Content and Total Crude Fibre Content have been represented in percentage in Table 1.

Discussion

The preliminary phytochemical screening of crude drug indicated the presence of saponins, essential oils, athraquinones, sterols, coumarins, flavonoids, steroids, tannins and glycosides. Quantitatively alkaloids, resins, tannins, crude fibre, saponin, phenolic, flavonoids, carbohydrate and saponins were determined by different methods. Proteins were found to be negative. Total phenolic compounds have been reported as 70.9 ± 0.53 (µg gallic acid equivalent/ml of extract) for methanol extract. The flavonoid content was found to be 34.27 ±0.61 (µg quercetin equivalents/ml of

Table 1: Quantification of phytoconstituents of *C. acuminatum*

S. No.	Class of Constituents	Quantity of phyto-constituent
1.	Total Fat Content	1.45 ± 0.13%
2.	Total Alkaloid Content	5±0.52%
3.	Total Resin Content	0.9±0.1 %
4.	Total Tannin Content	9.25±0.21%
5.	Total Crude Fibre Content	5.1±0.69%
6.	Total Saponin Content	2±0.48%
7.	Total Carbohydrate Content	112±1.7 (µg Glucose equivalents/ml of extract) in 80% ethanol extract
8.	Total Saponin Content	184±1.2 (µg saponin equivalents/ml of extract) in aqueous extract

Quantification of phytochemicals in the pseudobulbs

extract) in methanol extract (Arora, *et al.,* 2017[c]) Compounds that were reported earlier are alkaloid, carbohydrates, flavonoids, resin, saponin, steroids, tannins, whereas triterpenoids and proteins were found to be negative qualitatively (Sharma, *et al.* 2009). Essential oils such as Limonene, eugenol, citronellal, 1-8-cineole, piperitone and p-cymene were reported by TLC (Gupta *et al,* 1978). Caryophyllene 74.89% ; Eugenol 5.56%; Humulene 5.48% ; phenol, 2,4 bis (1,1 dimethylethyl) 5.57%; Caryophyllene oxide 5.33%; 2,5 Octadecadiynoic acid, methyl ester 3.16% are reported by method after GC-MS. (Arora et al., 2017[d]). Fingerprint profile of various phytochemicals have been reported by HPTLC (Arora, *et al.,* 2018). In another report beta-sitosterol, cetyl alcohol, glucose, rhamnose were reported (Bhatnagar, *et al*. 1970). Volatile oils are estimated as 0.54+-0.28(%v/w) (Sharma*, et al*., 2009). In one recent study (Bose *et al*. 2017), fatty acids, alpha –hydroxy acids, phenols, sterols, amino acids, sugars and glycoside were reported in this plant by GC-MS. Lohani, *et al*. (2013) analysed quantitatively the metal content and volatile constituents of the plant by Atomic absorption spectrophotometer (AAS) and GC-MS, they also reported α-tocopherol, β-tocopherol and terpenoids. Adam *et al.* (2018) showed the presence of acidic polysaccharides, anthocyanins, lignin, phenolic substances, cutin, suberin, lignin and starch. Bag *et al*. (2014) confirmed the presence of polyphenols, flavonoids, while synthesising gold nanoparticles of the extracts of pseudobulbs, whereas we have quantitatively measured the phytoconstituents as shown in Table 1. Quantity of phytoconstituents depends upon various factors.

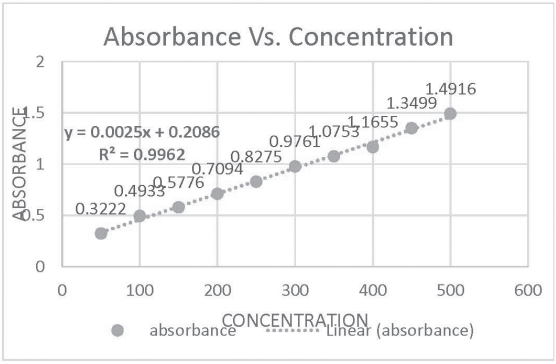

Figure 1: Standard plot of lucose

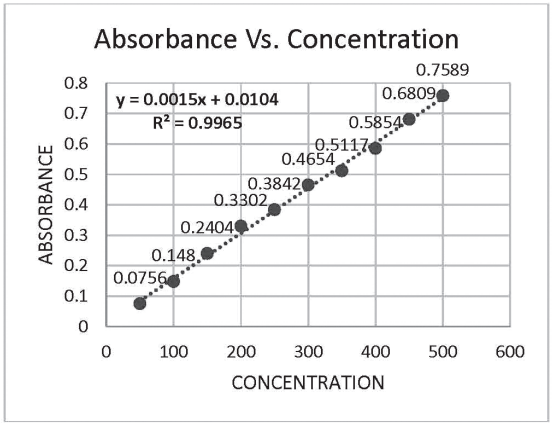

Figure 2 : Standard plot of Saponin

The presence of these metabolites suggests great potential of *C. acuminatum* as a source of useful phytomedicines as these secondary metabolites are accountable for the therapeutic activity of plants (Rabe and Van staden, 2000).

Phytcohemicals isolated from this plant(Kannan, 2008; Sharma *et al*., 2011; Singh, *et al.,* 2017) (ACD/ChemSketch) are shown below in figure 3.

Conclusion

Quantification of phytoconstituents of fats, carbohydrates, alkaloids, saponins, flavonoids, phenolics, crude fibres, tannins, and resins were attained. This investigation confirmed that the plant hold plenteously phytochemicals, which can further be isolated.

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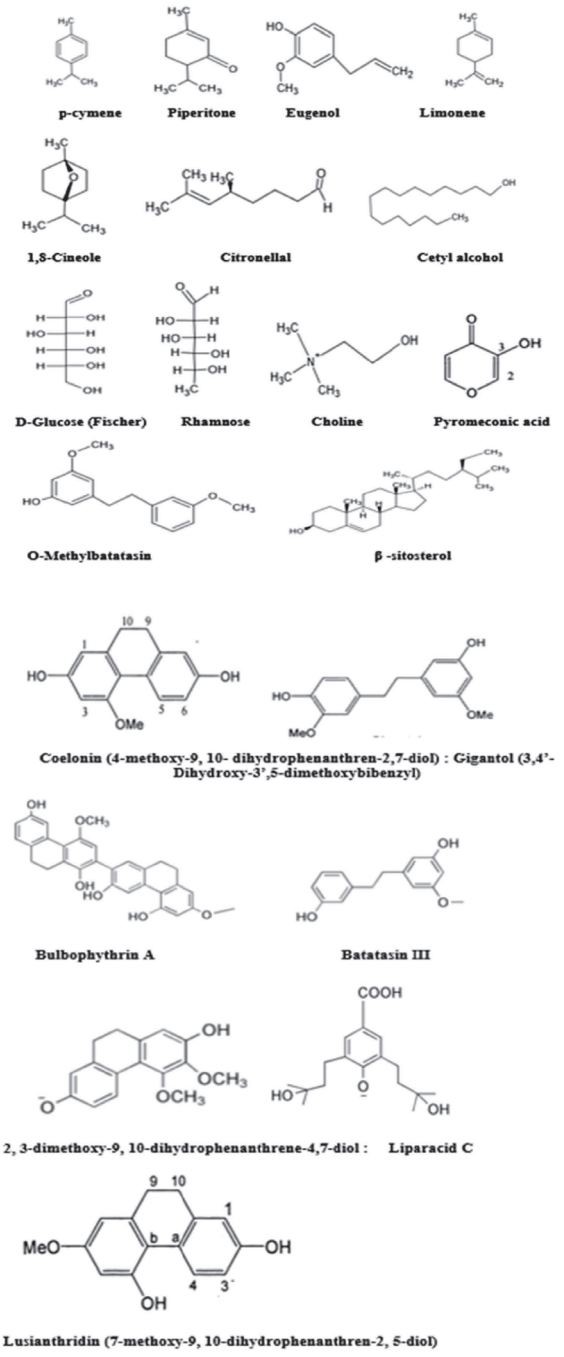

Figure 3: Phytochemicals Isolated From Plant

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Untapped Potential of Salicylic Acid, Jasmonic Acid and PGPRs to Develop Abiotic Stress Resilience in Crop Plants

Raghvendra Saxena[1], Manish Kumar*[1], Anurag Jyoti[1] and Rajesh Singh Tomar[1]
[1]Amity University Madhya Pradesh, Maharajpura Dang, Gwalior (MP)-474005
*Corresponding author: mkumar@gwa.amity.edu

Abstract

Abiotic stresses are the major limiting factor for crop productivity and food security. There are several regions in the world which have been affected by changing environmental scenario and facing a serious decline in agricultural productivity. Abiotic stresses are responsible for major crop yield loss in the field. Numerous studies suggested that plant growth and developmental processes are directly or indirectly regulated by plant growth regulators and phytohormones. These phytohormones strike balance between plant growth and adaptations in abiotic stress. The increasing number of studies in crop plants revealed a potential role of salicylic acid (SA) and jasmonic acid (JA) in activation of abiotic stress tolerance apart from their role in biotic stress resistance. Evidently, the endogenous level of salicylic acid and jasmonic acid in plants increased during abiotic stress. The present review article is mainly focussed on the role of two phytohormones; salicylic acid and jasmonic acid in inducing tolerance against drought and salt when applied exogenously and their endogenous level in crop plants. On the other hand, the role of PGPRs in plant growth and development activities are widely accepted that holds great significance.

Key words: Drought stress, salt stress, crop plants, phytohormones, salicylic acid, jasmonic acid.

1. Introduction

Environmental stresses including abiotic or biotic are inevitable constraints which significantly decline crop productivity and yield. A number of environmental factors viz., climate change, drought, salinity, temperature and others drastically affect the plants' functions. Abiotic stresses adversely affect plant growth and productivity by influencing the basic metabolic processes in different developmental stages from germination to maturity (Mantri et al., 2012). In the present scenario, the environment is frequently facing such incidences due to both natural as well as anthropogenic activities leading to additional load on food production to balance the increasing demand of food supply globally. Abiotic stress i.e., drought, salinity, cold and extreme temperature etc. are responsible for more than 50% loss in productivity in most of the crop plants (Wang et al., 2003). In view of this, the major concern in today's context of food security is to enhance the crop productivity by increasing crop tolerance against environmental stresses (Kumar et al., 2015). This can be achieved by adopting the scientific advancement along with integrated management practices to develop more tolerant and adaptable crop varieties. Parallel to crop improvement strategies, attention may also be focussed on the development of integrated management practices.

A number of studies have been reported on plant growth regulators and phytohormones, exploring their potential role in enhancing defence against abiotic stress in plants. This offers the huge potential of plant hormones that could be exploited to mitigate the deleterious impact of abiotic stresses (Kohli et al., 2013; Wani et al., 2016; Zhang et al., 2017). Further identification and investigation of such compounds that can ameliorate the adverse effect of environmental stress in plants would be a great importance in agriculture practices and sustainability. Plant growth regulators especially Salicylic acid (SA)

and jasmonates (JAs) are among the key plant growth regulators (PGRs) which activate signalling pathways to modulate physiological, biochemical and metabolic cascade of reaction in plants to respond against abiotic stress. Therefore, understanding of signalling pathways and the interaction of SA and JAs in activation of defence response against abiotic stress may offer a plausible approach in this direction (Khan MIR et al., 2013; Khan and Khan 2013; Kohli et al. 2013; Khan 2018). Several investigations supported that plant growth regulators mediated their response in two ways, firstly their endogenous level increases in several plant species when plants are subjected to some kind of abiotic stress and secondly the exogenous application of such plant growth regulators in appropriate concentration, which boosts abiotic stress tolerance to a significant level in several plant species (Pal et al., 2013). Earlier it was thought that phenolic compounds are not important for the vital functioning of plants, therefore they were considered as secondary metabolites, and however, several other studies later suggested that phenolic compounds are involved in important vital plant physiological, biochemical and metabolic processes. It was demonstrated for the first time that SA is involved in important plant physiological processes, which regulate plant growth (DeKock et al., 1974) and induce flowering etc. (Cleland and Ajami, 1974). Plant defence mechanism depends on the precise and timely detection of stress signals by plants, consequently activation of defence system which leads to activation of several signaling pathways delicate cross-talk with other molecules. The plant defense system involves activation of oxidative enzyme system to quench free radicals, ROS and induction of important phytohormones including SA and JA and activation of specific ion channels etc. Plant responses vary from one type of stress to another and plant species (Chinnusamy, et al., 2004; Laloi, C et al., 2004; Spoel, et al., 2008; Qamar, et al., 2009; Fraire-Velázquez et al., 2011; Verma et al., 2016). Therefore, all round activation of defence machinery leads to increased plant adaptation against abiotic stress in order to minimize adverse impact crop damage caused due to abiotic stress (Fujita et al., 2006). Plants response to various stresses involves complex interaction and crosstalk of several signaling pathways to cumulatively augment tolerance in plants against adverse environmental conditions (Ines Ben Rejeb et al., 2014). Salicylic acid and Jasmonic acid are recently added into the classical list of phytohormones i.e., Auxin (IAA), Cytokinin, Gibberellin, Cytokinin, ABA (Abscisic acid) and others. The function of salicylic acid, Jasmonic acid and ethylene are well documented and studied in several crop plants. They are involved in the induction of ISR and SAR in plants to develop resistance against pathogens (biotic resistance) in plants. Although, ISR and SAR are both activated and mediate their effect following distinct signaling routes. Primarily SAR in plants mediates through SA whereas ISR induction is mediated by JA and ethylene signaling routes (Van loon, et al., 1998; Pieterse et al., 2012).

Initial studies by Timmusk and Wagner (1999) revealed that selected PGPRs (*Paenibacillus polymyxa*) exhibit the ability to improve drought tolerance in *Arabidopsis thaliana*. In the last decade the term induced systemic tolerance (IST) was introduced to define the PGPR mediated enhancement of plant defence against abiotic stress through activation and modulation in several biochemical and physiological processes (Yung et al., 2008). However, increasing number of studies in the last decade supports the involvement of salicylic acid and jasmonic acid in adaptive responses under adverse conditions due to the interaction of salicylic acid (SA) with major biomolecules including secondary metabolites, osmolytes and other phytohormones. The involvement of salicylic acid in ROS-signaling and regulation of antioxidant enzymes gives a further boost in plant adaptive responses against abiotic stress. The effect of SA on abiotic stress tolerance in highly concentration dependent and effect varies with SA concentration and plant species. Therefore plants exhibit diverse effects under different conditions and concentrations (Yuan and Lin, 2008). It has been observed that deficiency of SA and

overproduction of SA may exhibit adverse impact on plant growth under abiotic stress (Yuan et al., 2007). Although, jasmonic acid is mainly known to act in inducing disease resistance in plants; however, its role in abiotic stress resilience in plants is also explored (Vos et al. 2013).

The applications of PGPR have also shown potential benefits in this direction where plant microbial interaction could be exploited in stress agricultural biotechnology. In the recent years, the potential of beneficial plant microbial interaction is much explored to promote environment-friendly strategies in sustainable agricultural practices. Therefore, continuous research and identification of new area are required to address technological challenges and yield barrier by adopting resource use efficiency and development of environmentally accepted green technology. Efforts have also been concentrated to introduce the beneficial plant microbes as mitigation measure on climate resilient agriculture. Parallel to this, the research efforts are further needed by research scientists to look for the suitable strategy to induce resistance in plants to mitigate adverse climatic conditions.

2. Abiotic stress

Abiotic stresses are experienced, largely due to unpredictable fluctuations of the environment, which are further aggravated by climate change. There are several agricultural regions worldwide exposed by one or more different types of environmental stresses leading to reduced crop growth and yield. Countries lose a big part of income every year by the reductions in crop yield caused by abiotic stresses (Kazan, 2015). It is estimated that more than 50% of crop yield loss may be encountered; depending on crop type and magnitude of exposure of environmental stress (Christensen, et al 2007). Among abiotic stress, drought is the most frequently experienced stress by many regions of the world, which occur every year under arid and semi-arid climates. A plant is very sensitive towards environmental perturbance and the majority of functions are affected by environmental stresses namely drought, heat, temperature, cold, salinity, heavy metals and pathogens, which are major constraints to limit crop production and yield (Feller, et al., 2014). Among several environmental stresses, drought and salinity are the most severe stress and frequently experienced in the world, hitting the agricultural crop productivity worldwide. Drought stress severely affects the vital physiological and biochemical processes of the plant. Abiotic stress limits the plant growth by altering several processes like photosynthesis, pigment contents, osmotic adjustment water relations etc. (Sanghera, et al., 2011; Pathak et al., 2014).

Drought alters the physiological and biochemical processes in plants and adversely limits ionic distribution and homeostasis in a cell leading to depressed plant growth and yield. It also adversely affects pigment biosynthesis, photosynthesis, osmotic adjustment, stomatal exchange and cell turgor (Khan et al., 2015). As drought, salinity is also the widest spread and prominent abiotic stress which also influences physiological and biochemical pathways in plants. Salinity causes reduced water uptake, an imbalance in nutrient uptake etc., leading to a significant loss in plant yield (Tuteja et al., 2012). Microbial communities which are an inhabitant of plant root system impart their beneficial effect on plant development and growth through the plant - microbe interaction. This interaction improves abiotic resistance (drought) in plants by modulating metabolic pathways (Schmidt et al., 2014). Moreover, the study revealed that bacterial isolates collected from dessert improve drought tolerance in plants by increasing the water uptake ability when inoculated with bacterial strains (Marasco et al., 2013).

3. Plant growth regulators or phytohormones

Phytohormones are key bioregulators for the regulation of various vital cellular processes and signaling pathways in plants under abiotic stresses. They are an important group of endogenous signal molecules that impart their effect in very small quantity and modulate host response to various abiotic stresses. Several studies revealed the huge potential of plant

hormones that could be exploited to mitigate the deleterious effect of abiotic stresses (Horvath, et al., 2007; Nazar et al., 2011; Khan et al., 2013). Phytohormones are important signaling molecules that are essential to sustain plant developmental activities. The classical list of phytohormones includes Auxin (IAA), Cytokinins (CKs), Abscisic acid (ABA), Ethylene (ET) and Gibberellins (GAs). Salicylic acid (SA), Brassinosteroids (BRs), and Jasmonates (JAs) have also been included in the phytohormones. Recently, the strigolactone (SL), comparatively new plant growth bioregulator is included into the list of phytohormones. The recent studies revealed that phytohormones could activate plant defence responses against biotic or abiotic stress. They can cross-talk with other signaling molecules, and could be important target molecules for metabolic engineering in order to ameliorate tolerance against environmental stresses in plants (Wani et al., 2016).

In the recent years researches, compounds like salicylic acid (SA), jasmonic acid (JA) and its derivative methyl jasmonate, (MeJA) have shown potential to reduce plant sensitivity and circumvent the negative impact of abiotic stress in plants and proved to enhance crop resilience under abiotic stresses (Walia, H. et al. 2007; Du, H et al., 2013; Qiu, Z. et al., 2014; Zhao, Y. et al 2014; Salimi, et al; 2016). Compounds like salicylic acid (SA) and jasmonic acid (JA) are now known as plant growth regulators, which can modulate plant's physiological and biochemical processes under abiotic stress including, drought, salinity, heat and other stresses etc. to adopt adverse conditions (Pedranzani, H. et al 2003; Ding H. et al. 2016; Fang L. et al., 2016). Recently, Verma, et al. (2016) have shown the significant roles of salicylic acid, jasmonate and ethylene in ameliorating plant's defence response under environmental stress namely drought, salt etc. Moreover, these phytohormones communicate with each other which involved several signaling pathways to efficiently execute the defence in plants under abiotic stress.

3.1 Salicylic acid (SA) in drought and salinity tolerance : *Ortho*-hydroxybenzoic acid or Salicylic acid (SA) is a small phenolic compound, which is ubiquitously present in the plant kingdom. It is synthesized via the phenylpropanoid pathway (Raskin et al., 1990; Metraux, 2002). Salicylic acid is well-known phytohormone, which is most widely studied for its role in inducing biotic resistance in plants, where it acts as a signalling molecule in elicitation of defence response against pathogens (Chandra et al., 2007). It is also known to elicit induced systemic resistance (ISR) as well as Systemic Acquired Resistance (SAR) in the plants under adverse environmental conditions and protects the plants during adverse conditions (Pieterse et al., 2012). Salicylic acid (SA) has shown beneficial effects on plants either in optimal or stress environments when applied exogenously. Since SA is an important signal molecule, it imparts its effect on several metabolic processes in plants and leads to the controlled production of various osmolytes and secondary metabolites in response to stress. Salicylic acid is also known to maintain plant-nutrient status hence, protects plants under biotic as well as abiotic stress (Khan, 2014). Although, salicylic acid is widely accepted to play a vital role in pathogen resistance in plants its role in abiotic stress tolerance is also evident. There is an increasing number of studies which indicate that salicylic acid enhances the plant defence against abiotic stress. It is due to its interaction with major osmolytes, secondary metabolites, mineral nutrients and other phytohormones. Role of Salicylic acid in ROS-signaling and modulation of antioxidants further indicate its ability to mitigate adverse effects of abiotic stress. Salicylic acid is gaining much attention in modulating the responses of the plant against abiotic stress like drought, salinity, heavy metal etc. (Fayez and Bazaid, 2014; Zhang et al., 2015; Singh et al., 2015). A study indicated that, when SA is applied exogenously, it may lead to induced tolerance in bean and tomato under salinity and drought stress (Szepesi, A. et al., 2005). Effect of exogenous application of SA (50 ìM) on drought-stressed seedling of Mustard (*Brassica juncea L.*) exhibited the improved tolerance by improving the oxidative defence and glyoxalase system which is due to

increased RWC, Chlorophyll content and enhanced activities of MDHAR, DHAR, GR, GPX, CAT, Gly I, and Gly II (Alam et al., 2013). The application of SA on the seeds of different verities of bean (*Phaseolus vulgaris L.*) have shown growth promotion effects under cold stress, which further suggested the impact of SA on the induction of growth aspects in seed germination and seedling growth leading to mitigate impact of cold stress (Gharib et al., 2010). Although, most of the studies indicated that exogenous application of SA in optimum concentration augment stress tolerance in plants, but it may be contradictory in some of the cases. Some studies revealed that same SA may promote stress tolerance in different plants in different concentrations. Moreover, the response of SA applied in the same concentration in the plant also contradicts for one type of stress to another (Nemeth et al., 2002; Yang et al., 2004). The involvement of SA in abiotic stress tolerance is further supported by an increased level of SA endogenously under abiotic stress including drought, salt and heavy metal, which triggers the accumulation of SA endogenously leading to illicit defence in plants to cope up the adverse impact of abiotic stress (Rodriguez-Serrano et al., 2006). Several studies supported that effect of Salicylic acid concentration dependent and responses vary with a concentration in different plant species and different stress conditions. Although, it is ubiquitously present in plant kingdom its concentration varies with species. Hao, et al (2011) conducted a study in cucumber to show the effect of SA on protein expression. The study revealed that there were approximately 59 proteins identified, which expressed in response to SA in cucumber. These proteins were actively involved in regulation of the various cellular processes i.e., in anti-oxidative reactions, cell defence responses, carbohydrate metabolism, respiration, photosynthesis, energy homeostasis, etc. (Hao JH, et al., 2011). Exogenous application of SA in 0.5 mM concentration in moong bean (*Vigna radiata* L.) has been reported to be effective in increasing tolerance in salt stress. Further, the study revealed that SA application leads to increased glutathione (GSH) level and accumulation of glycine betaine, which corroborated the improved salt tolerance in moong bean by improving photosynthesis and growth under salt stress (Khan, MI.et al., 2014). Similarly, application of SA (0.5 mM) exhibited the positive influence on mustard (*Brassica juncea* L.) plant growth and photosynthesis under salt stress, moreover, the results showed that Salicylic acid (SA) positively induced ascorbate-glutathione pathway, leading to enhanced GSH production. Thus, reduces the level of oxidative stress and mitigate the deleterious effect of salt stress maintaining the plant growth (Nazar et al., 2015). The role of SA mediated defence response was investigated in rapeseed (*Brassica napus*) under salt stress when applied exogenously. The results revealed the enhanced activity of the antioxidant system and glyoxalase enzyme in rapeseed providing better tolerance under salt stress (Hasanuzzaman et al., 2014).

3.2 Jasmonates (JAs) mediated abiotic stress tolerance: Plants respond to stress including both biotic and abiotic to maintain the growth by regulating several cellular processes through a small group of signaling molecules, including phytohormones, they are an important group of signal molecules that exhibit their effect in micro quantity and help plants to enhance tolerance against abiotic stresses like, drought, salinity, cold etc. Jasmonates (JAs) are collectively known for jasmonic acid and its conjugates. The most commonly used conjugates are jasmonoyl-isoleucine (JA-Ile) and methyl jasmonate (MeJA), which act as important plant growth regulators. Jasmonic acid and its derivatives are reported in several plant species that are involved in plant growth and development activities. They are known to regulate cellular responses of plants under the adverse condition to optimize the plant development. JAs are also involved in cross-talk with other phytohormones to modulate the plant responses against stress (Tasir et al., 2018). Jasmonates are well known small lipid-derived signal molecules that are involved in plant responses toward biotic as well as abiotic stress and regulate plant adaptations under such conditions (Table 1). There are an increasing

number of studies, which indicated that JAs are involved in several plant growth and developmental processes (Huang et al., 2017). The methyl jasmonate (MeJA) is a volatile compound discovered in jasmine flowers (*Jasminum grandiflorum*) (Demole et al., 1962). Methyl jasmonate (MeJA) act as a stress modulator which is reported to increase abiotic stress tolerance in plants significantly at various levels either by up-regulation of secondary metabolism or by control of photosynthesis and antioxidant system (Chen et al., 2011; Maserti, et al., 2011). Although, JA influence several developmental processes and growth responses in plants, but its role in enhancing adaption and augmenting abiotic stress tolerance in plants is also gaining acceptance under different abiotic stresses i.e., drought, salt stress, heat etc. although the exact mechanism is weekly understood (Zhang et al., 2009; Wasternack, C. 2014; Wasternack, C., Strnad, M., 2016, Sharma and Laxmi, 2016). Jasmonic acid is considered as endogenous plant growth regulator but recently, Qui, Z. et al (2014) reported the positive impact of exogenous application (foliar spray) of 2.0 mM JA on wheat seedlings under salt stress (150 mM NaCl). Results exhibited the enhanced activities of antioxidant enzymes activities, decreased the level of MDA and H_2O_2 which clearly indicated that exogenous application of JA has the ability to alleviate the salt stress effect in wheat seedlings (Qui Z. et al., 2014). Jasmonic acid (JA) is known to participate in several metabolic reactions i.e. conjugation with amino acids, carboxylation, glycosylation, hydroxylation, sulfation and methylation leading to fine tune of several metabolic reactions resulting into formation of metabolic compounds with changed biological activities. These metabolic compounds may be more active, less active or sometimes inactive in order to mediate different response under stress. Therefore, JA mediating plant responses to biotic and abiotic stresses vary and respond specifically towards adverse environmental conditions (Wasternack, C., Strnad, M., 2016). Moreover, plant response against abiotic stress includes a number of plant growth regulators and signaling molecules including phytohormones. However, one of the strategies which include the increase in the endogenous level of JA in plants under stress could be a most effective measure to augment plant adaptation against abiotic stress and may provide a potential tool in agricultural practices (Piotrowska, A. et al., 2009; Wasternack C. 2014). Noshin Ilyas, et al. (2017) conducted the study on wheat to monitor the effect of SA and JAs on germination and plant growth under drought stress. The study revealed that wheat seed primed with jasmonic acid (100µM) and salicylic acid (10Mm) have shown better tolerance under drought stress and exhibited better germination and growth under drought stress, which further indicated the crucial role of SA and JA in amelioration of abiotic stress tolerance in plants. It was observed that the efficacy of JA was higher than SA in inducing abiotic stress tolerance (Noshin Ilyas et al., 2017). Recently the role of jasmonic acid (JA) was examined in the maize seedlings under alkaline condition. The results suggested that exogenous application of JA acid exhibited the better tolerance in maize seedlings under (alkaline stress) Na_2CO_3-stress. Further, the study indicated that JA mitigates the toxic effect of alkali stress by induced antioxidant activity, decreased sodium uptake and reduced accumulation of reactive oxygen species (Mudaser Ahmad Mir et al., 2018). Another study revealed that the combination of drought stress and exogenous application of MeJA (0.25¼M) on wheat (*Triticum aestivum* L.) improved the adaptability of the plant to withstand drought stress. Further, the study indicated that exogenous application of MeJA (0.25¼M) increased the activities of antioxidant enzymes, enhanced photosynthesis and induced stomatal closure when the plant was subjected to drought stress leading to mitigate the adverse effect of drought stress (Ma. et al., 2014). Anjum et al., (2016) also reported the ameliorative effect of exogenous application of methyl jasmonate (0.5mM) on wheat plants under drought stress leading to improved growth and yield attributes under drought stress. One study (Alam et al., 2014) revealed the JA mediated improved tolerance in ten days old *Brassica* species seedling under

drought stress, which showed enhanced antioxidant and glyoxalase system. The results corroborated that application of JA (0.5mM)along with osmotic stress (15% PEG) in *Brassica* species seedlings have shown an enhanced level of glutathione reductase, (GR), Glyoxalase I and II, Glutathione peroxidase (GPX) and MDHR activities leading to boasting drought stress tolerance especially *B. Juncea* had shown better tolerance as compared other species (Alam, et al., 2014). Recently transcript profile analysis was conducted to compare and identify the differentially expressed gene under salt stress in salt sensitive and salt tolerant varieties of sweet potato. it has been shown that Jasmonic acid-mediated up-regulation of genes involved in salt responses in salt-tolerant variety (ND98) as compared to salt-sensitive variety (Lizixiang) of sweet potato. The results clearly showed that jasmonic acid (JA) cell signaling pathway plays a vital role in sweet potato to mitigate salt tress effect. Moreover, increased accumulation of JA in plants leads to a higher degree of stomatal closure and lowering the sodium ion takes place in salt tolerant variety i.e. ND98 (Zhang et al., 2017).

4. Phytohormones producing PGPRs mediated abiotic stress tolerance in plants:

Adverse climatic conditions and subsequent development of diverse environmental stress are among the most important critical and limiting factors, which negatively affects plant growth and yield. Plants possess the inherent capabilities to respond against adverse external conditions created due to climatic or edaphic factors and frequently cope up with changing environmental conditions by modulating their internal cellular metabolic processes (Simontacchi et al., 2015). To cope up with abiotic stress, a plant has developed intrinsic abilities to mitigate adverse effect. Microbes are the natural alleys of plant-microbial interactions which comprise complex and interacted metabolic cellular process. Millions of microbes inhabit in the rhizospheric zone and form a complex ecological community that can affect plant metabolic and cellular activities leading to influence on plant development and growth activities, moreover positive impact on abiotic stress tolerance in plants is also widely noticed (Schmidt et al., 2014). Microbes exhibit capabilities to mitigate adverse effects on plants by modulating several cellular processes in plants (Meena et al., 2012, Meena et al., 2017; Kumar et al 2019). However, despite wide progress and understanding of the physiological and molecular mechanism of abiotic stress tolerance in plants, the exact mechanism of bacterial-mediated tolerance in plants is still not very clear (Dimkpa, 2009). Increasing knowledge of beneficial plant microbes interaction may provide an alternative strategy to use of beneficial plant-microbe interactions in developing environmentally friendly and sustainable agricultural practices. Several studies indicated that plant growth promoting rhizobacteria can help to reduce damage occurred due to frequent incidences of various environmental stresses including various abiotic stresses by agriculture sector (Verma, et al., 2016; Vurukonda et al., 2016). The term Induced Systemic Tolerance (IST) has been referred to the microbial induced enhanced abiotic stress tolerance in the plants due to the microbial induced physical and chemical alterations in plants (Yang et al., 2008). Microbes possess immense potential to mitigate abiotic as well as biotic stress. Therefore, utilization of plant - beneficial microbe's interaction may offer a most promising solution to deal with changing environment and their impact on crop productivity. Microbes play a vital role in decreasing the adverse effect of abiotic stress and enhance plant's capability to cope the stress without affecting growth and productivity (Kumar and Prakash, 2018; Hassan and Dinesh, 2018). Being sessile organism, a plant is very sensitive for environment fluctuations and their cellular metabolic processes are directly affected by changing environmental conditions, such as drought, salinity, soil pH, etc. which frequently limits crop production. In the present scenario, it is essential to realize the immense potential of microbes in the direction of developing tools to mitigate the impact of changing environmental changes. Therefore, the identification of the useful aspect of PGPRs and

their implementation in modern agricultural practices could be one of the strategies in the direction of increasing crops adaptability for abiotic stress. Recombinant DNA technology further provides the tools to manipulate the metabolic pathways of useful PGPRs which may be helpful in diminish limiting effect of abiotic stress, However, the availability of scanty information regarding the applications and use of many beneficial PGPRs in crop plants is still beyond the knowledge of ecologists and agriculturists. Plants possess inherent ability to actively respond to a large number of chemical stimuli (signal molecules) secreted by rhizobacteria. These chemical signals can either elicit or fine-tune plant host defenses ability and resulting into enhanced defense ability against biotic and abiotic threats. Bacterial strains with plant growth promoting attributes isolated from the rhizosphere could be most promising as seed inoculants in innovative agricultural practices to boost plant adaptability against abiotic stress and reduce damage caused due to plant diseases.

Among several strategies adopted by PGPRs to impart their growth promoting effect on plants, production and modulation of phytohormones (i.e., Auxin, cytokinins, gibberellins, ABA and ethylene, etc) hold great importance in abiotic as well as biotic stress tolerance, as phytohormones strike the balance between growth and development under normal as well as in stressed conditions and help plants to tolerant stressful condition(Egamberdieva, 2013; Fahad et al., 2015).

There are reports which indicated that some of the bacterial strains namely *Pseudomonas spp., Bacillus* spp. and *Trichoderma* spp. have shown to strongly enhance plant host defenses. They could be a promising candidate as a biocontrol agent (Harman, 2004; Haas and Defago 2005). Recently the Tiwari et al. (2017) conducted the study which revealed, that PGPR bacterial strain *Bacillus amyloliquefaciens* NBRI-SN13 (SN13) have shown ability to induce abiotic stress (i.e., salt, drought, and heat) tolerance in rice seedlings (Rice cv. Saryu-52). The study further elaborated that the exogenous application of SA, JA, and ethylene on rice seedlings along with SN13 strain. It is found to be more effective in developing abiotic stress tolerance in rice by increasing osmolytes accumulation and up-regulating stress-responsive genes. This suggests that use of beneficial PGPRs in combination with phytohormones may offer a potential approach toward sustainable crop-management practices. Therefore, phytohor-mones producing PGPRs may impart their effect by modulating phytohormones level endogenously in plants resulting in improved abiotic stress tolerance (Tiwari et al., 2017).

Further, the application of improved Salicylic acid (SA) producing bacterial strain will activate plants innate immunity against biotic and abiotic threats. Literature survey revealed that SA also leads to enhanced abiotic stress tolerance in plants to respond against a variety of abiotic stresses, ie., drought, salinity, osmotic stress, temperature chilling etc. several physiological and developmental processes in plants i.e., photosynthesis, respiration, Seed germination and senescence etc are considered to be regulated by salicylic acid directly or indirectly (Rivas-San Vicente and Plasencia, 2011). The genus Bacillus has been focussed in last decades to focussed as a potential genus in order to induce host defense pathway (Brain et al., 2004). Several bacterial isolates were reported namely of *B. amyloliquefaciens, B. cereus, B. mycoides, B. pumilus, B. sphaericus and B. subtilis* have involved in eliciting strong host resistance pathways in plants locally as well as systemically (Kloepper et al., 2004). Zhang et al. (2002) assessed the effect of the bacterial isolate (*B. pumilus* strain Se34) on endogenous SA level in plants. The results indicated that *B. pumilus* strain Se34 treated plant showed an elevated level of SA in contrast to untreated plants. Therefore, Salicylic acid (SA) is considered a big contributor and essential signal molecule to induce resistance involved in the initiation of series of events that occur during the defense response in plants against stress (Carl et al., 2005). In this regard, Indira Gandhi et al. (2008) have also reported that

certain bacterial strains exhibit exogenous production of salicylic acid, which is considered as an important signal molecule for initiation of defense response in plants. The study further elaborated that three bacterial strains namely, *Serratia sp.* PSGB13, *Acinetobacter sp.* PRGB16 and *Pseudomonas sp.* PRGB06 exhibited to produces exogenous salicylic acid. In another study enhanced of abiotic stress tolerance was observed in tomato plants due to the manifestation of the plant-microbial interaction of (*Trichoderma spp*. –Plant-interaction) which is involved in plant growth and development stress tolerance (Tucci et al., 2011). There are studies, which indicated that microbes can produce SA acid and are capable to induce more SA production in plants under adverse conditions which subsequently attributed to improved tolerance in plants against abiotic stress. Some plant growth promoting bacteria (PGPB) exhibits capability to secret minute quantity of salicylic acid in the rhizosphere, resulting into activation of SA mediated defense signaling pathway in plants to improve tolerance against stresses (De Meyer and Hofte, 1997). Shanmugam and Narayanasamy also reported that rhizobacterial strain *Bacillus licheniformis* MML2501 exhibited the property to produce high salicylic acid in high concentration., therefore, it is further suggested that these SA producing PGPR strains can be applied in enhancing crop protection against stress.

Kang SM et al. (2014) studied those selected PGPR strains contribute significantly to mitigate the adverse effect of salinity and drought stress in Cucumber (*Cucumis sativus* L.).The inoculation of PGPR strains (*Promicromonospora* sp. SE188 *A. calcoaceticus* SE370 and *B. cepacia* SE4) with cucumber plants revealed the induction of phytohormonal regulation in plants leading to respond against drought and salt stress. The study revealed the higher endogenous level of SA and GA. This indicated the ameliorative effect of PGPRs on plants under abiotic (Kang et al., 2014).

Conclusion

Although there are several studies available indicating the role of SA and JA acid in abiotic stress tolerance, but how these two hormones modulate the metabolic pathways are still in the early stage. Therefore this is required to focus on biosynthetic pathways of hormones along with manipulation of metabolic pathways in order to sustain abiotic stress tolerance in plants towards agricultural sustainability. At the same time application of PGPRs are widely accepted in integrated nutrient management but PGPRs are useful in the induction of abiotic stress tolerance in crops is still need more investigations to elucidated signal transduction pathways to commemorate the induces systemic tolerance (IST) in plants under adverse environmental conditions. The future research in the direction of application of phytohormones and treatment of PGPR with owing the ability to elicit the endogenous level of phytohormones in plants to induce induces systemic tolerance (IST) will be employed in to bring down the magnitude of the problem. Moreover, a better understanding of SA and JA mediated signal transduction and cross-talk mechanism with other phytohormones may be a useful tool to develop tolerant crops in the field of abiotic research and agricultural sustainability.

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Antibacterial Activity of *Carica papaya* against Methicillin resistant *Staphylococcus epidermidis* Isolated from Wards Surfaces of Kampala International University TeachingHospital, Bushenyi, Uganda

Abubakar Sunusi Adam*[1], Ibrahim Ntulume[1], Rasheed Adeyemo[1], Saheed Akinola[1], Ibrahim Jatau Abubakar[2], Adamu Almustapha Aleiro[1,3], Sarah Onkobah[1], Lisa Micheni[1], Alice Namatovu[1,4]

[1]Department of Microbiology and Immunology, Faculty of Biomedical Sciences, Kampala International University Western Campus, Ishaka, Uganda
[2] Division of Pharmacy, College of Health and Medicine, University of Tasmania, Hobart, Australia
[3]Department of Biological Sciences, Faculty of Science, Kebbi State University of Science and Technology, Kebbi State, Nigeria
[4]Department of Biotechnical and Diagnostic Sciences, College of Veterinary Medicine, Animal Resources and Biosecurity, Makerere University, P. O. Box 7062, Kampala, Uganda,
*Corresponding author: abubakarsunusi@studwc.kiu.ac.ug, fidab63@gmail.com

Abstract

This study aimed at determining the antibacterial activity of *Carica papaya* against Methicillin resistant *Staphylococcus epidermidis* isolated from doorknobs, bed rails, floors and walls of Surgical, Medical, Maternity, Pediatrics, Accident and Emergency, Semiprivate and Private wards of Kampala International University Teaching Hospital. The bacteria were isolated from the wards surfaces and identified using biochemical tests, Desferroxiomine and Fosfomycin antibiotics. Disc diffusion method was used to detect methicillin resistance in *S. epidermidis* using Cefoxitin (30 µg) disc. Fresh leaves and seeds of *C. papaya* was processed and extracted using standard methods. Antibacterial activities of the methanol, acetone and aqueous crude extracts were assayed using the agar well diffusion method. Phytochemical analysis, Minimum Inhibitory and Bactericidal concentration of the crude extracts were determined using broth dilution methods. Both *C. papaya* leaf and seed crude extract exhibited antibacterial activity against MRSE with MICs and MBCs ranges of 250 to 31.3mg/ml and 125 to 31.3 mg/mL for leaf and seed extracts respectively. This study concludes that *C. papaya* leaf and seed crude extracts were effective against Methicillin resistant *S. epidermidis.*

Introduction

Staphylococcus epidermidis (*S. epidermidis*) is one of the human normal flora, typically the skin flora, and less commonly the mucosal flora (1). *S. epidermidis* possesses a wide variety of surface expressed molecules, some of which are likely to have important roles in the survival and adhesion of the skin (2). Transmission of *S. epidermidis* in the health care setting arises through contact with contaminated surfaces in the environment (3). *S. epidermidis* have the capability to survive within hospital settings surroundings on medical devices and medical equipment such as patient care equipment, uniforms, computer keyboards, cellular phones, bed rails, door knobs, table tops and identification badges for weeks to months (4). *S. epidermidis* and similar organisms have the ability to adhere to the surfaces, as a result of their unique pathogen-host-environment relationships (5).

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Staphylococcus epidermis is one of the major causes of nosocomial infections, which affects two million people annually, with a 25 % mortality rate, and prolong hospital stay among 5 to15% of patients worldwide (6,7). *S. epidermidis* strains circulating in hospitals have been established to be methicillin-resistant (8). The methicillin-resistance to *S. epidermis* are being usually due to the mica gene, which is carried by staphylococcal cassette chromosome mec (SCCmec), and produces a Penicillin binding protein 2A (PBP2A) with low affinity for ß-lactams (9). A previous study recommends the use of Vancomycin, Gentamicin, Cefazolin, Linezolid and Telavancin for the treatment of Methicillin-resistant *S. epidermidis* (10). However, most of the antibiotics recommended are the second line drugs and are also expensive; hence a need for an alternative measure against infections, especially from natural sources such as Ethno-medicinal plants.

Medicinal plants such as *Carica papaya (C. papaya*) are rich sources of medicine and can provide possible inexpensive alternatives in the treatment of resistant microbial strains, due to the presence of a multitude of phytochemical compounds which are linked to antimicrobial activities (11,12). *C. papaya* L. (Pawpaw), which belongs to the family Caricaceae, is a medicinal plant recognized as an effective natural medicine in controlling both oedema and inflammation associated with surgical operations (13). Phytochemicals, such as tannins, alkaloids and phenolic compounds present in different parts of *C. papaya*, have been shown to treat different ailments (14). The leaf and seed extracts of *C. papaya* has been reported to inhibit growth of several pathogens, including Coagulase-positive *S. aureus* and Coagulase-negative Staphylococci and also used as soap substitute for the treatment of skin infections (15,16). The effect of *C. papaya* seeds extracts on *S. epidermidis,* however, has not been widely explored. Hence a need to evaluate the antibacterial activity of *C. papaya* on *S. epidermidis* isolated from Hospital surfaces.

Materials and methods

Study area and Sampling site : This study was conducted at the Faculty of Biomedical Sciences and School of Pharmacy, Kampala International University. Isolation and Identification of Methicillin resistant *S. epidermidis* and determination of antibacterial activity of *C. papaya* was conducted at the laboratory of Microbiology department, while extraction and phytochemical screening of *C. papaya* leaves and seeds were performed at the laboratory of Pharmacology department. The plant samples were collected from Kigondo village in Ishaka municipality, Ishaka-Bushenyi, Uganda (GPS location latitude 050'0"S, Longitude 31Ú33'0"E). The Methicillin-resistant *S. epidermidis* were isolated from wards surfaces (bed rails, doorknobs, floors and walls) of Kampala International University-Teaching Hospital by swabbing the surfaces using a sterile cotton swab and brought to the department of Microbiology and Immunology laboratory within the University for Identification.

Isolation and identification of Methicillin-resistant Staphylococcus epidermidis : Swabbed samples of the bacteria were collected from wards surfaces (door knobs, bedrails, floor and walls) of Kampala International University Teaching Hospital (KIU-TH) and transported to the Microbiology laboratory at the Department of Microbiology and Immunology using transport media in an ice box cooler. The samples were inoculated on a sterile agar plates containing Mannitol salt agar. Pinkish colonies were sub-cultured on new Mannitol salt agar plates and confirmed using microscopy, catalase and coagulase test (17). The suspected colonies were further confirmed using Desferroxiomine (200 µg) and Fosfomycin (200 µg) antibiotics and affirmed with Cefoxitin (30 ìg) antibiotics (18).

Collection and Identification of plant samples : The samples of *C. papaya* which is commonly known as papari in Luganda were taken to a Botanist at the Department of Biology and science laboratory technology at Mbarara University of Sciences and Technology (MUST), Uganda, for

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identification. Thereafter, fresh leaves and seeds (from ripe pawpaw) were collected from Kigondo village early in the morning and transported in a sterile nylon bag to the Pharmacology laboratory, Department of Pharmacology, Kampala International University Western Campus (KIU-WC) where they were shade-dried and extracted (19).

Extraction of the plant samples : Extraction was carried out using the maceration method as described by20 Gideon (20). One hundred grams (100 g) of the leaves and seeds powder was weighed separately. Each was put in three different beakers (1000 mL size) and dissolved in 500 mL of absolute methanol, acetone and distilled water, with a polarity index of 5.1, 4.1 and 10.2, respectively. The mixture was allowed to mix for 48 hours, with frequent shaking using a vibratory sieve shaker to avoid pouring or evaporation of the mixture before extraction is complete. The crude extracts were filtered using a clean cotton cloth, followed by use of Whatman filter paper number 1. The filtrate was distilled and then evaporated to remove the solvent.

Determination of percentage yield extract : The percentage yield extract was obtained using the formula: W_2-W_1/W_0× 100

Where: W_2 is the weight of the extract and the container, W_1 the weight of the container alone, and W_0 the weight of the initial dried sample (21).

Phytochemical screening of the crude extracts : The crude extracts of *C. papaya* was screened to check the presence of phytochemicals, such as: flavonoid, tannins, terpenoids, cardiac glycosides, saponins, and steroids using the standard procedures described by Gideon (20).

Antibacterial activity assay

Preparation of extracts concentration : Five hundred milligrams (500 mg) of each extract were dissolved in 1 mL of 20% Dimethyl Sulfoxide (DMSO) to obtain the concentration of 500 mg/mL which was used for the determination of antibacterial activity as described by Gideon (20).

Antibacterial screening of the crude extracts : The antimicrobial activity of the extracts was demonstrated using the agar well diffusion method as described by Ogutu (22). Sterile Mueller Hinton agar plates were inoculated with the standardized suspension (0.5 McFarland standards) of the Methicillin resistant *S. epidermidis*. Five wells (5 mm each) were punched into the agar plates using a sterilized cork borer (5 mm). Using a micropipette, 100 ìL of both extracts were added to the first, second and third well accordingly. A concentration of 7 ìg/mL of vancomycin was prepared according to Johnson (23) and100 ìL of the prepared vancomycin was added to the fourth well as positive control while DMSO was added to the fifth wells as negative controls. Plates were incubated at 37^{ú}C for 24 hrs. The diameter of the zone of inhibition was measured and the results interpreted according to the Clinical and Laboratory Standard Institute guidelines (24).

Determination of minimum inhibitory and bactericidal concentrations of the active crude extracts : The minimum inhibitory concentration (MIC) of the crude extracts was determined according to Ogutu (22). Two-fold serial dilution of the extract was carried out in a series of sterile tubes containing 1ml of nutrient broth to obtain different concentrations (500, 250,125, 625, 31.25 and 15.63 mg/mL). One milliliter (1 mL) suspension of the test organism compared with 0.5 McFarland standard was added to each tube. This method was modified by preparing two sterile tubes: one containing only nutrient broth and test organism without the extract, to serve as negative control; and the other containing only the broth and extract without the test organism, to serve as a positive control. Each of the tests were done in triplicate in order to minimize errors.

The diluted tubes and the plates were incubated overnight at 37ÚC. After incubation, the turbidity from each diluted tube was compared with the control tubes and the highest dilution without turbidity was considered as the MIC and interpreted in mg/mL.

The result of MIC was used to determine Minimum Bactericidal Concentration by sampling

clear tubes. A loopful of broth from each clear tube was inoculated onto the nutrient agar in triplicate. The nutrient agar plate was streaked with the test organism to serve as controls. All the plates were incubated at 37ÚC for 24 hrs. After incubation the concentration at which no visible growth is seen was taken as the MBC (25).

Data analysis : The raw data was entered in excel, edited, cleaned and analysed using statistical package for social sciences (SPSS) version 21 software. One way Analysis of Variance (ANOVA) was used to compare the activity provided by leaves extract (Methanol, Acetone and Aqueous) and seeds (Methanol, Acetone and Aqueous) while t-test was used to compare the activity of Methanol leaf with that of Methanol seed extracts and Acetone leaf with that of Acetone seed extracts respectively. And p value of d"0. 05 used to determined significance between the activities of the extracts.

Ethical approval : Permission was sought from the management of Kampala International University Teaching Hospital for collecting samples from the wards surfaces. The resistant bacteria (MRSE) were handled with care following the guidelines provided by Clinical Laboratory Standard Institute (24).

Results

In the leaf and seed of *C. papaya,* methanolic crude extract gave the highest yield of 9% and 6.4%, respectively, while the aqueous gave the least yield of 5% and 4.2% respectively as shown in **Table 1.**

The phytochemical analysis of the plant extract carried out in this study revealed the presence of tannins, terpenoids, cardiac glycosides, alkaloids, phenols and triterpenoids in both leaf and seed extract. However, seed additionally had flavonoids, saponins and steroids as shown in **Table 2**.

Table 6 shows the antibacterial activity of methanol, acetone and aqueous crude extracts of *C. papaya* leaf and seed against antibiotic resistant *S. epidermidis* isolated from wards surfaces of KIU-TH. The seed crude extracts showed more activity than the leaf. Both seed and leaf aqueous crude extract had no activity while methanolic crude extract had the highest activity with an inhibition zone diameter of 23 mm and 16.5 mm respectively. Vancomycin (positive control) had an inhibition zone diameter ranging from 23 to 13 mm. The difference between the activity of the extracts from both leaf and seed is statistically significant at p-value < 0.05. However, there was no statistical difference between the activity of acetone and methanol leaf extracts at p-value = 0.6650. Furthermore, there was no significant difference between the activity of acetone seed extract and acetone leaf extract at p-value =0.0559 but the difference between the activity of methanol seed extracts and methanol leaf is statistically significant at p-value= 0.0040.

Table 4 showed the minimum inhibitory concentration and minimum bactericidal concentration of methanol and acetone crude extracts of *C. papaya* leaf. The minimum inhibitory as well as the minimum bactericidal concentrations ranges from 250 to 31.3 mg/mL respectively. The Methanolic leaf crude extract had the lowest MIC values (31.3 mg/mL) against PD23B, MT5W, SG11B and highest MIC values (125 mg/mL) against MD11B, AE48F, SG17B and SG3D. Acetone extract gave highest MIC value (125 mg/mL) against AE48F and lowest MIC values (31.3 mg/mL) against MD11B, PD19B, MD53F and AE28B. The highest MBC value (250 mg/mL) of the methanolic leaf extract was shown against SG17B while the lowest (62.5 mg/mL) was against MT13W, PD23B, PD19B, MT5W, MD53F and SG11B. Acetone extract gave highest MBC values (125 mg/mL) against MT13W, AE48F, SG17B and SG11B, and the lowest values (31.3 mg/mL) against MD11B, PD19B and MD53F.

The results on the minimum inhibitory concentration and minimum bactericidal concentration of methanol and acetone crude extracts of *C. papaya* seed was shown in **Table 5.** The minimum inhibitory and the minimum bactericidal concentrations ranged from 125 to 31.3 mg/mL respectively. The Methanolic seed

Table 1: Percentage yield of leaf and seed crude extract of *Carica papaya* in different solvents

Crude extract	**Leaf (%)**	**Seed (%)**
Methanol	9	6.4
Acetone	6.4	5.2
Aqueous	5	4.2

Table 2: Phytochemical analysis of *Carica papaya* leaf and seed crude extracts

Phytochemicals	Test performed	Leaf crude extracts			Seed crude extracts		
		ME	AE	AqE	ME	AE	AqE
Flavonoids	Lead acetate test	-	-	-	+	-	-
Tannins	Ferric chloride test	+	+	+	+	+	-
Terpenoids	Sulphuric acid test	+	+	-	+	+	-
Cardiac Glycosides	Borntragor's test	+	-	-	+	-	-
Saponins	Water test	-	-	-	-	-	+
Steroids	Chloroform test	-	-	-	+	+	-
Alkaloids	Wagner's test	+	+	+	+	+	-
Phenols	Ferric Chloride test	+	+	+	+	+	+
Triterpenes	Salkovaski's test	+	+	-	+	+	-

Key: ME= Methanol extract, **AE=** Acetone extract, **AqE=** Aqueous extract, **+** represents positive, **-** represents negative

Table 3: Antibacterial activity of leaf and seeds crude extracts of *Carica papaya* on Methicillin resistant *Staphylococcus epidermidis*

Methicillin resistant isolates of *S. epidermidis*	Ward Surfaces	Mean inhibition zone of leaf crude extracts diameter (mm)			Mean inhibition zone of seed crude extracts diameter (mm)			Mean inhibition zone of positive control diameter (mm)
		AE	ME	AqE	AE	ME	AqE	Vancomycin
MT13W	Wall	12	11.5	-	15	16	-	16
PD23D	Doorknob	11	13	-	12	14	-	14
MD11B	Bedrail	13	14	-	16	18.5	-	23
PD19B	Bedrail	10.5	11	-	13	15	-	17
MT5W	Wall	13	14	-	14	21	-	14
AE48F	Floor	16	12.5	-	18	19	-	16
MD53F	Floor	14	16.5	-	21	23	-	14
SG17B	Bedrail	15	17	-	18	20.5	-	13
AE28B	Bedrail	10.5	11	-	11.5	13	-	15
SG3D	Doorknob	11	13	-	13.5	16	-	18
SG11B	Bedrail	0	11	-	10	12.5	-	20

Key: ME=Methanolic extract, **AE=** Acetone extract, **AqE=** Aqueous extract, - represents absence.

Table 4: Minimum inhibitory and minimum bactericidal concentration (MIC and MBC) of leaf extracts of *Carica papaya* on Methicillin resistant *Staphylococcus epidermidis*

Methicillin resistant *S. epidermidis isolates*	Wards Surfaces	MIC values (mg/mL) Methanolic extract	Acetone extract	MBC values (mg/mL) Methanolic extract	Acetone extract
MT13W	Wall	62.5	62.5	62.5	125
PD23B	Bedrail	31.3	62.5	62.5	62.5
MD11B	Bedrail	125	31.3	125	31.3
PD19B	Bedrail	62.5	31.3	62.5	31.3
MT5W	Wall	31.3	62.5	62.5	62.5
AE48F	Floor	125	125	125	125
MD53F	Floor	62.5	31.3	62.5	31.3
SG17B	Bedrail	125	62.5	250	125
AE28B	Bedrail	62.5	31.3	125	62.5
SG3D	Door	125	62.5	125	62.5
SG11B	Bedrail	31.3	62.5	62.5	125

Key: MIC= Minimum Inhibitory Concentration, **MBC=** Minimum Bactericidal Concentration.

Table 5: Minimum Inhibitory and Minimum Bactericidal Concentrations (MIC and MBC) of seed extracts of *Carica papaya* on Methicillin resistant *Staphylococcus epidermidis*

Strains of *S. epidermidis*	Wards Surfaces	MIC values (mg/mL) Methanolic extract	Acetone extract	MBC values (mg/mL) Methanolic extract	Acetone extract
MT13W	Wall	62.5	125	125	125
PD23B	Bedrail	31.3	62.5	62.5	62.5
MD11B	Bedrail	31.3	62.5	62.5	125
PD19B	Bedrail	125	62.5	125	62.5
MT5W	Wall	31.3	62.5	31.3	62.5
AE48F	Floor	62.5	125	62.5	125
MD53F	Floor	31.3	62.5	62.5	125
SG17B	Bedrail	62.5	62.5	125	62.5
AE28B	Bedrail	62.5	31.3	125	62.5
SG3D	Doorknob	31.3	62.5	62.5	125
SG11B	Bedrail	62.5	125	62.5	125

Key: MIC= Minimum Inhibitory Concentration, **MBC=** Minimum Bactericidal Concentration.

Table 6: Statistical analysis between the activities of *Carica papaya* leaf and seed crude extracts

Turkey's multiple comparisons test	Leaf	Seed	P-values Acetone leaf and seed Methanol leaf and seed	
Acetone vs. Methanol	0.6650	0.3753	0.0559[a]	0.0040[a]
Acetone vs. Water	< 0.0001	< 0.0001		
Methanol vs. Water	< 0.0001	< 0.0001		
Acetone vs. Positive control	< 0.0001	< 0.0001		
Methanol vs. Positive control	< 0.0001	0.0002		
Water vs. Positive control	< 0.0001	< 0.0001		

KEY: superscripts a = one sample t-test.

crude extract had the lowest MIC values (31.3 mg/mL) against PD23B, MD11B, MT5W, MD53F and SG3D and highest MIC values (125 mg/mL) against PD19B. Acetone seed crude extract gave highest MIC value (125 mg/mL) against MT13W, AE48F and SG11B and lowest MIC values (31.3 mg/mL) against AE28B.

The highest MBC values (125 mg/mL) of methanolic seed crude extract was observed against MT13W, PD19B, SG17B and AE28B while the lowest (31.3 mg/mL) against MT5W. Acetone seed crude extract gave highest MBC values (125 mg/mL) against MT13W, MD11B, AE48F, MD53F, SG3D and SG11B and the lowest values (62.5 mg/mL) against PD23B, PD19B, MT5W, SG17B and AE28B.

4. Discussion

The antibacterial activity of methanolic seed extracts of *C. papaya* against Methicillin resistant *S. epidermidis* is in support of the studies by Ayanfemi and Bukola (26) as well as Egbuonu (27) which showed seed extracts to be more effective than the leaf against all the organisms tested in their study. The variation of antibacterial activities of the different extracts depends on the polarity of the solvents used, concentrations of the compounds being extracted from each solvent and in addition to their extrinsic bioactivity and by their ability to dissolve or diffuse in the media used in the assay (28). Methanolic extracts were more effective in this study, this could be because it contained more phytochemicals (Flavonoids, Tannins, Terpenoids, Cardiac glycosides, Steroids, Alkaloids, Phenols and Triterpenes). However, water extracts exhibited activity against Methicillin resistant *S. epidermidis,* this could be attributed to the low quantity of phytochemicals (Phenols, Saponins, Tanins and Alkaloids) in the extracts. Plant extracts have the ability to either inhibit or completely kill the bacterial cell under study; this can be examined through the determination of minimum inhibitory and minimum bactericidal concentration of the active extracts.

The results of MIC and MBC from this study (250 to 31.3 mg/mL for leaf and 125 to 31.3 mg/mL for seed) were in line with the discoveries of Ayandele and Oluwaseun (29) which reported the MIC's and MBC's values of *C. papaya* leaf and seed extracts against many bacterial isolates including *Staphylococcus aureus* ranging between 200 to 150 mg/mL and 200 to 175 mg/mL respectively. However, the MIC's and MBC's values were higher compare to other studies reported by Mwesigwa (30) with MIC values ranging between 100 to 3.12 mg/mL against *E. coli* and Okunola (31) reported the MIC of *C. papaya* leaf extract against *E. coli, Salmonella, S. aureus* and *Streptococcus pyogens* ranged between 100 to 75 mg/mL. The high MIC's and MBC's values

observed with extracts against test isolates might be an indication of low effectiveness or that the isolates have the potential for developing resistance to the bioactive compounds (32). Therefore, the high MIC's and MBC's values observed in this study with both the *C. papaya* leaf and seed extracts may be due to the fact that the organism used in this study were resistant isolates of *S. epidermidis*. The bioactivity of plant extracts is dependent upon its phytochemical constituents.

Conclusion

The research concluded that the leaf and seed methanolic and acetone crude extracts of *C. papaya* had an antibacterial activity against the Methicillin-resistant *S. epidermidis* isolated from KIU-TH wards surfaces. When compared, the methanolic crude extracts of both the leaf and seed had more activity than acetone crude extract. However, the aqueous crude extracts exhibited no activity against the resistant bacteria. This study recommended that *C. papaya* leaf and seed crude extract could be a potential source of novel antibiotic that can be utilized in the management of infections caused by *S. epidermidis*.

Conflict of interest

All authors have declared no conflict of interest

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Screening of Antifungal Potential of Leaf Extracts from *Albizia lebbeck* (L.) Benth

Md. Nazneen Bobby[1] , S. Krupanidhi[1] , K. Abraham Peele1 , M. Indira[1] , E.G.Wesely[2] , A. Ranganadha Reddy[1], T.C Venkateswarulu[1*]

[1, 1*]Department of Biotechnology, Vignan's Foundation for Science, Technology & Research, Vadlamudi-522213, Andhra Pradesh, India

[2]Department of Botany, A.A. Govt. Arts College, Namakkal-637002, India

* Corresponding Author: venki_biotech327@yahoo.com

Abstract

The antifungal activity of *Albizia lebbeck* Benth. (L.) was performed against selected pathogenic fungal strains namely *Aspergillus niger, Aspergillus flavus, Penicillium citrinum* and *Rhizopus oryzae.* Crude extracts from leaves of *Albizia lebbeck* Benth showed significant antimicrobial effect. Among different extracts, a methanolic extract of *A. lebbeck* showed highest zone of inhibition. The order of antifungal activity, expressed as minimum inhibitory concentration (MIC) of Methanol > Ethyl acetate > Petroleum ether observed for fungal strains tested. Methanol and ethyl acetate extracts exhibited significant antimicrobial activity than petroleum ether extract and may be suggested for use as natural antibiotic administration for the fungal diseases.

Key words: Antifungal properties, MIC, Pathogenic fungal strains

Introduction

Albizia lebbeck Benth. (L.) (AL) commonly known as Shirish has number of therapeutic properties (1). AL is an economically important plant for industrial and medicinal uses. The leaves are good fodder with rich protein content (2). The plant contains saponin, macrocyclic alkaloids, phenolic glycosides and flavonols (3). In ayurvedic medicine, it is considered as an antidote against all types of poisons (4). The ayurvedic formulation of shirish like Panch shirish agada and Mahagandhahasti agad etc. has been indicated in poisoning. However, it has been established that no part of the plant has any antidotal value against either snake or scorpion venoms. In addition, the bark decoction of AL possesses anti-anaphylactic, anti-asthmatic activity and these potentials can be assumed as supportive measures in poisoning treatment (5). Saponin isolated from AL bark and methanolic pod extract of AL possess antispermatogenic effect. AL also has analgesic, anti-inflammatory, anti-diarrheal, anxiolytic and nootropic activity (6). The bark of *A. lebbeck* has been previously shown to possess antimicrobial activities against E. coli, *S. typhi*, *P. aeruginosa*, *S. aureus*, *Bacillus cereus*, *Klebsiella aerogenes*, *Proteus vulgaris*, *Shigella boydii*, *Aspergillus fumigatus*, *Aspergillus flavus*, *A. niger*, *C. albicans*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Shigella dysenteria*, *Shigella flexneri*, *C. albicans*, *Candida tropicalis* and *Candida kruse* (7-9). Earlier investigations, a number of plants have been reported for antimicrobial properties across the world (10-12). The extract from leaves of *Albizia lebbeck* Benth was not reported for antifungal activities. Hence, the present study was focused on investigation of antifungal study from the leaves of *Albizia lebbeck* Benth.

Materials and methods

Plant material and preparation of crude extract : Aerial parts (leaf) of *Albizia lebbeck* plants were collected from botanical garden of MCAS, Rasipuram. The collected plant material was shade dried and ground well in a grinder with 2 mm diameter mesh. The dry powered plant materials (50 g) were extracted successively with 200 ml of petroleum ether, ethyl aceate, methanol

by using soxhlet apparatus for 48 hrs at a temperature not exceeding the boiling point of the solvent. The aqueous extracts were filtered using whatman filter paper (No.1) and then concentrated in vacuums at 40 ºC using rotary evaporator. The residues obtained were stored in a freezer -80 ºC until further tests (13, 14).

Agar disc diffusion method : The crude extracts were used for bioassay against the fungal species which includes, *Aspergillus niger* (IMI no: 500308), *Aspergillus flavus* (500309), *Penicillium citrinum* (500310) and *Rhizopus oryzae* (500312). Inoculums were prepared from the 24 hours old culture of standard fungal isolates in Potato dextrose broth. Potato dextrose plates were prepared and the inocula were seeded by spread plate method. In the prepared Potato dextrose agar plates the well was prepared with equal distance in the size of 4mm.The prepared wells were loaded with 250, 500, 1000 and 2000 µg of plant extracts. The plates were incubated at 37 °C for 24 - 48 h (15). Antifungal activity was evaluated by measuring the inhibition zone in millimeter in diameter and tabulated. All the samples were done in triplicate. Both positive and negative controls were determined, for negative control the three solvents (petroleum ether, ethyl acetate and methanol) were also used to determine their effect on test organisms.

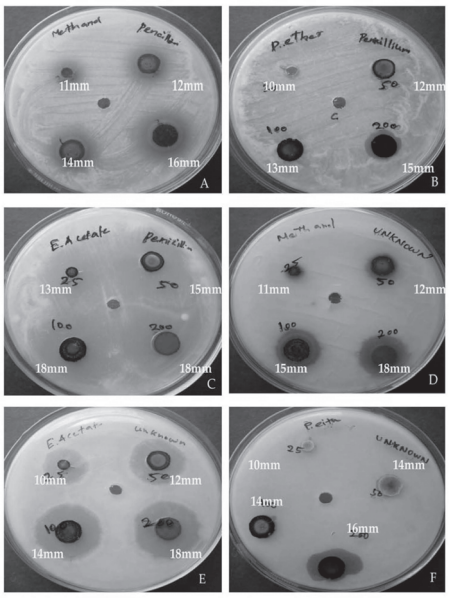
Results and discussion

Plants and their preparations have been used as medicines against infectious diseases. The plants are rich in secondary metabolites which include alkaloids, glycosides, flavonoids, insecticides, steroids, related active metabolites which are of great medicinal value and have been extensively used in the drug and pharmaceutical industry (16). The antifungal study results revealed that methanolic extracts of *A. lebbeck* conferred widest spectrum activities that inhibited the growth of all studied pathogens with zone of inhibition. The methanolic extracts of *A. lebbeck* illustrated the highest zone of inhibition against the plant pathogens *Aspergillus niger* (17 mm), *Aspergillus flavus* (18 mm), *Penicillium citrinum* (16 mm), and *Rhizopus oryzae* (18 mm). The ethyl acetate extracts demonstrated maximum zone of inhibition against *Aspergillus niger* (18 mm), *Aspergillus flavus* (16 mm), *Penicillium citrinum* (18 mm) and *Rhizopus oryzae* (18 mm). The petroleum ether extracts demonstrated maximum zone of inhibition against *Aspergillus niger* (19 mm), *Aspergillus flavus* (17 mm), *Penicillium citrinum* (15 mm) and *Rhizopus oryzae* (16 mm). (Table 1).

Table 1 Anti-fungal activity of leaf extracts of *Albizia lebbeck* Benth against the fungal pathogens

	Zone of inhibition(mm) Solvent extract											
Organisms	**Petrolium Ether (µg)**				**Etheyl Acetate (µg)**				**Methanol (µg)**			
	250	**500**	**1000**	**2000**	**250**	**500**	**1000**	**2000**	**250**	**500**	**1000**	**2000**
Control	-	-	-	-	-	-	-	-	-	-	-	-
A. niger	11	13	16	19	10	12	14	18	11	13	15	17
A. flavus	11	12	15	17	10	11	15	16	12	14	16	18
P. citrinum	10	12	13	15	13	15	18	18	11	12	14	16
R.oryzae	10	12	14	16	10	12	14	18	11	12	15	18

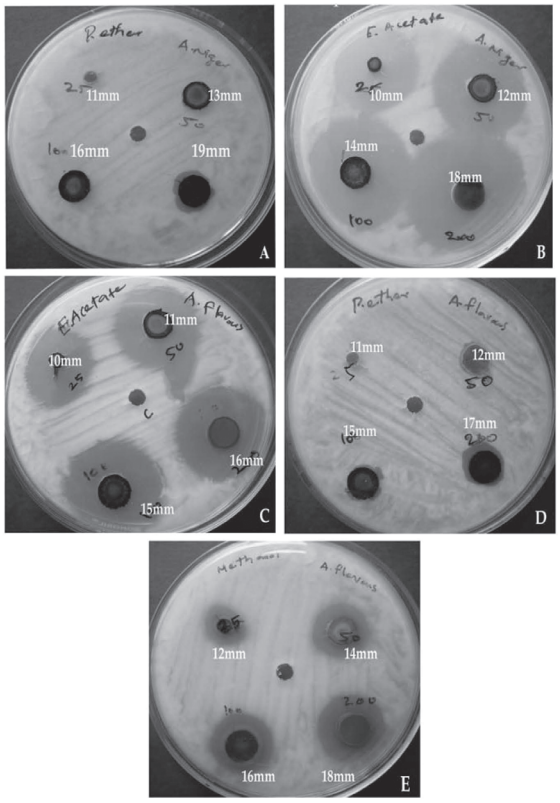
The results of the present study confirm the antifungal activity of *A. lebbeck* and the antifungal effects of leaf extracts were shown in the Figure 1 & 2.



A - Antifungal activity of methanol towards *Penicillium citrinum*
B - Antifungal activity of petroleum ether towards *Penicillium citrinum*
C - Antifungal activity of ethyl acetate towards *Penicillium citrinum*
D - Methanol extract showing zone of inhibition against *Rhizopus oryzae*
E - Ethul acetate extract showing zone of inhibition against *Rhizopus oryzae*
F - Petroleum ether extract showing zone of inhibition against *Rhizopus oryzae*

Figure 1 Antifungal activity of *Albizia lebbeck* Benth against *P.citrinum* and *R.oryzae*

In previous studies, Shahid and Firdous in 2012 tested the order of antifungal activity, observed in *A. lebbeck* benth. was seed> pod> flower > roots against six fungal strains such as *Aspergilus parasiticus, Aspergilus Niger, Candida albicans, Aspergillus effusus, Fusarium solani* and *Saccharomyces cerevisiae* and compared with Itraconazole and AmphoteracinB. Similarly, Mohammed Nazneen Bobby et al (2012) reported antibacterial effect of *Albizia lebbeck* against the bacterial pathogens and stated that methanol and ethyl acetate extracts exhibited slightly higher efficacy than petroleum ether extracts. However, the antifungal activity from leaf extracts of *A. lebbeck* was not reported and hence, the present study provides desirable data which is used in formulation of therapeutic products from plant origin. Johnson et al (2010) have reported that methanol was the most effective solvent for plant extraction than hexane and water (17). Uzama



A-Antifungal activity of Petroleum ether towards *A.niger*
B-Ehtyl aceatate showing zone of inhibition against *A.niger*
C-Antifungal activity of E.acetate towards *A.flavus.*
D-Antifungal activity of P.ether towards *A.flavus.*
E-Methonolic Extract showing fungal inhibition against *A.fla*

Figure 2 Antifungal activity of *Albizia lebbeck* Benth against *A.niger* and *A.flavus*

Danlami and Envuladu Patience Elisha (2017) reported that the *Albizia lebbeck* and its mistletoe leaves extracts have good potency for antimicrobial activity and found greater zone of inhibition against the fungal strains such as *Aspergillus fumigatus* (16 mm), *Aspergillus niger* (14 mm), *Fusarium oxysforum* (9 mm) from the ethanol extract of mistletoe leaves of *Albizia lebbeck* (18). Similarly, Ali et al (2018) reported that the petroleum ether, ethyl acetate and methanol bark extract of *A. Lebbeck* have shown the significant antimicrobial activity against the test organisms namely, *Candida arrizae*, *Aspergillus fumigatus*, *Aspergillus*

niger, *Rhizopus oryzae*, *Candida albicans*, *Saccharomyces cerevisiae*, *Candida krusei* and they found that among all the ethyl acetate extracts of *A. Lebbeck* have the most potential antimicrobial (19). However, the findings of the present study proved that the leaf extract of *A. lebbeck* can be used as potential source antifungal studies.

Conclusion

A. lebbeck is the potential source of bioactive compounds that could be used to formulate potent antimicrobial drugs of natural origin. The findings of this study proved that the extracts of *A. lebbeck* has antifungal activity and which might be helpful in preventing the diseases caused by pathogenic organisms and can be used as alterative system of medicine.

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Conflict of interest None declared

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Improvement in the production of L-Lysne by ENU treated Chemical mutagenesis of *ddh* gene recombinant strain of *Corynebacterium glutamicum MTCC25069*

Bhushan Vanasi[1], Ramesh Malothu*[1]
[1]Department of Biotechnology, Institute of Science Technology, Jawaharlal Nehru Technological University:Kakinada, Andhra Pradesh, India.
*Corresponding Author : rames-h_biotech@jntuk.edu.in

ABSTRACT:
Auxotrophic mutant formed from ddh gene recombinant MTCC25069with blocked homoserine dehydrogenase showed an increased yield of L Lysine of 24.89 g/l from normal ddh gene recombinant MTCC25069strain which had a yield of 20.66 g/l of L Lysine. The maximum yield of L-lysine for the auxotrophic mutant is attained at 7.5 pH, 300C of temperature and an incubation time of 96 hrs. The Auxotrophic mutant of ddh recombinant C. glutamicum showed nearly 6.52 g/l more amount of l lysine than Auxotrophic mutant of wild type with 18.57 g/l of L Lysine. The Chemical mutagen ENU caused mutation in the *Homoserine serine dehydrogenase* enzyme diverted the Aspartyl â semialdehyde to bind with *2,3Dihydrodipicolinate synthase* to participate in the L Lysine synthesis through 2,3 meso-Diaminopimelate (Meso-Dap). Being a recombinant for *diaminopimelate dehydrogenase (ddh)* the auxotrophic mutant for the homoserine dehydrogenase follows the ddh pathway by overexpression of ddh by deviating the Acetyltransferase and Succinyl transferase is the reason for the high yield of L Lysine production.

Keywords: Aspartyl â semialdehyde, *2,3 Dihydrodipi-colinate synthase*, *Homoserine dehydrogenase, Diaminopimelate dehydrogenase (ddh). L, L-diaminopimelate (2, 3-meso DAP).*

INTRODUCTION:

L Lysine is one of the most important essential amino acids which could be used in many biophysical mechanisms in the living organisms. Corynebacterium glutamicum is used to produce L Lysine commercially[1]. L-Lysine is an essential amino acid which is utilized in many biochemical reactions like phosphorylation and also used as an additive for fodder crops[2]. Annually around 80, 00, 00 tones were produced which made L Lysine second among global amino acid synthesis at industrial scale[3,4]. Chemical synthesis, enzymatic method, fermentation, extraction from protein Hydrolysate, genetic engineering and protoplast fusions were several kinds of technologies employed in L Lysine synthesis from Corynebacterium glutamicum[3,26]. L-lysine is one of the most deficient components found in the food of both human and animals. Animal feed generally contains a less quantity of L-lysine and is not synthesized by cattle, poultry or other livestock, so L-lysine will be added as a food supplement for animals to meet feed requirements[6]. L-Lysine, one of the eight essential amino acids for animals and humans which is used as feed additives, dietary supplements and also as an ingredient of pharmaceuticals and cosmetics[7].

Corynebacterium glutamicum is a non-lethal and non-emulsifying gram-positive bacterium. It exhibits a low protease activity in the culture and can secrete protease-sensitive proteins into the culture supernatant[14]. C.glutamicum is a gram-negative bacteria with the absence of lipopolysaccharide removed in the production of therapeutic proteins[15] increases the yield by reducing the purification steps. C. glutamicum is

generally recognized as safe (GRAS) for the industrial biochemical production of L Lysine and L glutamate[16].

Corynebacterium glutamicum is one of the major microorganisms used in amino acid synthesis. The Corynebacterium glutamicum is a rod-shaped bacteria, aerobic and gram-positive bacteria grows in the soil, on the surfaces of vegetables and fruits[17]. *C.glutamicum* has the capability to metabolize glucose, fructose, and sucrose[18, 29]. *C. glutamicum* utilizes many different kinds of carbohydrates, organic acids, and alcohol as a carbon and energy source for rapid microbial growth and for many amino acids synthesis[24, 25]. The glucose, or sucrose or any carbon source is utilized by the *Corynebacterium glutamicum* for l lysine synthesis by fermentation[28]. The time of incubation is reported for maximum L Lysine is between 48 hrs to 72 hrs[30, 31]. The ddh recombinant *Corynebacterium glutamicum* MTCC25069 produces more amounts of L Lysine compared to Wild type. This is because of the expression of more amount of ddh which acts as an enzyme for the substrate 2,6 dicarboxylic acid with the participation of less number of enzymes. Chemical mutagenesis with ENU increased the yield of L Lysine in the mutant than the Wild type strain[27]. The ENU causes insertion or deletion mutation and shows its effect on protein synthesis. The ENU causes mutation in Homoserine dehydrogenase gene to cause the Homoserine Auxotrophsof C. glutamicum[21].

Generally, the Aspartyl â semialdehyde is produced in two ways. In the Krebs cycle of *Corynebacterium glutamicum*, the Oxaloacetic acid (OAA)[19,20] undergoes transamination reaction with the presence of *glutamate: oxaloacetate: transaminase* enzymes produce aspartyl â semi aldehyde which further produces homoserine and L L diaminopimelate (2,3 meso- DAP) by two different pathway[1]. The Aspartyl â semialdehyde is also formed from *Aspartate dehydrogenase* from Aspartyl phosphate which was formed from Aspartate by *Aspartate kinase*[2,19]. The aspartyl â-semi aldehyde acts as a common substrate to produce L Lysine through L L diaminopimelate (2,3-DAP) and Methionine or threonine through homoserine[1,28]. The aspartylâ-semi aldehyde converts to Homoserine by reacting with *homoserine dehydrogenase*[4] which participates in the Homoserine pathway in the production of Threonine and Methionine[22,23]. Homoserine reacts with *MetA*[6] and produces O-Acetylhomoserine which reacts with *Met B* synthesize Cystathionine further reacts with *C*[7] to produce Homocysteine finally reacts with *Met E* or *Met H* to produce methionine or Homoserine reacts with homoserine kinase produces L homoserine phosphate and converts to threonine[11] by Threonine synthase in Homoserine pathway. Aspartyl â semi aldehyde reacts with *2,3 Dihydrodipicolinate synthase* produces 2,3 Dihydropicolinate which further reduces to 2,6 Dicarboxylic acids by 2,3 *Dihydrodipicolinate reductase*. Corynebacterium glutamicum chose three kinds of enzymes namely *Acetyltransferase* or *Succinyl Transferase* or *diaminopimelate dehydrogenase (ddh)* to produce L L diaminopimelate (2,3 meso DAP). The LL diaminopimelate (2,3 meso DAP) converts to L Lysine by *Lysine synthase*. By Recombination with ddh gene with a constitutive promoter enhances the productivity of L Lysine by diverting the acetyltransferase and succinic transferase pathway to ddh pathway. The Chemical Mutagen N-nitroso-N-ethyl urea **(**ENU)[3,9] has the capability to cause deletion or insertion mutation in the *Homoserine dehydrogenase*[18] enzyme and blocks the Homoserine Pathway which generally leads to the production of threonine and Methionine. This block in the homoserine pathway diverts the aspartic â-semialdehyde to react with 2,3 Dihydrodipicolinate synthase the enzyme to produce more amounts of "-diaminopimelate (" DAP) through ddh pathway.2,3 Dihydrodipicolinate synthase [35] converts aspartic â-semialdehyde to 2,3 Dihydropicolinate. In the presence of reductase 2,3 Dihydropicolinate reduces to Piperidine 2,6, dicarboxylic acid. The formation of DAP will be done by binding of Piperidine 2,6, dicarboxylic acid with three different enzymes acetyltransferase or Succinyl transferase or diaminopimelate dehydrogenase enzymes leads to three different pathways for the L Lysine production through DAP.

2,6 dicarboxylic acid reacts with *acetyl transferase* produces the N – acetyl 2 –amino 6-keto L-pimelate which reacts with the enzyme *aminotransferase* produces N- Acetyl- L- L - diaminopimelate produces L- L- DAP by *deacetylase* in the acetyltransferase pathway which is a three-step pathway. 2,6 dicarboxylate reacts with Succinyl transferase to produce N Succinyl 2- amino 6 keto L pimelate which reacts with *dap C* Produces N Succinyl L-L diaminopimelate which again reacts with *dap E* gives L L diaminopimelate is also a three step pathway. The ddh recombinant strain produces more L L diaminopimelate (2,3 meso DAP) by overexpression of ddh enzyme which follows ddh pathway for the production of L L diaminopimelate (2,3 meso-DAP) by reacting with 2,6 Dicarboxylic acid as substrate by eliminating the more number of reactions that were in the remaining *acetyltransferase* and *Succinyltransferase* pathways by overexpression of *ddh* by the ddh recombinant strain of C. glutamicum MTCC25069strain with constitutive promoter. The general events that took place in the Lysine production are illustrated in fig 1.

MATERIALS AND METHODS:

Bacterial cell cultures: The bacterial strain used in this research is a recombinant of ddh gene with a constitutive promoter of C. *glutamicum* (MTCC25069) developed by cloning[32] in the lab of Ramesh Malothu, school of biotechnology, JNTUK which had an increased yield of 20.66 g/L of l-lysine.

Recombinant strain developed in our laboratory from the MTCC25069wild type C. *glutamicum*, by recombining *ddh* gene with a constitutive promoter was utilized in the process of chemical mutagenesis with-nitroso-N-ethyl urea (ENU). The mutagenic power of N-ethyl-N-nitrosourea (ENU) stems from the generation of diazomethane[33, 34].

Chemical mutagenesis method: Seed culture medium (D-Glucose 10 g/l, Peptone 5 g/l, Yeast extract 3.75 g/l, NaCl 5 g/l, $(NH4)2SO4$ 17.5 g/l, K_2HPO_4 25 g/l, KH_2PO_4 25 g/l, Threonine 20 g/l, Methionine 20 g/l, $ZnSO4$0.5 g/l, $MgSO4.7H_2O$ 25 g/l, $FeSO4.7H_2O$ 1 g/l and $MnSO4.5H_2O$0.5 g/l), LB medium (Tryptone 10 g/l, NaCl 10 g/l and Yeast 5 g/l). *The ddh recombinant MTCC25069* strain were grown on LB medium and collected into test tubes with 3 ml each into 5 test tubes which were used for inducing the chemical mutation. Then the cell cultures were incubated for 24 hrs. at 37^0C in an orbital shaker. Centrifuge the tubes at 10000xg for 5mins and collect the pellets. These collected pellets were suspended in 3 ml sodium citrate buffer and again centrifuge these pellets at 10000xg for 5 mins. The pellet is collected and resuspended into 3 ml buffer containing sodium citrate buffer with a PH of 4.1 and 1.2 ml of N-nitroso-N-ethyl urea (ENU) with 100 nM concentration. The cultures were incubated separately for 0,5,10,15,20,25 and 30 min and centrifugation has done at 10000xg for 5 mins after the stipulated time. The pellets were collected and suspended in 3 ml sodium citrate buffer to wash the mutagen ENU and the resulted pellets were again suspended in the 3ml sodium citrate buffer to remove the traces of ENU. These pellets are incubated at 30^0c for 3 days in the threonine and Methionine enriched seed culture media.

Isolation of Auxotrophs: The growth obtained after 3 days of incubation was inoculated in 1 ml seed culture media without Threonine or Methionine and in 300c in an orbital shaker. 50 units of penicillinase were added to each tube and left for 10 minutes. 100µl of this growth inoculated on to seed culture media with threonine and methionine and also onto the seed culture media without threonine or methionine and incubated for 3 days at 300C in the orbital shaker. After the time of incubation is completed the samples were screened for Lysine production. The samples were centrifuged at 15000xg for 10 min. The supernatant is collected for lysine analysis. Quantitative analysis of L Lysine was done by SDS PAGE.

Optimization of fermentation parameters: Different parameters like Concentration of ENU, Time of exposure of ENU, PH of the culture media, Temperature and time were tested to find the better growing conditions of the recombinant strain when

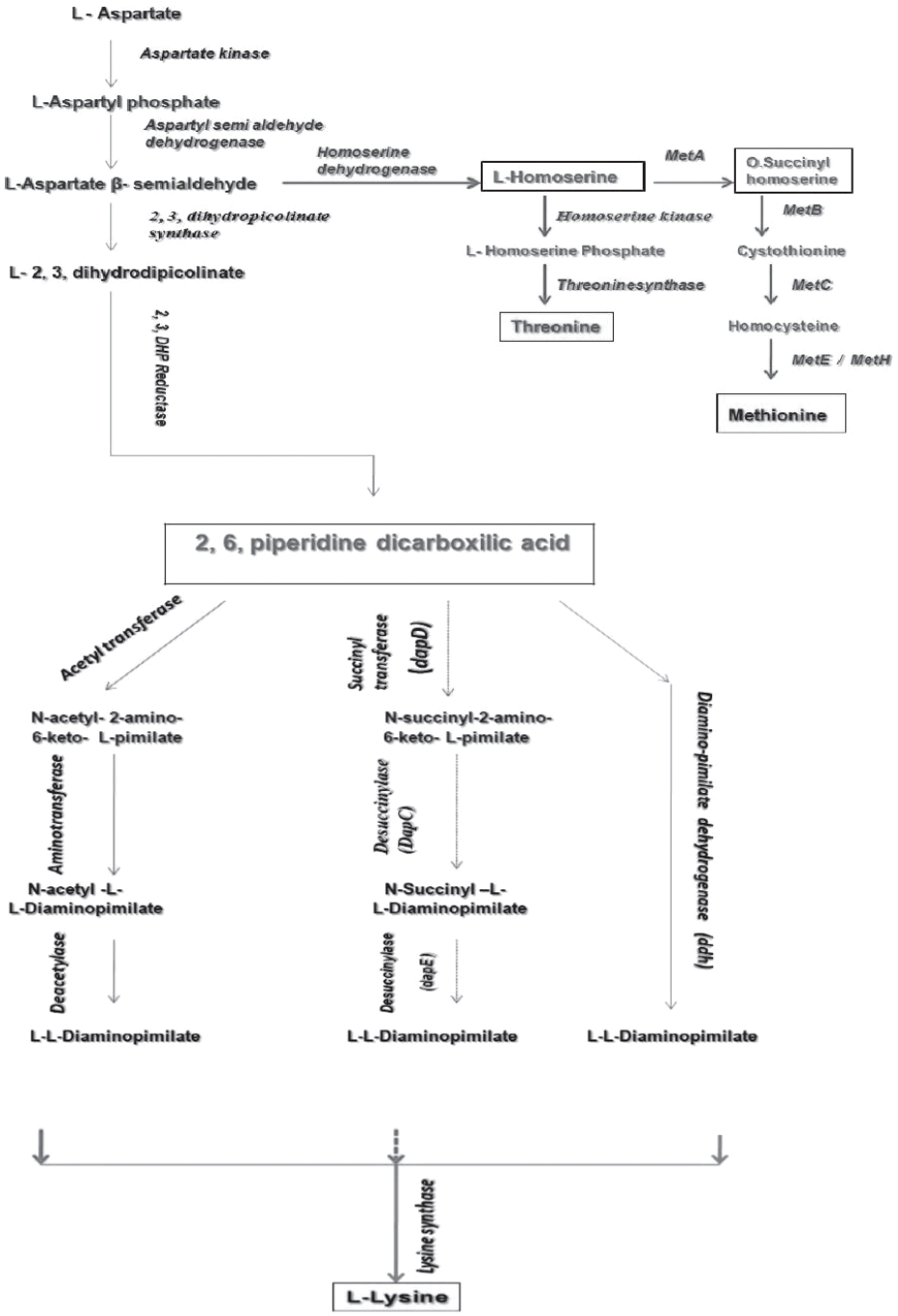


Fig 1: The Homoserine pathway for Threonine and Methionine synthesis and Acetyltransferase, Succinyl Synthase and diaminopimelate dehydrogenase pathways in the synthesis of L Lysine through L L diaminopimelate (2,3 meso- DAP).

treated with ENU for the high amount of production of L Lysine was tested.

In the chemical mutagenesis, we tested for different concentrations of 25, 35, 50, 75, 100 and 120 nm of the concentration of ENU and different times of exposure of ENU of 5, 10, 15, 20, 25 and 30 minutes to the Bacterial strain were tested at Constant temperature, Time, and pH. Which are optimized conditions of wild type *C. glutamicum* for L-Lysine production (13). The recombinant strain after treating with ENU, temperature and time of incubation was kept constant and tested for different pH values of 6, 6.5, 7.0, 7.5, 8 and 8.5. After checked for different PH the ENU treated ddh recombinant cultures were tested for different temperatures of 10, 25, 29, 30,31 and 35 by keeping the Volume, pH and time of incubation constant. The 100 nm ENU treated recombinant *C. glutamicum* was tested for different time periods of incubation 24, 48, 72, and 96 hrs by keeping the pH at 7.5 and temperature 300C constant.

By checking for all the parameters we chose the best-adapted values of chemically mutated recombinant strain which had given a high amount of yield of L Lysine to culture the recombinant strain and compared with Wild Type C. Glutamicum for productivity of L Lysine.

Culturing the Chemical Mutagen ENU (100 NM) Treated ddh Recombinant strain of C. glutamicum MTCC25069under Optimized Conditions: After optimizing the different conditions of Temperature, PH and time of incubation 20 min chemical mutagen ENU (100 NM) exposed ddh gene recombinant strain MTCC25069was cultured in under these conditions. The ENU treated ddh recombinant was cultured at 300C of temperature, 7.5 PH and with an incubation time of 96 hrs. was tested for the productivity of L Lysine from mutant ddh recombinant strain compared with normal wild type MTCC25069C. glutamicum mutant.

Molecular Docking analysis of Aspartyl â semi aldehyde with Homoserine dehydrogenase and 2,3 Dihydrodipicolinate Synthase: Molecular docking was performed for Aspartyl â semi aldehyde with Homoserine dehydrogenase and 2,3 Dihydrodipicolinate synthase to find the bonding interactions between the Protein and ligand. The amino acid sequence of Homoserine dehydrogenase enzyme of C. glutamicum MTCC25069with accession number NP_600409.1 and 2,3Dihydrodipicolinate synthase enzyme of C. glutamicum MTCC25069with accession number NP_601846.1 was collected and from NCBI and checked verified in the UniProt and the sequences from UniProt is used to build a protein model by Homology modeling in the SWISS-MODEL Server belongs to Swiss Institute of Bioinformatics (SIB). Protein model quality built by Swiss model server analyzed through the PDBsum database. After checking the Ramachandran plot and RMSD values we choose the protein models of Homoserine dehydrogenase and 2,3 Dihydrodipicolinate synthase to dock with ligand Aspartyl â semialdehyde in the PyREX software. Finally, the image analysis and amino acid interactions in the Protein ligand are generated in the discovery studio.

RESULTS AND DISCUSSION:

Chemical mutagenesis:

Effect of Concentration of ENU on the recombinant strain of C. glutamicum: The amount of ENU used to treat plays a pivotal role in causing Mutagenesis in the bacterial species. The recombinant C. glutamicum showed high productivity of L Lysine of 23.28 g/l of yield by keeping the temperature, time of incubation, and PH constant at 100 Nm concentration of ENU. Optimized conditions to grow the wild type C. glutamicum MTCC25069strain for maximum yield of L Lysine of 96 hrs. time of incubation, 30 C of temperature and PH 7.5. Kept constant by checking the yield for 25,35,50,75, 100 and 120 nm of concentration produced. 20.66 g/l, 20.11g/l, 21.05 g/l, 22.10 g/l, 23.28 g/l and 18.17 g/l of L Lysine respectively. We got high productivity of L Lysine at 100 nm concentration of ENU 23.28 g/l for Auxotrophic ddh recombinant mutant and a high yield of 16.23 g/l for Auxotrophic mutant of wild type C. glutamicum when compared to 35 nm, 50 nm,75nm concentrations of ENU.

Table.1: Table for L Lysine production at different ENU concentrations.

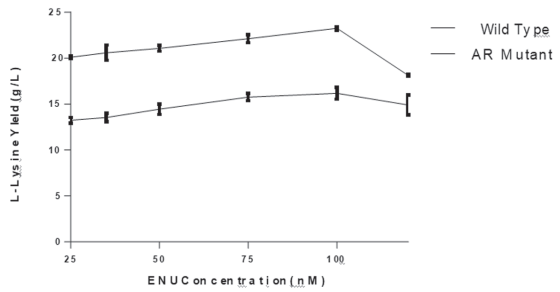
S.No	Time of Incubation (hours)	Temperature (0^0 c)	Concentration of ENU treated	pH	Lysine concentration Auxotrophic Wild Type	Lysine concentration Auxotrophic Mutant
1	96	30	25	7.5	13.26	**20.11**
2	96	30	35	7.5	13.50	**20.66**
3	96	30	50	7.5	14.40	**21.05**
4	96	30	75	7.5	15.80	**22.10**
5	96	30	100	7.5	16.23	**23.28**
6	96	30	120	7.5	14.99	**18.17**

Table.2: Table for L Lysine production at different times of exposure of ENU to ddh recombinant strain.

S.No	Time of Incubation (hours)	Tempe-rature (0^0 c)	Time of Exposure of ENU in Mins.	Concentration of ENU in Nano molars	pH	Lysine concentration Auxotrophic Wild Type	Lysine concentration Auxotrophic Mutant
1	96	30	5	100	7.5	13.05	20.22
2	96	30	10	100	7.5	14.78	22.67
3	96	30	15	100	7.5	15.23	23.17
4	96	30	20	100	7.5	16.48	24.02
5	96	30	25	100	7.5	15.54	22.23
6	96	30	30	100	7.5	14.23	21.56

Table 3: Table for L Lysine production at different times of exposure of ENU to ddh recombinant strain.

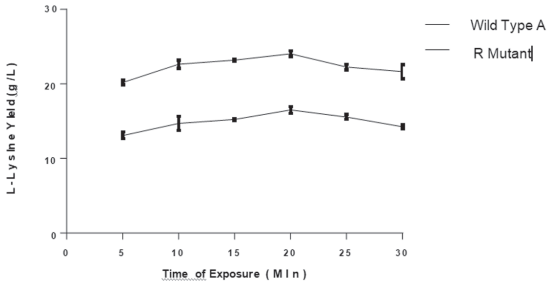
S.No	Time of Incubation	Temperature	pH	Lysine concentration Auxotrophic Wild Type	L-Lysine concentration Auxotrophic Mutant
1	96	30	6.0	14.78	16.50
2	96	30	6.5	15.02	18.17
3	96	30	7.0	16.35	22.16
4	96	30	7.5	17.02	24.32
5	96	30	8.0	16.21	19.17
6	96	30	8.5	13.23	17.29



Graph 1: A plot between Concentration of ENU exposed vs Concentration of L Lysine produced. The L-Lysine concentration showed an increased pattern with an increase in the concentration from 25 nm till 100 nm with a maximum yield of 23.28 g/l and decreased at 125 nm. concentration at constant values, time of Incubation 96 hrs., Temperature 30^0C and pH 7.5.

The amounts of L Lysine produced shown increased trend from 25 nm concentration up to a maximum amount of L Lysine achieved at 100 nm concentration of ENU 23.28 g/l which is increased from 20.66g/l represented in table 1 and plotted in the graph 1 by taking L Lysine concentration on Y-axis and Concentration of ENU on X-axis. The pattern of increase of L Lysine production shown illustrated in graph 1.

The effect of time of exposure of ENU on Recombinant C. glutamicum ATCC 13032: The ENU treated recombinant C. glutamicum showed maximum yield of L Lysine of 24.02 g/l for 20 mins of exposure of chemical mutagen at 100 nm of concentration were compared to different times of exposure chemical mutagen at 100 nm concentration for 5, 10, 15, 25 and 30 mins. The effect of ENU on the L Lysine is recorded maximum at 20 min of exposure before washing with citrate buffer in the process of creating the Auxotrophic mutant[4] from the recombinant ATCC 13032. The increased productivity of L Lysine Yield by taking the time of exposure of ENU is represented in the graph 2 by plotting the graph between Time of exposure of ENU on X-axis and Concentration of L Lysine on Y axis from the values of table 2 got for different times of exposure of ENU by keeping Time of incubation 96 hrs., Temperature 30 C and 100 NM Concentration of ENU and pH of 7.5 Constant. The trend of the graph increased from 5 mins of exposure till 20 mins of exposure recorded 20.22 g/l, 22.67 g/l, 23.17 g/l and 24.02 g/l for 5 mins, 10mins, 15mins and 20 mins of exposure to ENU respectively. The maximum yield of L Lysine achieved by the Auxotrophic mutant of ddh recombinant is 24.02 g/l and for Auxotrophic mutant of the wild type strain is 16.48 g/l at 20 mins of exposure of ENU. After 25 mins of exposure decreased the amount of L Lysine yield to 22.23 g/l and at 30 min of exposure, it was 21.56 g/l.



Graph 2: A Plot between Time of Exposure of 100 n M ENU and L. LysineYield. The L Lysine concentration showed an increased pattern with an increase in the Time of exposure of ENU from 5 mins. To 20mins. with a maximum yield of 24.02 g/l and 16.48g/l for Recombinant and wild type mutants respectively and decreased at 25 mins. of ENU exposure at a constant time of Incubation 96 hrs., Temperature 30^0C and PH 7.5.

Isolation of Auxotrophic Mutant: After three days of incubation, the strains that show growth in the presence of Methionine or Threonine or the presence of both the amino acids were considered as auxotrophic mutants and were isolated and cultured. Due to lack of homoserine dehydrogenase, the mutant strains utilize the Threonine and methionine supplemented in the media. The auxotrophic mutants require more quantity of threonine and methionine in their media signifies the lack of Homoserine pathway that could

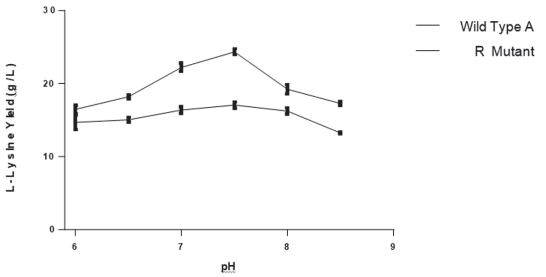
occur in the normal strains of both Wild type as well as ddh recombinant varieties of C. glutamicum ATCC 13032. These auxotrophic mutants were used further to know the optimized conditions of PH, Temperature and time of incubation.

The Chemical mutagen had shown its maximum activity at 100 nm by blocking the Homoserine dehydrogenase a key enzyme in the production of Threonine and methionine. The ENU caused mutation in Homoserine dehydrogenase gene which generally combines with Aspartyl B Semi aldehyde to produce the homoserine. This blockage in the Homoserine pathway favors the DAP pathway by combining with 2,3, Dihydrodi-picolinate synthase produced 2,3, Dihydrodi-picolinate which reduces to 2,6, a dicarboxylic acid.

Optimization of PH, Temperature and Time of Incubation for Chemical mutagen ENU treated *ddh* recombinant strain of *C. glutamicum ATCC 13032*:

Effect of pH on the Recombinant strain of *C. glutamicum*: The ENU concentration used to mutate the recombinant strain is 100 nm which was an optimized condition for high productivity of L Lysine was used which was treated on Recombinant strain for 20 mins. The recombinant strain has shown maximum lysine productivity of 22.16 g/l at 7.5 pHs when compared to ENU mutated wild type strain MTCC25069at the same 7.5 pHs. The Productivity of l lysine is increased considerably in the recombinant strain for all the Ph values when compared with wild type strain. But both the strains had shown maximum productivity at 7.5 pH.

The trend of production of L Lysine increased from PH 6.0 to 7.5 and shown decreased productivity for PH 8 and PH 8.5. The L Lysine yield recorded maximum at PH 7.5 with an L Lysine concentration of 24.32 g/l. 16.50 g/l, 18.17 g/l, 22.16 g/l, 24.32 g/l, 19.17 g/l and 17.29 g/l of L Lysine yield was reported for 6.0,6.5 7.0, 7.5, 8.0 and 8.5 PH values respectively represented in the table 3. The L Lysine yield recorded maximum at 7.5 PH with 24.32 g/l for Auxotrophic recombinant mutant and 17.02 g/l for an auxotrophic mutant of wild type C. glutamicum. The graph was plotted by taking PH values on the X-axis and Yield on the Y-axis and illustrated the trend of increase in the productivity of L Lysine in graph 3.

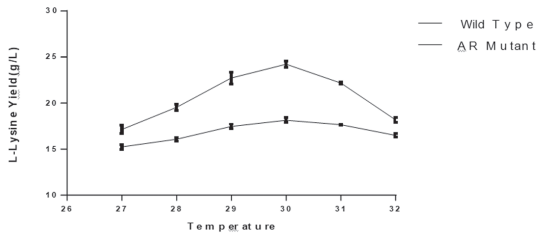


Graph 3: Plot between a PH of the Media and L Lysine Yield. The L Lysine concentration showed increased pattern with increase with the increase in value of PH, from 6.0 to 7.5 reached the maximum yield of 24.32 g/l L-Lysine and decreased for further increase in the PH by keeping time of Incubation for 96 hrs., Temperature 30^0C constant after treating with ENU (100 NM) with 20mins of exposure before washing.

The PH showed its impact on media utilization and glucose consumption by maximum uptake of glucose at 7.5 pH had supported the increased participation of the C. glutamicum in the Dap pathway for the L- Lysine synthesis. The PH had shown its impact on the fluidity of bacterial cell wall and Plasma membrane of the C. glutamicum at 7.5 which helped in the glucose consumption.

Effect of Temperature on the Recombinant strain of C. glutamicum: Here the strain treated with 100 Nm concentration of ENU which was exposed to ENU for 20 mins was tested. The incubation was kept for different temperatures and PH of 7.5 and time of incubation 96 hrs. was kept constant. The recombinant strain showed increased productivity of L Lysine with the maximum amount of L Lysine was 24.20 g/l at 30 C of temperature. All the recombinant strains

showed an increased pattern in the L Lysine production till 30 C and productivity decreased for 31C and 32 C of temperature. L Lysine yield of 17.11 g/l, 19.52 g/l, 22.78 g/l, 24.20 g/l, 22.16 g/l and 18.17 g/l for temperatures 27.0^{0}C, 28.0^{0}C, 29.0^{0}C, 30.0^{0}C, 31.0^{0}C and 32.0^0 C respectively was reported is represented in the table 4. The Auxotrophic mutant of ddh recombinant strain had shown the maximum L Lysine productivity of 24.20 g/l and wild type auxotrophic mutant shown 18.13 maximum L Lysine yield at 30^{0}C. The trend for the increase in the productivity of L Lysine is illustrated in graph 4 by taking the PH on the X-axis and L Lysine Yield on Y axis.

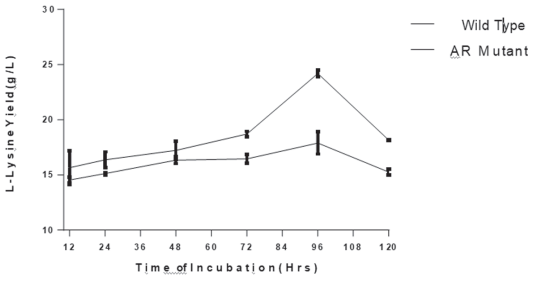


Graph 4: A plot between Change in the Temperature VS L Lysine Yield. The L Lysine concentration showed an increased pattern with an increase in temperature, from 27^{0}C to 30^{0}C reached the maximum yield of 24.20 g/l and 18.13 g/l L- Lysine respectively for Auxotrophic mutants of ddh recombinant and wild type and decreased for a further increase in the temp. by keeping the time of Incubation for 96 hrs., PH 7.5 constant after treating with ENU (100 NM) with 20 mins of exposure before.

The temperature had shown its significant effect on the rate of metabolism favoring the enzymes and substrates that participates in the synthesis of Amino acids. The temperature of 30^{0}C had favored the enzyme-substrate complex formations and showed its impact on the increased rate of reactions by reducing the activation energies of catalytic enzymes that were involved in the L Lysine pathway by keeping the Time, Volume and PH are constant.

3.2.3 effect of Time of incubation on ENU treated recombinant strain ofC. glutamicum: The recombinant strain has shown maximum productivity of 24.16 g/l after 72 hrs. and also shown increased productivity of when compared with ENU mutated wild type strain. Time of incubation is 96 hrs. for wild type strain which is also same for both Recombinant and ENU treated Recombinant strains of C. glutamicum MTCC25069. The Time of incubation signifies the growth kinetics of C. glutamicum. Further incubating the strain shown the decrease in the quantity of L lysine production due to the strain entering into death phase between 96 to 120 hrs. of incubation.

The L Lysine productivity increased while the time of incubation is being increased. The L Lysine productivity for 12 hrs.,24 hrs.,48 hrs., 72 hrs., 96 hrs., and 120 hrs. was 10.23 g/l, 13.21 g/l, 17.16 g/l, 18.68, g/l, 24.16 g/l and 18.17 g/l respectively represented in the Table 5 and plotted in graph 5 by taking Time of incubation on the X-axis and L Lysine yield on the Y-axis. The trend of the time of incubation increased from 12 hrs. to 96 hrs. reached the maximum L Lysine concentration of 24.16 g/l for Auxotrophic recombinant mutant and 17.98 g/l for Auxotrophic mutant of L Lysine further decreased to 18.17 g/l and 15.23 for Recombinant and Wild type mutants respectively at 120 hrs. of incubation.



Graph 5: A Plot between Time of Incubation for *ddh gene* Recombinant C. glutamicum and L Lysine yield. The L Lysine concentration showed an increased pattern with an increase with the increase in time of incubation, from 12 hrs. to 96 hrs. reached the maximum yield of 24.16 L and 17.98 g/l Lysine for Auxotrophic recombinant

TABLE 4: Table for L Lysine production at different temperatures of ENU treated ddh recombinant C.glutamicum.

S.No	Time of Incubation	Temperature	pH	Lysine concentration Auxotrophic Wild Type	Lysine concentration Auxotrophic Mutant
1	96	27.0	7.5	15.23	17.11
2	96	28.0	7.5	16.07	19.52
3	96	29.0	7.5	17.45	22.78
4	96	30.0	7.5	18.13	24.20
5	96	31.0	7.5	17.66	22.16
6	96	32.0	7.5	16.52	18.17

TABLE 5: Table for L Lysine production at different times of incubation of ENU treated ddh recombinant C.glutamicum.

S.No	Time of Incubation (hours)	Temperature (0^{o} C)	pH	L-Lysine concentration Auxotrophic Wild Type	L-Lysine concentration Auxotrophic Mutant
1	12	30	7.5	14.56	15.78
2	24	30	7.5	15.11	16.43
3	48	30	7.5	16.32	17.16
4	72	30	7.5	16.43	18.68
5	96	30.0	7.5	17.98	24.16
6	120	30.0	7.5	15.23	18.17

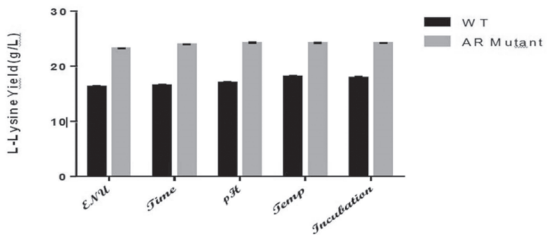
TABLE 6: Table for L Lysine production for ddh recombinant mutant and wild type mutant at the optimized parameters of ddh recombinant mutant.

S.no	Optimized Parameters	L Lysine in Wt.mutant (g/l)	L Lysine in AR Mutant(g/l)
1.	Conc. Of ENU (100 nM)	16.12	23.28
2.	Time of exposure (20 min.)	16.48	24.02
3.	PH (7.5)	17.02	24.32
4.	Temperature (30^{0}C)	18.13	24.20
5.	Time of incubation 96 hrs.	17.98	24.16

mutant and auxotrophic wild type mutant respectively and decreased for a further increase in the time by keeping temperature 30^0C., PH 7.5 constant after treating with ENU(100NM) with 20 mins of exposure before washing.

The Corynebacterium glutamicum had entered the stationary phase after the 96 hrs. of incubation is the reason for and production of L Lysine increased substantially after the logarithmic phase and reached the maximum amount of L Lysine production between 72 hrs. to 96 hrs. of incubation.

Comparative analysis of maximum Yields of L Lysine for Auxotrophic ddh recombinant mutant and Auxotrophic wild type mutant at Optimized Conditions: By optimizing the growth of ENU treated ddh Recombinant MTCC25069strain of C. glutamicum we got 23.28g/l of L Lysine and 16.2 g/l of L lysine for wild type auxotrophic mutant for 100 NM concentration of ENU. By treating the ENU (100 NM) concentration for 20 minutes we got the better yield of 24.02 g/l for ddh recombinant strain and 16.48 g/l for the Wild type. After isolating the Auxotrophic mutant for Threonine and Methionine after exposing to ENU (100 NM) for 20 mins of time of exposure of ENU before washing with Citrate buffer was checked for different PH, different Temperatures and for different times of incubation for ddh gene recombinant ATCC auxotrophic mutant of C. glutamicum and wild type auxotrophic mutant. we got a better yield of L Lysine of 24.32 g/L for the ddh Recombinant auxotrophic mutant and 17.02 g/l for wild type auxotrophic mutant at 7.5 PH. We got a better yield of 24.20 g/l of. Lysine for Auxotrophic mutant of ddh recombinant C. glutamicum and 18.13 g/l for wild type auxotrophic mutant at 30^0C of temperature and high productivity of 24.16 g/l and 17.98 g/l of L Lysine respectively after 96 hrs. of time of Incubation. Hence The best optimizing conditions for ENU treated ddh Recombinant mutant strain of Corynebacterium glutamicum shown for high yield of L Lysine at the 100 NM concentration of ENU with an exposure time of 20 mins created an Auxotroph which show a better yield of L Lysine at 7.5 PH, 30^0C of temperature and 96 hrs. of Incubation. The Optimized Parameters and amount of L Lysine Produced for Recombinant mutant and quantity of L Lysine produced by wild type auxotrophic mutant at the same conditions of recombinant strain was tabulated in table 6.



Graph 6: Maximum Yield of L Lysine for auxotrophic wild type mutant (WT) and auxotrophic ddh recombinant mutant (AR Mutant) based on Optimized parameters of Recombinant strain.

Molecular Docking analysis of Aspartyl â semi aldehyde with Homoserine dehydrogenase and 2,3 Dihydrodipicolinate Synthase:

Molecular docking analysis of Aspartyl â semi aldehyde ligand with Homoserine dehydrogenase protein: Homology model is developed in the SWISS-MODEL server for the amino acid sequence collected from UniProt [40]. The Protein model built in the Swiss model [36]. collected in the pdb format. The protein model is further analyzed in PDBsum[37]. database to check the protein quality for docking. The Ramachandran plot had shown nearly 91.5% of residues in the favorable regions and RMS distance from planarity is around 2 signified good protein quality. The ligand of aspartyl semi aldehyde collected from PubChem is used to dock in the PyREX [39] software with the Modeled Homoserine dehydrogenase protein. The ligand Aspartyl â semi aldehyde had shown its interaction with glycine GLY (151) with a hydrogen bond and also carbon-hydrogen bond with glycine GLY (288). Proline PRO (B: 287) Alanine ALA (B:289), tyrosine TYR (B: 178,155), Glycine GLY (B:268, 151), Asparagine ASN (B:270) and Leucine LEU (B: 154) are the interacting amino acids with the ligand in

Homoserine dehydrogenase. Homoserine dehydrogenase enzyme is with one conventional hydrogen bond. The Homoserine dehydrogenase showed a good interaction with the ligand Aspartyl â semi aldehyde with a binding energy of -5.2 Kcal. Homoserine dehydrogenase had nine interacting amino acids is the reason for having high binding energy even though having only a hydrogen bond and a Carbon-hydrogen bond.

Molecular docking analysis of Aspartyl â semi aldehyde ligand with 2,3 Dihydrodipicolinate Synthase: Homology model is developed in the SWISS-MODEL server for the amino acid sequence 2,3 Dihydrodipicolinate synthase collected from UniProt. The Protein model built in the Swiss model collected in the .pdb format. The protein model is further analyzed in the PDBsum database to check the protein quality for docking.

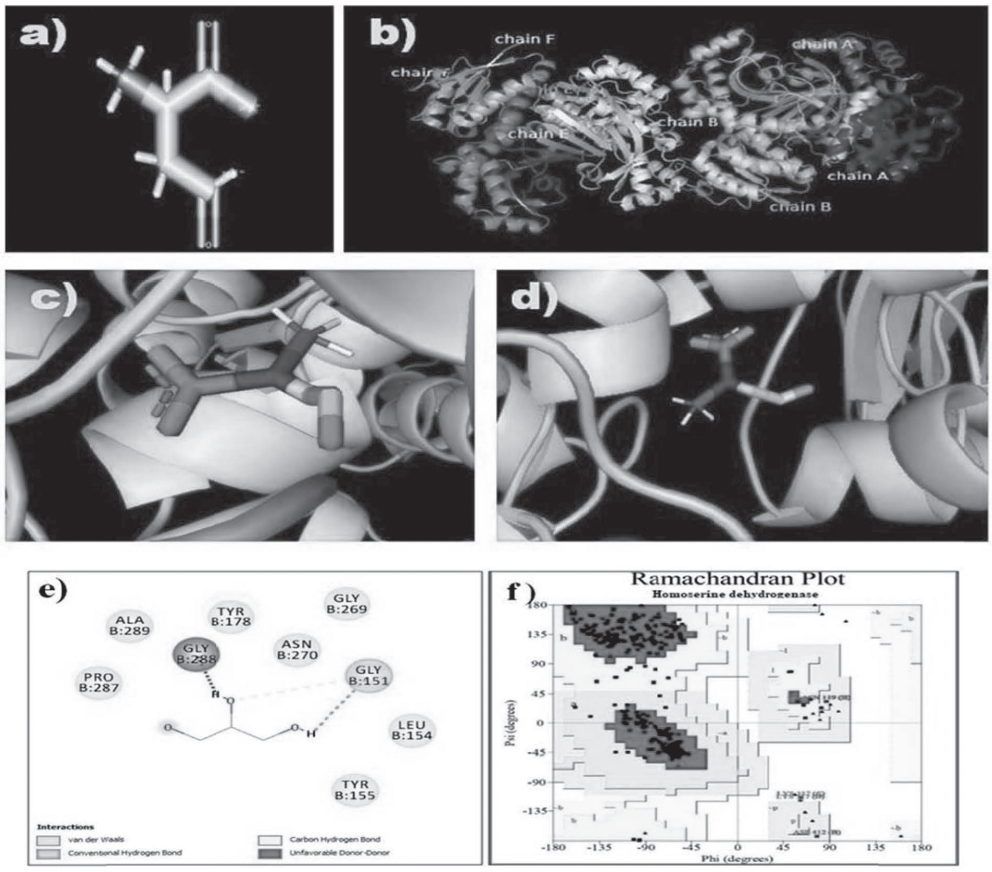
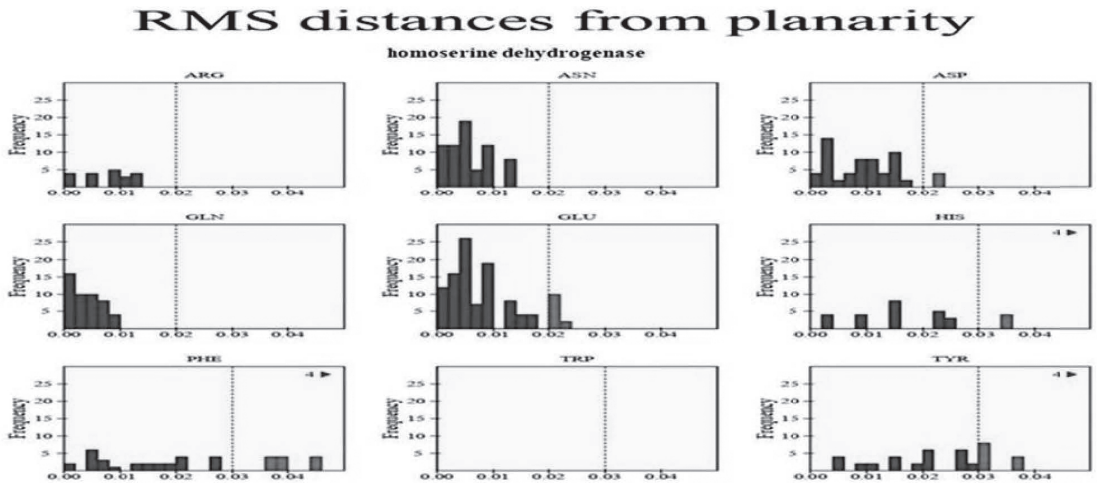


Fig: a) Ligand Aspartyl â semialdehyd e**)** Protein Homoserine dehydrogenase **c&d)** Protein-ligand interaction (Docking in Pyrex)**e)** lid plot analysis (PDBsum) of Ligand Aspartyl â semi aldehyde with Protein Homoserine dehydrogenase: GLY (151), H-bond and C-H bond with GLY (288). PRO (B: 287), ALA (B:289), TYR (B: 178,155), GLY (B:268, 151), ASN (B:270) and LEU (B: 154) are the interacting amino acids in protein Homoserine dehydrogenase with the ligand Aspartyl â semi aldehyde.**f).** Ramachandran plot for HSD with 91.5% favored regions, **h)** RMSD value of HSD between 1.5 to 2.0 for HSD signifying the good quality of Protein to be docked with the ligand.

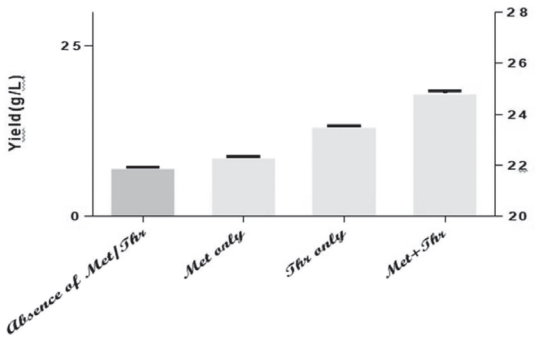


The Ramachandran plot had shown nearly 90.05% of residues in the favorable regions and RMS distance from planarity is between 1.5 to a signifying good quality of modeled protein. The ligand of aspartyl semi aldehyde collected from PubChem is used to dock in the PyREX software with the Modeled 2,3 Dihydrodipicolinate synthase protein. The ligand Aspartyl â semi aldehyde had shown its interaction with Leucine (B:206), Serine (B:261), Aspartate ASP (B:205) and Isoleucine (B:257) with a conventional hydrogen bond. 2,3, Dihydrodipicolinate synthase is with 4 conventional hydrogen bonds. Leucine LEU (B:206), Aspartate ASP (B:205), Serine SER (B:261) and Isoleucine ILE (B:257) were the interacting amino acids of 2,3, Dihydrodipicolinate synthase. The 2,3 Dihydrodipicolinate synthase showed a good interaction with the ligand Aspartyl semi aldehyde with a binding energy of -4.4 Kcal. Even though it had less amino acid interactions but due to the presence of 4 hydrogen bonds it can able to show considerable binding with the Ligand.

The aspartyl b semi aldehyde had shown higher binding energy of -5.2 k. cal with homoserine dehydrogenase with 9 interacting amino acids and two hydrogen bonds than 2,3 Dihydrodipicolinate synthase enzyme with - 4.5 k.cal of binding energy, 4 interacting amino acids with 4 Hydrogen bonds. As the chemical mutagen, ENU caused mutation in the homoserine dehydrogenase caused protein not to express or might have produced a protein which could not bind properly with Aspartyl B semi aldehyde diverted the Aspartyl semialdehyde to bind with 2,3 Dihydrodipicolinate synthase enzyme entered the dap pathway. As the binding energy is more for homoserine dehydrogenase enzyme it showed higher reactivity with the Aspartyl B semi aldehyde but lack of it caused the Aspartyl semialdehyde to bind with2,3 Dihydrodipicolinate synthase which directed to L -Lysine synthesis through producing L L diaminopimelate. Being a ddh recombinant strain the Corynebacterium glutamicum produced more ddh which deviated the 2,6 dicarboxylic acid away from reacting with acetyltransferase or Succinyl transferase to produce L Lysine.

The L Lysine yield in the newly developed auxotrophic mutant of ddh recombinant strain of Corynebacterium glutamicum MTCC25069 strain in the presence of Homoserine based amino acids:

Auxotrophic ddh recombinant strain in the presence of methionine and threonine had produced g/l of L Lysine, in the presence of Threonine only had produced 22.34 g/l and 23.54 g/l in methionine only and 22.92 g/l of L Lysine in the absence of both methionine and Threonine from the auxotrophic recombinant mutant.



Graph 7:**L Lysine yield of Auxotrophic mutant of ddh Recombinant strain of C. glutamicum.**

In the presence of both Methionine and Threonine, the L Lysine Yield is 24.89 g/l, in the presence of threonine only the L Lysine yield is 23.54 g/l, methionine only is 22.34 g/l and in the absence of Methionine and threonine is 21.92 g./l for the Auxotrophic mutant of ddh recombinant MTCC25069strain of C. glutamicum.

Discussion:

The ddh Recombinant mutant produced more amounts of L Lysine under because of effect chemical mutagenesis of ENU by blocking the *Homoserine dehydrogenase* of homoserine pathway diverting the aspartate â-semialdehyde to bind with 2,3 Dihydrodipicolinate synthase. The auxotrophic recombinant mutant had shown a yield 24.89 g/l L Lysine in the presence of threonine and Methionine.23.54, g/l in the presence of threonine only and 22.34 g/l in the methionine only and 22.92 g/l without threonine and methionine in the media signifies the mutation had occurred in the Homoserine dehydrogenase which participates in the Homoserine pathway of the auxotrophic mutant of ddh recombinant Corynebacterium glutamicum ATCC 13032. Binding energy for the protein homoserine dehydrogenase of – 5.2 K. Cal with one conventional hydrogen bond binds more strongly with -0.7 k.cal greater than 2,3 Dihydrodipicolinate synthase with -4.5 K.Cal with 4 conventional hydrogen bonds with aspartate â-semialdehyde. Due to the lack of Homoserine dehydrogenase in the auxotrophic mutant of ddh recombinant, the energy utilized in the synthesis of Threonine and Methionine by Homoserine pathway will be utilized in the L Lysine synthesis by binding with 2,3 Dihydrodipicolinate synthase to produce L Lysine through L L diaminopimelate (2,3 meso-DAP). Even the number of hydrogen bonds and interacting amino acids of the proteins play an important role in the diverting the Homoserine pathway to DAP pathway can be analyzed in the molecular docking analysis of Protein and ligand.

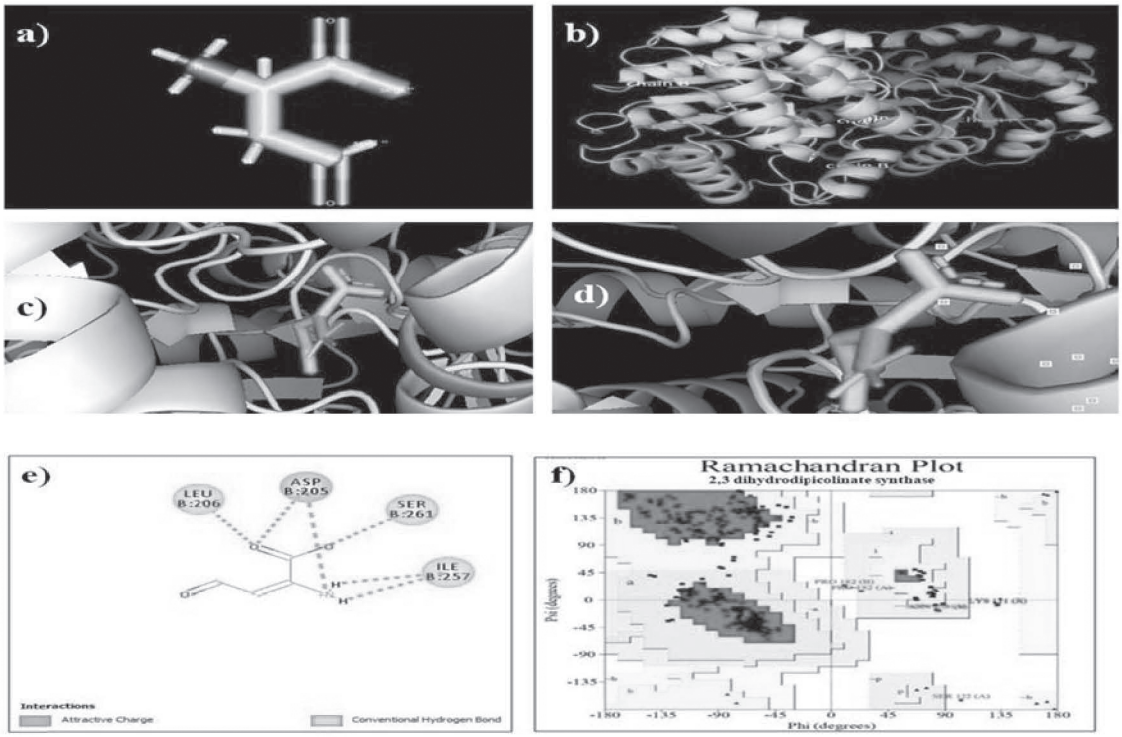
Being a recombinant for ddh gene, the recombinant mutant expressed more amounts of DDH enzyme which further diverts the strain from entering 3 enzymes involved pathways of acetyltransferase and Succinyl transferase to a single enzyme involved ddh pathway in the production of L L diaminopimelate (2,3 meso-DAP) for the production of High yield of L Lysine.

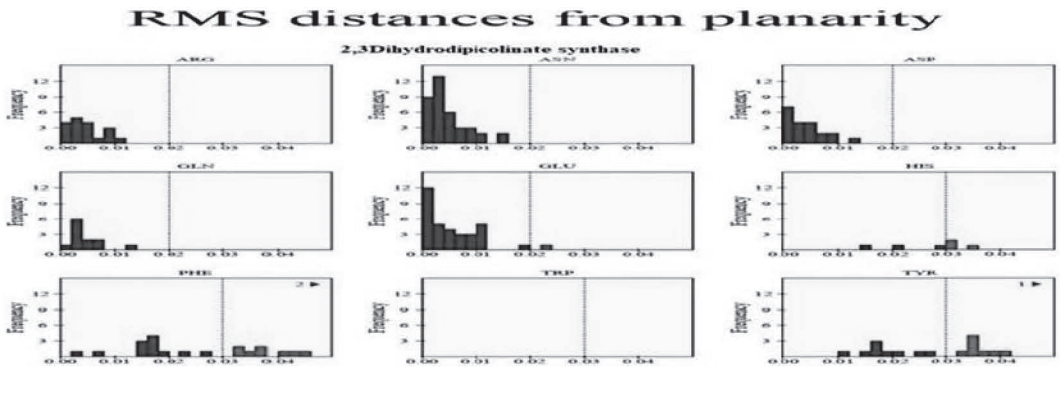
Table 7: Ramachandran plot statistics representing the quality of proteins used for Modeling

S.No	Plot statistics	Homoserine dehydrogenase		2,3 Dihydrodipi-colinate Synthase:	
1	Residues in most favored regions[A,B,L]	1124	91.5%	447	90.5%
2	Residues in additional allowed regions[a,b,l,p]	92	7.5%	42	8.5%
3	Residues in generously allowed regions [~a,~b,~l,~p]	8	0.7%	2	0.4%
4	Residues in disallowed regions	4	0.3%	3	0.6%
5	Number of non-glycine and non Proline residues	1228	100.0%	494	100%
6	A number of end-residues (excl. Gly and Pro)	8		4	
7	Number of glycine residues (shown as triangles)	92		24	
8	Number of Proline residues	48		18	
9	Total number of residues	1376		540	

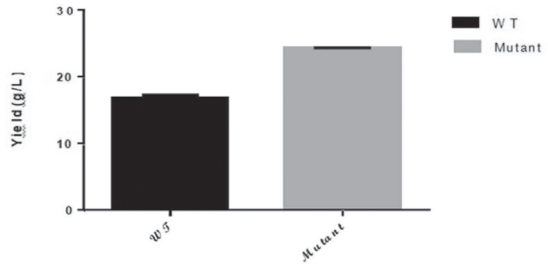
Conclusion:

By culturing the Chemically mutated ddh recombinant MTCC25069strain the L Lysine yield was increased to 24.89 g/l at optimized parameters of PH 7.5, temperature 30 C and 96 hrs. of time of incubation compared to an auxotrophic mutant of wild type strain with a yield of 18.57 g/l of L Lysine.





Improvement in the production of L-Lysne by ENU treated



Graph8: Yield of L Lysine of Auxotrophic mutants of ddh recombinant and Wild Type C. glutamicum at Optimized conditions of ddh Recombinant mutant. R- L Lysine in an auxotrophic mutant of ddh Recombinant C. glutamicum MTCC25069is 24.89(g/l). L -Lysine in an auxotrophic mutant of wild type C. glutamicum MTCC2506918.57 (g/l).

The wild type mutant and ddh recombinant mutant had Chemical mutagen ENU induced blockage in the homoserine pathway and overexpression of ddh gene directed DAP synthesis in ddh recombinant strain with the involvement of fewer enzymes compared with Acetyltransferase and Succinyl transferase pathways lead to increased production of L Lysine than auxotrophic mutant Wild Type of C. glutamicum. Nearly 4.23 g/l amount of L Lysine was increased in this newly developed Strain when compared with normal ddh recombinant strain which had a yield of 20.66 g/l L Lysine. The L Lysine Yield of Auxotrophic mutant of ddh recombinant is 6.32 g/l L Lysine Yield more than auxotrophic mutant wild type with a yield of 18.57 g/l L Lysine. In the case of Auxotrophic mutant of ddh recombinant strain shown the maximum yield in the presence of both threonine and methionine of 24.89 g/l L Lysine, 23.54 g/l L Lysine in the presence of threonine only, 22.34 g/lL Lysine in the presence of only methionine, and finally 22.92 g/l of L Lysine in the absence of both methionine and threonine. From this study, the amount of lysine production enhanced is discussed on the basis of molecular docking which further supported our results. Hence we developed an auxotrophic mutant from the ddh gene recombinant of Corynebacterium glutamicum MTCC25069which will be helpful in industrial L Lysine Production through this new Auxotrophic mutant.

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Conflict of Interest: The authors declare that they have no conflicts of interest.

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Extraction of phycocyanin from *Arthrospira* Sp. and screening of its antimicrobial potency against the selected pathogenic strains

T.C. Venkateswarulu[1], S. Krupanidhi[1], M. Indira[1], P. Sudhakar[2], P. Bangaraiah[3], Md. Nazneen Bobby[1], D. John Babu[1] A.Venkata Narayana[1] A.Ranganadha Reddy[1] K. Abraham Peele[1*]
[1,1*]Department of Bio-Technology, Vignan's Foundation for Science, Technology & Research, Vadlamudi-522213, India.
[2]Department of Bio-Technology, Acharya Nagarjuna University, Guntur-522510, Andhra Pradesh, India.
[3]Department of Chemical Engineering, Vignan's Foundation for Science, Technology & Research, Vadlamudi-522213, India.
*Corresponding author: karlapudiabraham@gmail.com

Abstract

Phycocyanin is mainly produced by *Arthrospira* Sp., which is cultured in open ponds. Algal screening for identifying anti-infectious agents is fundamentally novel attempt in present research scenario. *Arthrospira* Sp. produces the bioactive molecules that are rich in protein content. In this work, we report the extraction and partial purification of Phycocyanin using ammonium sulphate precipitation and dialysis. Further, the partially purified phycocyanin is tested for the antimicrobial property against the selected pathogenic strains and found highest zone of inhibition on *E. coli* (22 ± 0.6 mm).

Introduction

Phycocyanin is the heterodimer consists of á and â subunits, formed by the assembly of monomer units of phycobiliproteins and chromophores linked through thioether bond. The C-phycocyanin can be stable up to around 70 °C, with light absorbing nature ranging between 20 and 70 °C. Phycocyanin is the major component of the phycobiliprotein family used as nutrient and food dyes and even in cosmetics [1-2]. Cyanobacteria are photosynthetic micro-organisms captures CO_2 from atmosphere and have wide assortment of applications. Cyanobacteria have a wide scope of pigmented compounds like phycobiliproteins. Spirulina (*Arthrospira* Sp.) is one of the most **common types of cyanobacteria rich in phycobilins**. *Spirulina* is consumed as the most nutrient-rich food all over the world. *Spirulina* is a planktonic photosynthetic filamentous rod shaped cyanobacteria. The protein in *Spirulina* is highly usable, in which the Phycobiliproteins play a major role [3]. Phycobiliproteins (PBPs) are large water soluble supra molecular proteins include 40– 60 % of the total dissolvable protein in these cells [4]. Their strong antioxidant properties come from fluorescent and chelating nature that can trap the free oxygen radicals of reactive oxygen species (ROS). Phycocyanin from *Arthrospira* Sp. produces phycobiliproteins that have promising positive results in pharmaceutical, dairy and agriculture [5]. Phycocyanins possess low toxicity and have antimicrobial, anti-inflammatory, anti-cancer and immune system boosting function. There, the current research focused on phycocyanin extraction from *Arthrospira* Sp.and further, its antimicrobial potency has been tested on the selected pathogenic strains.

Materials and methods

Isolation of microalgae and extraction of phycocyanin : Water sample for microalgae isolation was collected from site that appeared to contain algal growth in a water pond near Chavavaripalem, Tenali, Andhra Pradesh, India. The water sample was transferred to a 250 mL conical flask containing Zarrouk's medium and then incubated under continuous illumination using white fluorescent light. The algal isolate was

preliminarily, identified as *Arthrospira* Sp., based on their morphology, further the algal isolate was inoculated in Zarrouck medium for extraction of phycocyanin. The biomass was harvested and kept for drying in direct sunlight for two weeks. One gram of *Arthrospira* Sp. dry powder was transferred into a 100 ml potassium phosphate buffer (0.1 M, pH 7). Cells were lysed in a sonicator for 10 minutes and the suspension was centrifuged at 12000 RPM for 20 minutes at 4 ^{o}C and then, the supernatant was recovered [6].

Partial purification : The partial purification of crude extract containing C-Phycocyanin was carried out using ammonium sulphate precipitation (60% saturation), followed by centrifugation in a cooling centrifuge (14000 rpm for 10 minutes at 4 ^{0}C). The pellet was resuspended in 25 ml of 0.1M phosphate buffer and the protein concentration was estimated by Bradford assay. The dialysis was also performed against phosphate buffer at 4 °C overnight and filtered through 0.45 µm filter. Absorption spectrum was determined as a ratio of A 620/280 nm for the confirmation of C-Phycocyanin.

Antimicrobial activity of C-Phycocyanin : The pathogenic strains were selected for testing antimicrobial activity of phycocyanin. The samples were spread on Muller Hinton agar plate and the wells were made, followed by the addition of partially purified C-Phycocyanin extract at different concentrations and incubated at 37 °C for 24 hours, the results of antimicrobial analysis were observed as zone of inhibition [7].

Results and discussion

Identification microalgae : *Arthrospira* Sp. was identified by its morphological characters using Fluorescent microscope at 40x magnification. Short fragments of hormogonia like cells can be seen (Fig. 1). The biomass production after 30 days of culture reached a protein concentration of 2,720 mg/L

Extraction of Phycocyanin Pigment

The C- Phycocyanin concentration (CPC) was estimated to be 79 µg/ml after partial purification and the purity was assessed as a ration of optical densities at 620 and 280 nm. Partial purification of Phycocyanin followed precipitation of crude extract using ammonium sulfate and the total protein concentration was 420 µg/ml. The OD of partially purified C-Phycocyanin concentration is 3.97 after dialysis. The crude extract containing phycocyanin and extract after dialysis are showed in Fig. 2 (a&b).

Antimicrobial activity : The Phycocyanin pigment showed maximum inhibitory activity against *E.coli* (Fig 3a & 3b). There was no activity observed in two fungal pathogens, *Asperigillus niger and Asperigillus flavius* (Table 1). The significant antimicrobial activity has been found against *Kliebsella pneumoniae, Proteus vulgaris, Pseudomonas aeruginosa, Shigella flexineri, Bacillus cereus* and *Staphylococcus aureus.* Antimicrobial activity of ciprofloxacin (10 ìg/ml) used as a positive control.

Conclusion

The phycocyanin is a natural blue pigmented protein that is effectively active against human pathogens. The present study confirmed that the partially purified phycocyanin able to inhibit the growth of bacterial pathogens effectively, hence it can be developed as a lead drug against pathogenic infections.

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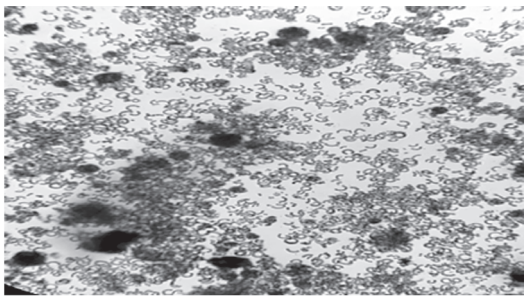

Fig. 1. Microscopic identification of *Arthrospira* Sp under Fluorescent microscope at 40X

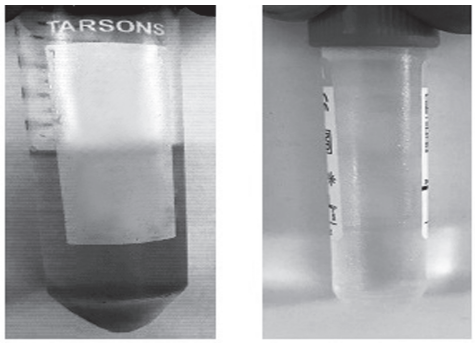

Fig. 2 (a) Crude protein sample after ammonium sulphate precipitation and (b) Partially purified phycocyanin after dialysis

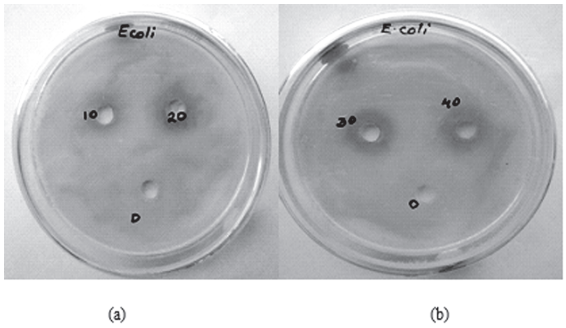

Fig. 3 (a) *E.coli* tested with 10 ìL and 20 ìL of partially purified phycocyanin and Fig. 3(b) *E.coli* tested with 30 ìL and 40 ìL

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Table 1 Antimicrobial activity of partially purified c-phycocyanin *(*10 µg/ml)

Microorganism	**C-phycocyanin(10 µg/ml)**	**Ciprofloxacin(10 µg/ml)**
	(zone of inhibition) (mm)	
Kliebsella pneumoniae	18 ± 0.6	24 ± 0.2
Escherichia coli	22 ± 0.6	26 ± 0.4
Proteus vulgaris	17 ± 0.6	23 ± 0.3
Pseudomonas aeruginosa	17 ± 0.7	21 ± 0.7
Shigella flexineri	16 ± 0.4	20 ± 0.5
Asperigillus flavus	—	12 ± 0.9
Bacillus cereus	20 ± 0.6	24 ± 0.4
Staphylococcus aureus	16 ± 0.7	22 ± 0.5
Asperigillus Niger	——	10 ± 0.4
Candida albicans	04 ± 0.2	16 ± 0.5
Candida tropicalis	06 ± 0.4	14 ± 0.4

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Novel Solidifying and Gelling Agents - Source, Properties and Prospective Application

Ami Patel*, Rashmi Gadhavi
[a]Assistant Professor, Division of Dairy Microbiology, Mansinhbhai Institute of Dairy & Food Technology-MIDFT, Dudhsagar Dairy campus, Mehsana-384 002, Gujarat state, INDIA.
*Corresponding author : amiamipatel@yahoo.co.in

Abstract

Some microbial and plant origin proteins and colloidal polysaccharides act as solidifiers or stabilizers for solid or semi solid media preparation. They form a continuous three dimensional molecular network and provide firmness to the culture medium. Gelatin and agar agar are widely employed as solidified agents in the preparation of microbiological media. However, few bacteria and fungi are able to degrade these complex compounds and thus, there is a need of alternative solidifying agents. Current text discusses development of newly discovered gelling agents, their source and properties, and prospective application as solidifying agent in the microbiological media preparation.

Key Words: *cassava starch; gellan gum; guar gum; pluronic polyol F127; solidifying agents*

Introduction

Growth medium or culture media are designed for the growth of cell (plant cell) or microorganisms. It contains all essential nutrients required to support the growth and multiplication of microbes and different microbial cells required different types of culture medium for their growth. Whole together, the microbiological media can be categorized in various ways, such based on their physical form (solid, semi solid or liquid), ingredients composition, and purpose or function of media. An undefined medium (also known as a basal or complex medium) usually contains a carbon source (such as glucose, starch, cellulose, etc.), a nitrogen source or source of amino acids, growth factors, various salts to fulfill the requirement of minerals, and most essentially water. In addition to these essential components, solid media contains a solidifying/ gelling agent. It is not possible to identify a specific microorganism in liquid media. Thus, for better detection and identification of microbial cell, especially bacteria it shall grow and cultivate on solid or semi solid media. The use of solid culture media has been of fundamental importance to microbiological research since the late nineteenth century. Earlier, Robert Koch introduced agar as a solidifying agent in culture media, since then it has become the primary material for solid media throughout the world.

Solidifying and Gelling Agents : Some microbial and plant origin proteins and colloidal polysaccharides act as solidifiers or stabilizers for solid or semi solid media preparation. They form a continuous three dimensional molecular network and provide firmness to the culture medium. Diffusion characteristics are also because of such solidifying agent however the diffusion rate is depending on viscosity of the medium which is varying with the concentration of solidifying agent. A characteristic feature of any solidifying agent is it would have physical state reversibility i.e. able to convert from solid state to liquid state and vice versa. In majority of the cases this attribute is based on the media temperature and several times it is based on the pH of the culture medium. A typical solidifying agent should be colorless, odorless, transparent, better moisture retainer, non-toxic, resistance to digestion by bacteria, lack of syneresis, able to form a reversible colloid, provide enough firm medium for streaking, spreading technique, inexpensive and easily available.

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Gelatin (the first gelling agent), agar agar (generally known as agar), xanthan gum, gellan gum, carrageenan, isubgol, guar gum, starch and pluronic polyol f127 are the example of solidifying agents which are used in the preparation of different growth media. Every solidifying agent has a specific range of pH and temperature for their optimum functioning and gelling. Due to lack of necessary biotic and abiotic factors, several mesophiles and extremophiles are difficult to cultivate (1). Now a day's many solidifying agents are available to work at different temperature, pressure and pH which can be useful to culture many new microorganisms including extremophiles. The chronological origin of various solidifying agents is diagrammatically represented in Figure 1. In the current text, we have discussed the origin, typical characteristics, merits and demerits, and use of different solidifying agents. Further, we have also compiled the information of newly discovered ingredients with their prospective application as gelling agents in the preparation of microbiological media.

1. Gelatin: Gelatin is animal origin gelling agent; it is extracted from collagen of animal bones and skin. Gelatin word is come from Latin word "gelatos" means "jellied froze". It was first introduced by Denis Papin, a Frenchman in 1682. It is obtained from baking/boiling of animal bones and skin. It 1754 its first patent was submitted in England and another in 1845 by Peter Cooper an industrial powdered gelatin mix was patented (US Patent 4084). It has no colour and odor so suitable for culture media preparation. It generally melts at 35 degree Celsius and solidifies at low temperature (25°C) based on the concentration; other characteristics have been summarized in Table 1.

2. Agar Agar: It is the most widely employed solidifying agent. It was first introduced in microbiology by a German microbiologist Walther Hesse in 1882 with the suggestion of his wife Fannie Hesse. Then after, very fast gelatin was replaced with Agar in microbiology work.

Table 1. General characteristics of Solidifying Agents

Agent	Year of Discovery	Approximate concentration* as compared to Agar	Melting Temperature *in f°C	Solidifying Temperature * in f°C	Cost for 100 gm* in INR
Gelatin	1881	1.8-4.5 %	>35	d" 25	200-300
Agar Agar	1882	0.8 to 2.0 %	>85	<40	400-500
Xanthan Gum	1995	1 to 2 %	>270	<50	100-125
Gellan Gum	1978	0.55 to 1.0 %	>110	<40	
Carrageenan	1977	2 to 3 %	>50	<25	80-100
Isubgol husk	1997	3 %	>100		100
Guar Gum	2005		>220	<70	50
Starch		10 %	>55-80	<37	80-100
Pluronic polyol F127		18-50 %	Solid at high temperature and liquid at low temperature; above 10°C stable gel can be formed		

*Melting and Solidifying temperature varies with concentration

It has jelly like structure and derived from cell wall of red algae, *Gelidium*, *Gracillaria* and *Pterocladia* known as agarophytes (*Rhodophyta*) phylum released on boiling. Agar is a mixture of heterogeneous galactans, mainly composed of 3,6-anhydro-L-galactoses (or L-galactose-6-sulfates) D-galactoses and L-galactoses (routinely in the forms of 3,6-anhydro-L-galactoses or L-galactose-6-sulfates) alternately linked by â-(1,4) and á-(1,3) linkages. Initially, commercial production of agar was only at Japan until World War II. In World War II many countries faced the scarcity of obtaining agar; thus, forced to establish domestic agar industries for use in research work.

Agar melts at 85°C and solidifies at 32-40°C therefore it is ideal for wide temperature range of bacterial growth. Agar has all necessary characteristics which a solidifying agent shall have to use as a culture media, such as it is transparent, indigestible by majority of bacteria and fungi, stable, and non toxic. However, it does not get properly solidify at low pH and many agarolytic microorganisms that can hydrolyze and metabolize agar as a carbon and energy source have been identified in seawater and marine sediments. Several marine bacteria (like *Vibrio* sp.) yeasts as well as few actinomycetes are able to produce á-agarase, â-agarase, and â-porphyranase (2). It is commercially used in dessert preparation as well as mainly for microbial culture medium solidifying agent.

3. Xanthan Gum: Xanthan gum is produced by plant pathogenic organism *Xanthomonas campestris.* It is a pentasaccharides made up of D-glucosyl, D mannosyl and D-glucuronyl acid residues. Few bacteria can degrade Xanthan gum i.e *Verrucomicrobium* sp. GD, salt tolerant Bacillus species, and Gram positive red pigmenting bacteria. It has soft texture and good thickening ability it is generally used as viscosifier in industry.

4. Gellan Gum: It is produced by bacteria *Sphingomonas elodea* (industrially, *Sphingomonas paucimobilis*). It is water soluble exo-polysaccharide. Industrially production of Gellan gum is depends on many factors including temperature, pH, oxygen transfer, stirring rate, and composition of the fermentative medium.

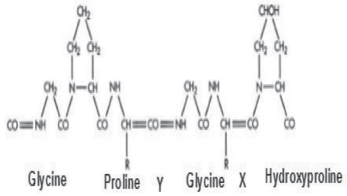
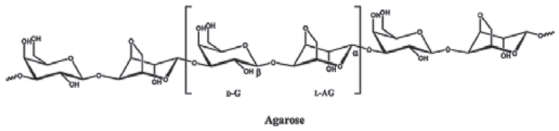
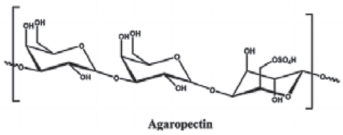
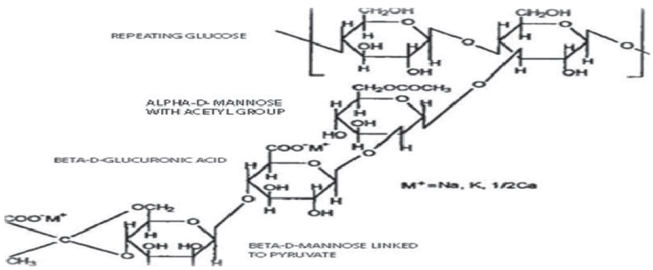
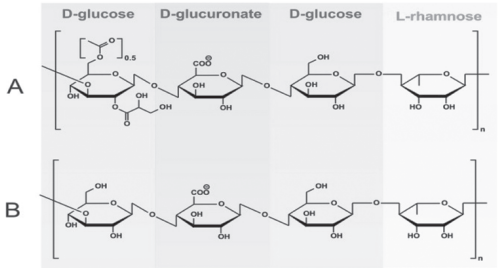
Gellan gum solidifies faster with higher clarity as compared to agar. Commercially it is available with different names as GELRITE and KELCOGEL. It is a good solidifying agent for the growth of thermophilic bacteria including *Methanobacterium* sp., and *Methanobrevibacter* sp. However, some bacterial species can degrade Gellan gum like *Verrucomicrobium* sp. GD, *Paenibacillus* sp., salt tolerant *Bacillus* species, and Gram positive red pigmenting bacteria. It has some other industrial applications also like dental and personal care products as well in capsules, films and fibers production.

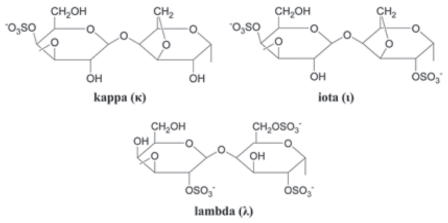
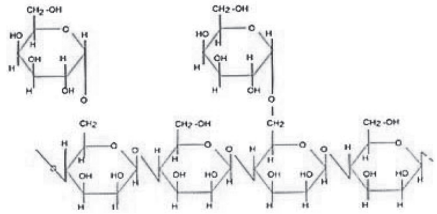
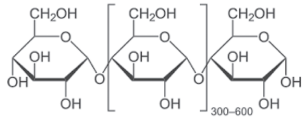
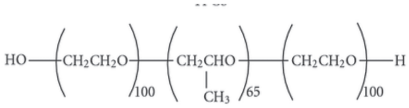
5. Carrageenan: It is extracted from the cell wall of marine algae *Chondrus crispus.* It is also used as agar replacer due to gelling ability (especially K salt of carrageenan). It has limitations for the growth of several marine bacteria, which can degrade carrageenan i.e. *Pseudoalteromonas carrageenovora* , *Alteromonas fortis*, *Zobellia galactanivorans*, a marine flavobacterium species *Delesseria sanguinea*.

6. Isubgul husk/Psyllium: It is plant origin gelling agent derived from seeds of *Plantago ovate* from *plantaginaceae* family. It is popularly known as Psyllium. It is generally used as a solidifying/ gelling agent in plant tissue culture. It has high viscosity at high temperature. Mucilage of Isubgol husk is colloidal and polysaccharide in nature, composed of Xylose, arabinose, galactouronic acid, rhamnose and galactose.Two polysaccharide fractions have been separated from the mucilage. One fraction is soluble in cold water and upon hydrolysis yields D-xylose (46%), aldobiouronic acid (40%), L-arabinose (7%) and an insoluble residue (2%). The other fraction is soluble in hot water, forming a highly viscous solution which sets to a gel when cooled and yields upon hydrolysis D-xylose (80%), L-arabinose (14%), aldobiouronic acid (0.3%) and traces of D-galactose (3).

7. Guar Gum: It is plant origin gelling agent composed of exo-polysaccharide. It can be used

Table 2. Structure or Building component of Solidifying Agents:

Agent	Structure / Building Component
Gelatin	
Agar Agar	 
Xanthan Gum	
Gellan Gum	

Carrageenan	
Isubgol husk	Contains Xylose, arabinose, galacturonic acid and traces of rhamnose and galactose in the structure
Guar Gum	
Starch	
Pluronic polyol F127	

to grow various fungi and bacteria. It has higher melting point up to 220°C so can be used for maintenance and isolation of thermophilic organisms. However, limitation for guar gum degrading bacteria as *Bacteroides ovatus* found in human colon (4). Other strains also capable to produce extracellular enzymes and degrade guar gum i.e *B. variabilis* and *B. uniformis*. If such extracellular enzymes are present in media other bacterial strains like *B. distasonis* and *B. thetaiotaomicron* can ferment guar gum.

It has high mobility and alone cannot make a stable gel so it shall be mixed with other gelling agent to form a stable gel. Addition of cations or borax to guar gum can improve the gelling properties. Use of guar gum is restricted due to its inefficiency of self gellation and its high viscosity at higher temperature, which renders the dispensing of media to the petri plate difficult (5). It has less clarity due to the presence of impurities. In addition, due to its high adhesive property, it also hampers the isolation of microorganisms from a culture medium (6).

8. Starch: It is a plant origin gelling agent. It is obtained from various sources i.e. corn, barley, potato, wheat rice and tapioca (Cassava Starch). It is either used alone or with other gelling agent for better output.

Cassava starch: Cassava starch is an acidic polysaccharide consisting in its powdered form of 77% carbohydrate, 21% lipid and 2% protein (7). A 10% cassava starch give satisfactory setting

typical of solid nutrient within 30 min to 1 h in both plates and slants. The method of autoclaving used in sterilizing the aqueous starch mixture was found to enhance its gelling quantity. The gelling property appeared to improve with age both in the hot air oven at 37-40°C and at room temperature up to six months. Both sets of plates at pH 7.3 and 3.9 did not support growth of *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeroginosa*, *Salmonella typhi*, *Aspergillus niger* and *Aspergillus flavus* (8)

9. Pluronic polyol F127: Pluronic polyol F127 a block copolymer of polypropylene oxide and ethylene oxide, the polymer chains of which form hydrogen bonds with water molecules, provided the temperature is low. As the temperature rises, the energy in the water breaks these bonds and the larger and less kinetically active polymers then bond to each other, forming a gel. It may be used to solidify culture media for the enrichment, isolation and growth of micro-organisms. A stable gel is formed at temperatures above 10°C, the exact temperature depending on the concentration of polyol used. The most important feature of the polyol gel is that it liquefies as th5e temperature drops below the critical value for the concentration used. The gel may be autoclaved, and will alternate repeatedly between liquid and gel form. It does not appear to be toxic to aquatic bacteria, is more transparent than agar and may be used to isolate heat-sensitive organisms (9).

10. Other Solidifying Agents: There are several other sources that can be explored for gelling agents. It is suspected that locust bean gum or carob gum, a galactomannan obtained from the seed endosperms of carob tree (*Ceratoniasiliqua*), which has a wide range of applications in food (10) and pharmaceutical industries (11,12) might find application as a gelling agent as well. Locust bean gum forms a viscous aqueous solution at relatively low concentration, which stabilizes emulsion and replaces fat in many food products. This unique characteristic makes it a very useful industrial gum. It is also non-ionic in nature and hence, solutions of locust bean gum are not influenced by pH, salts and heat treatment (13). Through its synergistic actions with hydrocolloids such as carrageenan and xanthan gum, locust bean gum forms a gel with more elasticity and strength (14,15). It has also been reported that it supports the growth of pluripotent embryonic stem cells in an undifferentiated state in mouse which makes it viable and a non-animal derived alternative to other gels (16).

Katira gum, a colloidal polysaccharide obtained from the bark of *Cochlospermum religiosum* is used for plant cell culture. It is transparent but its viscosity is significantly less than agar (17), suggesting that it can be likely used in combination with agar. Welan gum, synthesized from *Sphingomonas* sp. (18), used in cement industry (19), might also turn out to be a potential gelling agent. Moreover, starch sources from chickpea dextrose tapioca, corn, barley, potato and wheat have also been used as solidifiers for plant tissue culture (20-23) but have not been explored as gelling agents in microbial media.

Among other gelling agents who have been used for specialized applications are methyl cellulose (24), Polycell cellulose paste (25) and alginate gel (26). Semi-solid gels (27) and the introduction of precipitates such as A1PO4 (28) have also contributed towards the successful isolation of bacteria. Pluronic polyols have been used as defoaming agents, binders, stabilizers and gelling agents in agriculture, medicine, cosmetics, and the textile and food industry.

Future Prospect and Conclusion : Several published alternatives of agar have not been studied very extensively for their properties. In fact, several physicochemical properties of different gums remain to be studied. Also the diversity of microorganisms that can be grown on them has been relatively unexplored. This might be achieved by using different gelling agents in combination than a single gelling agent. For example, a blend of Xanthan gum and agar in the ratio of 6:4 is recommended as an alternative to agar because of its suitability comparable to agar and cost

Table 3 The source, advantages and their limitations of different solidifying agents

Solidifying agent	Source	Advantages	Limitation	Microorganism able to degrade
Gelatin	Collagen present in animal skin and bones	Clear, stable, non toxic and unable to digest by most organisms in culturing (except some organism)	It does not work on incubation higher than 25°C; it is hydrolyzed by proteolytic organisms able to produce gelatinase enzyme.	*Serratia marcescens, Bacillus sp., Staphylococcus* sp., *Algibacter lectus,* and majority of fungi
Agar Agar	Extracted from species of red algae genera *Gelidium, Gracillaria* and *Pterocladia.*	Highly stable, clear, non toxic and non degradable by most of organisms; used over a wide range of temperature	It is not suitable for thermophilic organisms. Not useful for agar degrading bacteria so called agarolytic microorganisms commonly produce agarases	*Vibrio* sp., *Agarivorans albus, Saccharophagus degradans, Streptomyces coelicolor* and other actinomycetes
Xanthan Gum	Bacteria *Xanthomonas campestris*	Cheap source of gelling agent	Unable to form a good stable gel alone so required to be used in combination with other gelling agent	*Verrucomicrobium* sp. GD, salt-tolerant *Bacillus* species,& Gram Positive red pigmentingbacteria
Gellan Gum	Aerobically from bacteria: *Auromonas (Pseudomonas) elodea* renamed *Sphingomonas paucimobilis*	Less quantity required as compared to agar; Easy judgment of various colonies on the plate	Some species of bacteria are able to degrade Gellan gum	*Verrucomicrobium* sp. *GD, Paenibacillus* sp.
Carrageenan	Obtained from the cell wall of marine algee: *Chondrus crispus*	Remains stable at high pH, so suitable for the growth of alkaliphiles	Does not suitable for Carrageen degrading bacteria	*Pseudoalteromonas carrageenovora*
Isubgol husk	Plant origin: *Plantago ovate*	No cracking or drying problem in plates on incubation; cheaper than agar; used in plant tissue culture media	Less effective as a solidifying agent than agar	Not reported
Guar Gum	Guar bean (leguminous plant *Cyamopsis tetragonoloba)*	It is a cheap alternative to agar; can be used for maintenance and isolation of thermophilic organisms.	It has inefficiency of self gellation	*Bacillus sonorensis, Clostridium butyricum, Bifidobacterium* sp., *Lactobacillus* sp.
Starch	Barley, corn, potato, rice, wheat, Cassava	It is not available as nutrient for microbial use.	Higher concentration is required for stable gel	*Algibacter lectus, B. subtilis, Aspergillusniger*
Pluronic polyol F127	Pluronic polyol F127 a block (Registered Product of BASF Canada).	More transparent than agar; use for heat sensitive bacteria and it is ideal for the growth of aquatic bacteria	Some fungi can metabolize Pluronic Polyol F127	Not reported

Novel Solidifying and Gelling Agents-Source

Table 4 Details of reference study available on solidifying agents

Agent	Major findings of the research work	References
Xanthan Gum	Eight bacteria and eight fungi were grown on media solidified with either agar (A, 1.5%), xanthan gum (X, 1%), or combinations of (0.9% X + 0.1% A, 0.8% X + 0.2% A, 0.7% X + 0.3% A, 0.6% X + 0.4% A). All fungi and bacteria exhibited normal growth and differentiation in all these treatments. Rather, growth of most of the fungi was better on xanthan (alone) and xanthan + agar media than agar medium.	Babbar and Jain, 1998 (29)
Gellan Gum	The gellan gum containing medium supported the growth of all 18 leptospiral strains studied. The colonial growth in gellan gum plating medium of six representative strains was consistent in colonial growth on agar plating media. In addition, gellan gum medium appeared to be an excellent medium for the recovery of leptospires from the blood, liver, and kidneys of hamsters experimentally infected with a virulent *Leptospira interrogans* serovar *bataviae* strain.	Rule and Alexander (30)
Carrageenan	No differences were observed in the firmness of the media plates containing carrageenan (2.0%) and media plate containing agar (1.5%). After overnight incubation at 37°C the patched colonies grew similarly on both master plates; in fact, the plates with carrageenan were clearer and the colonies were more readily distinguishable.	Watson and Apirion (31)
Isubgol husk	The suitability of 'Isubgol husk' as a gelling agent was investigated for shoot formation. The time of initiation of shoots (4 weeks) and their subsequent growth were comparable on agar and 'Isubgol'-gelled media.	Khan et al., (32)
Guar Gum	All fungi (12) and bacteria (11) exhibited normal growth and differentiation on the media gelled with guar gum. Microscopic examination of the fungi and bacteria grown on agar or guar gum gelled media did not reveal any structural differences. However, growth of most of the fungi was better on guar gum media than agar, and correspondingly, sporulation was also more advanced on the former. Bacterial enumeration studies yielded similar counts on both agar and guar gum.	Jain et al., (33)
Starch	A 10% cassava starch gave satisfactory setting typical of solid nutrient within 30 min to 1 h in both, plates and slants; gelling property appeared to improve with age both in the hot air oven at 37-40°C and at room temperature up to six months. Cassava-starch was not degraded as nutrient by *Staphylococcus aureus, Escherichia coli, Pseudomonas aeroginosa, Salmonella typhi, Aspergillusn niger* and *Aspergillus flavus* both at pH 7.3 and 3.9, a necessary attribute of any potential solidifying agent in nutrient media.	Dabai and Muhammad (7)
Pluronic polyol F127	Polyols formed a gel which was clearer than that obtained with agar; the bacterial colonies which developed were more discrete than those obtained on agar and spreading colonies were not observed on the gel surface. When CPS medium (Collins & Willoughby, 1962) was solidified with agar (held at 45°C and cooled rapidly) pour plate counts of bacteria ranged from 2.5 to 5.6 x 10^3 ml^{-1}; those in polyol ranged from 4.5 to 8-1 x 10^3 ml^{-1}.	Gardener and Jones (8)
Pluronic polyol F127	Majority of the fungi and bacteria grew as well in 20% polyol as in 1.5% agar media; conversely, various species of nematodes and plant seedlings or tissues exhibited differential sensitivities to different concentrations of the polyol.	Ko and Van Gundy (34)

advantage (35). Alternative gelling agents like cassava flour, rice flour, corn flour, and potato starch in combination with agar is considered to be suitable for *in vitro* root regeneration (36) and that they might have potential in microbial culture media, too. The blending different gelling agents like guar gum, xanthan gum or isubgol with agar increases the viscosity and firmness of the media (35).

Moreover, some hope for the speedy discovery of new gelling agents to cover the pH and temperature ranges that are not covered by the existing gelling agents. Work is required to find the reasons inhibition of certain microorganisms, if any, by the existing gelling agents and modifications to gelling agents to prevent their degradation by select group of microorganisms. Further research in the field is required to uncover newer insights of culturable microbes. In summary, there needs more progress in gelling agents to expand the range of culturable microbes and improve the quality of media.

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Modeling and Optimization of L-Asparaginase production from novel *Bacillus stratosphericus* by soft computing techniques

Madhuri Pola, Chandrasai Potla Durthi, Satish Babu Rajulapati*
Department of Biotechnology, National Institute of Technology Warangal, Telangana, India
*Corresponding author : satishbabu@nitw.ac.in

Abstract

Feed forward Artificial Neural Network (ANN) model and global optimization by Genetic Algorithm (GA) were employed on significant variables to enhance the production of L-Asparaginase from *Bacillus stratosphericus*. Using the experimental data, network was built with 4 inputs and 2 outputs along with 10 hidden neurons. Levenberg-Marquardt back propagation algorithm was employed to study the interactions between variables and their influence on L-Asparaginase and L-Glutaminase activity. The predicted enzyme activities were compared with the experimental data. The R^2 value was found to be 0.99419 from ANN and it was higher compared to Response Surface Methodology (RSM). GA optimization was employed on the quadratic equation obtained from RSM studies to find optimal solution. The optimal concentrations of L-Asparaginase and L-Glutaminase obtained from GA were 29.68 IU/ml and 0.12 IU/ml respectively. The optimal process variables were found to be Incubation time-55h, pH- 6.0 and Temperature-24°C and L-Asparagine-2.5 g/L.

Keywords: Artificial Neural Network; Genetic Algorithm; Global optimal solution; RSM; Statistical optimization.

Introduction

Diverse ranges of microbes produce different bioactive compounds which has medicinal as well as economic importance [1]. They were able to produce both extra-cellular and intra-cellular products [2]. Extra-cellular products are more advantageous because of easy downstream processing [3]. This study focused on extra-cellular L-Asparaginase production from endophytes of medicinal plants [4].

Tumour cells for their energy supplement depend on the external L-Asparagine, a non-essential α-amino acid with in the blood [5]. L-Asparaginase acts on free L-Asparagine catalysing L-Asparagine into L-Aspartic acid and ammonia [6]. The L-Aspartic acid formed as the result of enzyme catalysis cannot be used by the Cancerous cells. Hence, due to lack of energy supplement the Cancer cells death takes place [7-9]. L-Asparaginase is used in combination along with chemo therapy to treat Acute Lymphocytic Leukemia (ALL) and lymphosarcoma [10, 11]. Acrylamide formation due to Millard reaction of fried and baked foods can be prevented by pre-processing them with L-Asparaginase treatment before cooking [12, 13]. Currently Acrylaway (Novozyme) and preventASe are the FDA approved L-Asparaginases used in food industry, produced from *Aspergillus niger* and *Aspergillus oryzae* respectively [14]. The FDA approved Elspar and Erwinase are the native forms of L-Asparaginase [15-17] and PEG L-Asparaginase (modified *E.coli* L-Asparaginase) were used to treat cancer in pharmaceutical industry [18, 19]. Commercially available L-Asparaginases have drawbacks due to non-host interactions, drug resistance and the L-Glutaminase activity of L-Asparaginase [20, 21].

To reduce the side effects of the commercially available drugs, there is a need to identify the source which has capability to produce higher quantity of L-Asparaginase with minimal side effects [22].

Of all the biological enzymes produced by the industries for the use in various food and pharma industries, production of L-Asparaginase has gained importance in an economical manner. Kidd in 1953 reported L-Asparaginase in rat lymphomas [6] [23]. Both intracellular Type –II and extracellular Type-I bacterial Asparaginases has been identified [24]. Occurrence of L-Asparaginase has been reported in bacteria [25] (*E.coli, Erwinia sp., Bacillus sp., pseudomonas sp., Enterobacter sp*.), plants, animals, yeast etc [26, 27].

Enzyme technology helps us to screen the microorganisms which were able to produce L-Asparaginase [28]. Many optimization methodologies were developed by researchers to improve the production studies in an economical manner [29-33].

In this study, endophyte from *Ocimum tenuiflorum* was screened using M-9 media by rapid plate assay technique. The microorganism was identified as *Bacillus stratosphericus* [25]. Blind optimization followed by Stastical optimization using Response Surface Methodology (RSM) were studied [34].

ANN is based on parallel computing inspired by neurons in the brain and it has applications in many fields like manufacturing, telecommunications, electronics etc. whereas, genetic algorithm (GA) was employed to find the global optimal solution by employing the concepts of Darwin's theory of natural selection. Four variables i.e. Time, Temperature, pH and L-Asparagine were chosen to develop neural network model and to study multi objective GA optimization.

Materials and Methods

Endophyte isolation and culture conditions : Due to the medicinal values, *Ocimum tenuiflorum* was chosen for isolation of L-Asparaginase producing endophytes [34]. M-9 rapid plate assay and 16S rRNA sequencing [25] studies confirmed the L-Asparaginase producing endophyte as *Bacillus stratosphericus* [25]. The endophyte *Bacillus stratosphericus* was chosen because it produced maximum pink zone around the colony. Cultivation, submerged fermentation studies were conducted using M-9 media, Nesslerizartion studies were performed at regular intervals on the crude sample to estimate the L-Asparaginase and L-Glutaminase activities [34]. To develop ANN model the RSM data was taken and the regression co-efficient values for both RSM and ANN were compared. In addition the equations (L-Asparaginase and L-Glutaminase) generated from the RSM ANOVA acts as precursors to perform GA optimization.

ANN modelling

Developing model by ANN for nonlinear biological reactions has gained importance [35]. ANN is based on the human artificial intelligence which tries to mimic the human brain to develop model for the data supplied [36]. All the data supplied to the ANN were stored in the form of nodes [37, 38]. Input nodes feed the data to the network and the hidden neurons process the data by employing tan sigmoid function [39]. The processed hidden layer data was transferred to the output layer [40] with the linear transfer function to process the data and the final results were displayed at the output node [41]. Levenberg-Marquardt algorithm [42] was employed to minimize the error between the results obtained from RSM and predicted result from ANN by adjusting the weights [43]. The data supplied to the network was divided into training, test and validation [44-46].

In the current study, non-linear interactions between 4 input variables Time (30-60 h), pH (5.0-7.0), Temperature (20^{o}C-30^{o}C) and L-Asparagine (1-3 g/L) and 2 output variables (L-Asparaginase and L-Glutaminase activity) were taken from the stastical optimization-RSM and the limits for the input variables were shown in Table 1 [34] . The multilayer perceptron with 18 neurons (4-10-2-2) was built to train and simulate the network. The

regression co-efficient values from the RSM generated model and ANN generated model (Table 2) were compared and analysed.

Genetic algorithm optimization

GA was developed based on the Darwin's Survival of fittest theory finds the global optimal solution with in the desired space. [47] [48]. The Data points were considered as chromosome bit strings [49]. Each point on the chromosome represents gene and the solution generated is called population [50].

From Darwin's evolutionary concepts, genetic operators were employed for a specific population to get optimal solution [51, 52]. The genetic operators chosen were:

a. Selection : Best fit individuals from the given population were chosen as parents to next generation until best fit population was attained [53].

b. Crossover : Recombination of the data will takes place between the individual chromosomes to generate a new generation with better characteristics and fitness values [54, 55].

c. Mutation : Mutation leads to alter the genes at random to generate new solutions.

From the RSM studies [34], the ANOVA equations obtained from the RSM studies with 4 input variables Time (30-60h), pH (5.0-7.0), Temperature (20°C-30°C) and L-Asparagine (1-3 g/L) fed as input to the multi-objective genetic algorithm tool by using Matlab software. The limits of the variables chosen were based on the previous experiments (Table 2) [34]. The main objective of employing GA was to maximize L-Asparaginase production with minimal L-Glutaminase activity.

RSM ANOVA equations were supplied to GA for global optimization as follows

- y1=-374.98043+ 2.15508*x(1) +3.51766* x(2)+99.95797*x(3) +5.99492*x(4)-0.012023 *x(1)*x(2)-0.013862*x(1) *x(3)- 0.013971* x(1) *x(4) +0.11209* x(2) *x(3)-0.12409*x(2) *x(4) +0.013313* x(3)*x(4)-0.015065*(x(1)*x (1))-0.071645 *(x(2)*x(2))-8.64463*(x(3)*x (3))-0.47963*(x(4)*x(4))
- y1:L-Asparaginase Activity
- y 2 = + 2 9 . 5 4 4 9 4 - 0 . 8 7 3 9 4 * x (1) - 1.93078*x(2)+8.83928*x(3)-16.77692*x(4)-7.82500*10^-3 *x(1)*x(2)-0.025792*x(1)*x (3)+0.042458*x(1)*x(4)+3.37500 *10^-3*x(2)* x(3) +0.10262 *x(2)*x(4)+0.37062*x(3)* x(4)+0.015864*(x(1)* x(1))+0.044680* (x(2)*x(2))-0.73499 * (x(3)*x(3))+2.59451* (x(4)*x(4)) y2: L-Glutaminase Activity

RESULTS AND DISCUSSION

ANN modelling results : MATLAB R2013a was used to perform ANN modelling studies. Artificial neural network was built with four inputs - (Time (30-60h), pH (5.0-7.0), Temperature (20°C-30°C) and L-Asparagine (1-3 g/L)) and two outputs (L-Asparaginase and L-Glutaminase activity).The upper and lower limits taken for the four inputs were shown in the Table 1. Randomly the given data was distributed into training, testing and validation data by the algorithm. The interaction between the inputs and their influence on the enzyme activities (L-Asparaginase and L-Glutaminase activity) from RSM and ANN was compared and studied (Table 2). From ANN modelling performance plot curve (Fig. 1), 0.99572, 0.98494 and 0.99939 were the observed regression coefficients values obtained for training, test and validation data sets respectively. The predicted Regression coefficient value from ANN model (0.99572) is higher w.r.t. RSM predicted Regression coefficient (0.986 L-Asparaginase) indicates the superior prediction of ANN model for the L-Asparaginase production with minimal Glutaminase activity. From ANN modeling studies effective prediction was observed between the input variables and enzyme activities.

GA optimization

In this study, the genes were encoded in the form of binary digits. The parameters chosen for GA optimization chosen were as follows:

- **Population type** : Double vector
- **Population size** : 60(15* no. of variables)
- **Mutation** : Constraint dependent

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- **Crossover** : 1.0
- **Selection** : 2
- **Migration** : Forward
- **Generations** : 800
- **Pareto front population fraction** : 0.35

Within 800 generations the best fit optimum conditions were selected based on the given range of variables from RSM (Table 1) and ANOVA equations were supplied [34] as follows:

- y1 = -374.98043+2.15508*x(1)+ 3.51766*x(2)+99.95797*x(3) +5.99492*x(4)-0.012023*x(1)*x(2)-0.013862*x(1)*x(3)-0.013971*x(1)*x(4)+0.11209*x(2) *x(3)-0.12409*x(2)*x(4)+0.013313*x(3)*x(4)-0.015065*(x(1)*x(1))-0.071645 *(x(2)*x(2))-8.64463*(x(3)*x(3))-0.47963*(x(4)*x(4))

  y1 : L-Asparaginase Activity
- y2 = +29.54494-0.87394*x(1)-1.93078*x(2)+8.83928*x(3)-16.77692*x(4)-7.82500*10^-3*x(1)*x(2)-0.025792*x(1)* x(3)+0.042458*x(1)*x(4)+3.37500 *10^-3*x(2)*x(3)+0.10262*x(2)*x(4)+0.37062*x(3)*x(4)+0.015864*(x(1) *x(1))+0.044680*(x(2)*x(2))-0.73499 * (x(3)*x(3))+2.59451*(x(4)*x(4))

  y2 : L-Glutaminase Activity

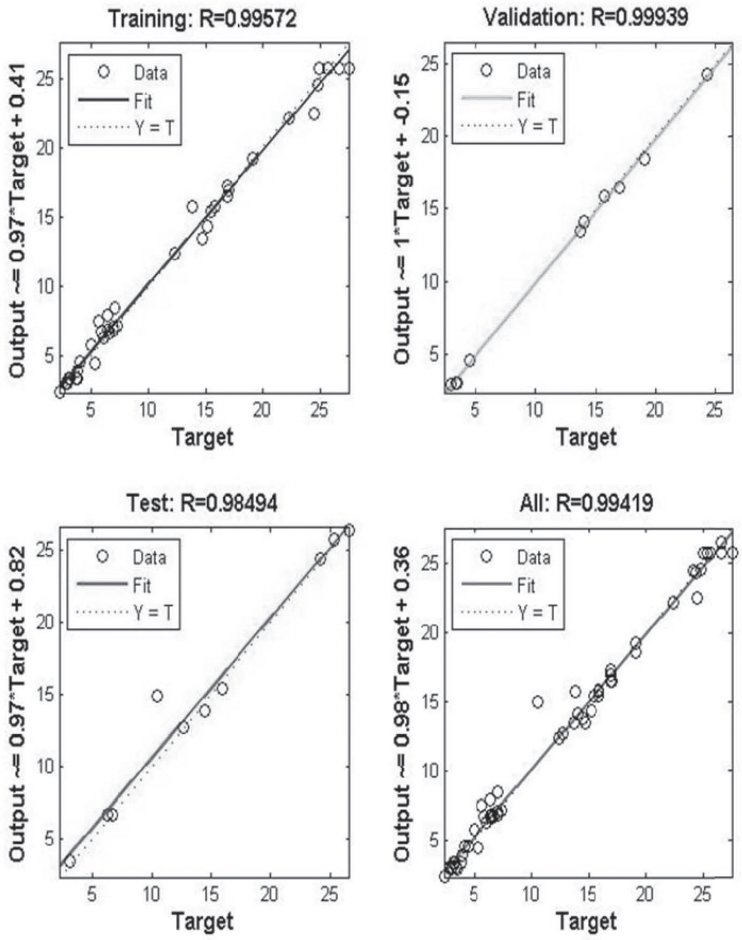

Fig. 1. Output vs. target regression plot obtained from ANN

Table 1. Limits of Input variables

Parameter	Lower Limit	Upper Limit
Time (h)	30 h	60 h
pH	5.0	7.0
Temperature °C	20 °C	30 °C
L-Asparagine (g/L)	1g/L	3 g/L

Table 2. Comparision of Experimental and Predicated enzyme activities from RSM and ANN

Run	Experimental	RSM Predicted Values	ANN Predicted Values Values	Experimental Values	RSM Predicted Values	ANN Predicted
	L-Asparaginase (IU/ml)	L-Asparaginase (IU/ml)	L-Asparaginase (IU/ml)	L-Glutaminase (IU/ml)	L-Glutaminase (IU/ml)	L-Glutaminase (IU/ml)
1	6.98±0.024	8.56	6.98	3.01±0.014	3.01	3.01
2	25.65±0.307	25.51	25.76	3.81±0.459	3.54	3.38
3	19.12±0.014	20.34	19.18	15.43±0.136	14.78	15.35
4	2.89±0.005	2.20	2.91	4.45±0.12	4.82	4.54
5	24.78±0.097	25.46	24.52	6.5±0.15	6.43	6.56
6	6.34±0.012	6.19	6.68	6.76±0.003	6.95	6.67
7	24.267±0.59	24.63	24.27	3.345±0.099	3.77	3.04
8	22.34±0.4	22.81	22.10	6.09±0.013	5.54	6.24
9	6.45±0.51	6.19	6.80	6.98±0.02	7.33	6.82
10	27.5±0.9	25.51	25.76	3.21±0.208	3.54	3.38
11	17±0.004	16.85	16.48	13.98±0.038	14.31	14.06
12	5.65±0.15	4.61	7.43	7.32±0.247	7.86	7.13
13	24.45±0.012	24.60	22.48	5.89±0.041	5.83	6.69
14	2.85±0.04	4.55	2.98	7.07±0.229	6.77	8.41
15	16.88±0.15	16.31	16.52	3.86±0.32	2.78	3.93
16	13.65±0.012	13.60	13.45	15.82±0.001	15.84	15.84
17	15.88±0.04	15.64	15.40	12.65±0.409	12.60	12.70
18	6.42±0.2	6.08	7.87	5.34±0.321	5.45	4.37
19	24.9±0.001	25.51	25.76	3.09±0.064	3.54	3.38
20	26.6±0.022	25.51	25.76	3.74±0.107	3.54	3.38
21	19.12±0.131	18.44	18.50	3.421±0.032	3.12	2.96
22	26.53±0.152	27.93	26.42	10.48±0.011	11.43	14.92
23	25.6±0.002	25.51	25.76	3.8±0.001	3.54	3.38
24	16.89±0.030	17.33	17.22	14.65±0.002	14.19	13.48
25	24.087±0.0012	21.99	24.43	14.45±0.002	14.89	13.81
26	15.14±0.002	16.01	14.32	16.95±0.005	16.47	16.95
27	4.98±0.005	4.74	5.77	4.06±0.001	3.86	4.55
28	13.78±0.017	15.29	15.71	2.31±0.002	2.49	2.33
29	15.78±0.0013	14.38	15.73	12.31±0.002	12.20	12.32
30	25.3±0.005	25.51	25.76	3.21±0.002	3.54	3.38

The Anova equations obtained from RSM was optimized using GA and the results obtained from GA optimization were shown in Table 3. 28.67 IU/ml and 0.09 IU/ml are the experimentally achieved activities of L-Asparaginase and L-Glutaminase from the optimum process conditions, i.e., incubation time 55h, pH 6.0 and temperature 24°C and L-Asparagine 2.5 g/L. The Pareto front graph for the two objectives (L-Asparaginase and L-Glutaminase) was shown in Figure 2. Plot functions enable to plot various aspects of genetic algorithm. From Pareto front graph we can infer with increase in the L-Asparaginase activity, the L-Glutaminase activity decreases. The final

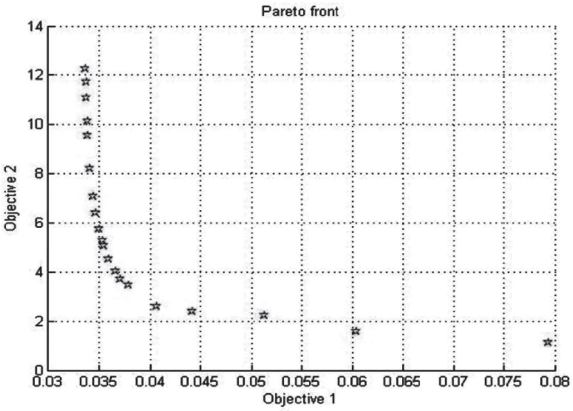

Fig. 2. Pareto front plot generated from GA

Table 3. Enzyme activities by GA optimization

S.no	Time (h)	Temperature (°C)	pH	L-Aspar agine (g/L)	Optimized values from GAL-Asparaginase (IU/ml)	L-Asparaginase Experimental (IU/ml)	Optimized values from GA L-Glutaminase (IU/ml)	L-Glutaminase Experimental (IU/ml)
1	38.2	22.71	7	1.93	12.59	10.13	0.88	2.42
2	58.69	22.35	5.9	2.55	29.69	27.32	0.09	1.21
3	46.36	22.43	5.9	2.24	27.29	28.23	0.25	3.34
4	48.1	22.78	5.92	2.09	27.84	28.99	0.22	1.21
5	**54.66**	**22.67**	**5.92**	**2.3**	**29.38**	**28.67**	**0.12**	**0.09**
6	49.18	22.54	5.95	2.16	28.18	24.34	0.2	2.67
7	58.7	22.01	5.9	2.68	29.7	25.78	0.08	1.09
8	41.29	22.27	6.41	2.23	22.64	19.34	0.42	3.57
9	42.23	22.18	6.18	2.09	24.61	23.56	0.38	0.98
10	52.7	22.62	5.93	2.3	29.08	27.64	0.14	0.58
11	38.58	21.52	6.75	1.93	16.56	17.43	0.64	4.32
12	50.65	22.51	5.94	2.17	28.58	27.43	0.17	1.65
13	38.2	22.71	7	1.93	12.59	10.98	0.88	1.21
14	43.07	22.7	6.75	2.03	19.47	18.34	0.45	0.57
15	45.78	22.39	5.9	2.12	26.99	27.01	0.27	0.39
16	51.86	22.4	5.94	2.18	28.85	28.49	0.16	0.32
17	57.68	22.37	5.9	2.58	29.67	29.41	0.09	0.25
18	56.03	22.6	5.9	2.48	29.57	29.14	0.1	0.19
19	44.31	22.29	5.98	2.34	26.42	26.01	0.29	0.41
20	56.1	22.37	5.9	2.63	29.59	29.71	0.1	0.26
21	49.49	22.65	5.85	2.2	28.31	28.43	0.19	0.34

optimized enzyme activities from RSM and GA were shown in the Table 4.

Conclusion

Input variables - Time, pH, Temperature and L-Asparagine were chosen for modelling and optimization of output variables i.e. L-Asparaginase and L-Glutaminase activities. The model obtained from the ANN significantly gave better predicted values (R^2 = 0.99419) incomparision with RSM model. 21 possible optimal solutions were drawn from GA optimization. Experiments were conducted and the best matched possible solution was found to be at time - 55h, pH - 6.0 and temperature - 24°C and L - Asparagine 2.5 g/L and activities obtained for L-Asparaginase and L-Glutaminase were 28.67 IU/ml and 0.09 IU/ml respectively. Predicted and experimentally determined enzyme activity by *Bacillus stratosphericus* is higher than the existing sources.

Table 4. Comparison of RSM and GA Optimization results

	L-Asparaginase (IU/ml)	L-Glutaminase (IU/ml)
RSM	25.28	3.09
GA	28.67	0.09

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Conflict of Interest

Authors have no conflict of interest regarding the publication of paper.

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Inflammation Iduced Obesity Associated Diseases: a Mini Review

G.V. Swarnalatha, * R. Senthilkumar
Department of Biochemistry, Rayalaseema University, Kurnool, AP, India.
Corresponding Author : :senthilanal@yhoo.com

Abstract

A variety of factors including pathogens, damaged cells and other toxic compounds can influence the immune system and this biological response is known as Inflammation. These factors may induce acute and/or chronic inflammatory responses in the heart, pancreas, liver, kidney, lung, brain, intestinal tract and reproductive system, potentially leading to tissue damage or disease. Inflammation is divided into two different formats: Acute and chronic inflammation. Acute inflammation that occurs over seconds, minutes, hours and days following the injury of tissues. The damage will be purely physical or it may involve the activation of an immune response. Chronic inflammation that occurs longer times, days and months. Inflammation is involved in impairment of energy balance as well as insulin resistance and heart diseases. Accumulation of fat in the body causes optimal status of human body health and the stage is known as obesity. Obesity damages several signalling pathways in the body and causes diabetes, some cancers, gall bladder diseases and gall stones, osteoarthritis, gout and breathing problems and asthma. In this minireview, we discuss the inflammation mediated obesity associated diseases.

Key words: Inflammation, Adipocytes, Obesity, Diabetes, Cancer

Introduction

Immune system is affected by few factors such as pathogens, malfunctioned cells and tissue, toxic compounds. This system is initiating the healing processes by removing injurious stimuli and this is processes known as Inflammation. Inflammation is playing a vital role in body defence mechanism and maintains the health status in the body. In acute inflammatory responses, the cellular signalling transduction minimizes the impending injury or infection, and these maintain the acute inflammation resolution and restoration of tissue homeostasis [1, 2]. However, uncontrolled acute inflammation may become chronic, contributing to a variety of chronic inflammatory diseases. At the tissue level, inflammation is characterized by redness, swelling, heat, pain, and loss of tissue function, which result from local immune, vascular and inflammatory cell responses to infection or injury. Important microcirculatory events that occur during the inflammatory process include vascular permeability changes, leukocyte recruitment and accumulation, and inflammatory mediator release [3-5] Fig.1.

Various pathogenic factors, such as infection, tissue injury, or cardiac infarction, can induce inflammation by causing tissue damage. The etiologies of inflammation can be infectious or non-infectious (Table 1). In response to tissue injury, the body initiates a chemical signalling cascade that stimulates responses aimed at healing affected tissues. These signals activate leukocyte chemotaxis from the general circulation to sites of damage. These activated leukocytes produce cytokines that induce inflammatory responses [6-8]. In this mini review, we reveal the inflammation induce obesity associated few diseases.

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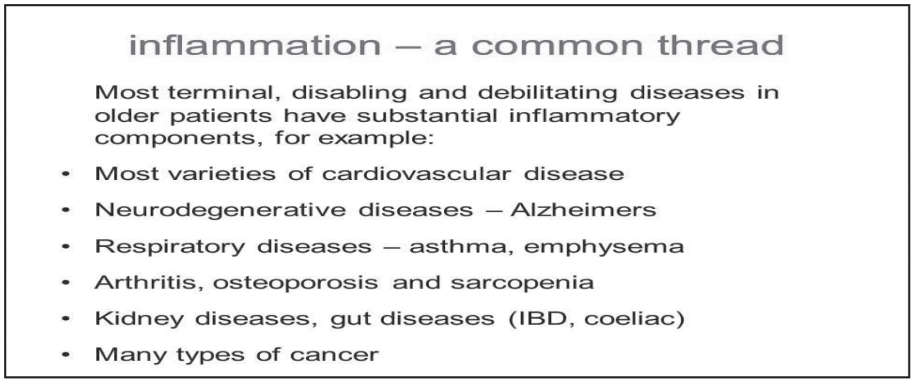
Fig.1. Iflammation induces many numbers of diseases in different organs



Obesity Causes Diabetes

Weight gain is a common symptom of Cushing's syndrome, a condition in which you are exposed to too much of the stress hormone cortisol, which in turn causes weight gain and other abnormalities. People who are overweight or obese often have health problems that may increase the risk for heart disease. These health problems include high blood pressure, high cholesterol, and high blood sugar. Obesity is a serious, chronic disease that can have a negative effect on many systems in our body. People who are overweight or obese have a much greater risk of developing serious conditions, including: Heart disease, Type 2 diabetes. bone and joint disease. Reduced physical activity during these days increases the calories that lead to obesity. Obesity induces a low grade inflammation which leads to production of several diseases such as cardiovascular, type 2 diabetes, dyslipidemia and neurodegenerative disorders [9, 10]. The efforts are going on to understand the mechanisms behind these diseases, as they are threat to world population. Hence, the global mortality and morbidity rate are increasing [11, 12]. Obesity-related inflammation also regulates systemic metabolism and energy homeostasis. Obesity is due to the monogenetic defects which are caused by mutated genes of brain. This regulates the food intake of brain which further controls energy homeostasis [13]. Effective and therapeutic measures are needed to emphasize this problem. In obesity and type 2 diabetes, the inflammatory mediators like tumour necrosis factor-á (TNF-á) and interleukin-6 (IL-6) are increased (Table 2).

Obesity results in chronic inflammation by increasing the plasma lipid levels and insulin resistance. This leads to progression of fatty liver disease, atherosclerosis and diabetes. It is known in mice that obesity increases the expression of TNF and inflammatory cytokine in adipose tissue [14]. In humans and animal models there is an inflammatory signalling in adipocytes due to obesity. The macrophages and adipocytes of adipose tissue secrete cytokines such as TNF á, IL-6, IL-1 and migration inhibitory factor. The visceral fat of obese humans show increased expression of inflammatory mediators [15, 16]. Short-term hyperlipidaemia and robust inflammatory response are the effects that aids in the resolution of the infection [17].

The insulin signalling is disrupted by the cytokines. Suppressors of cytokine signalling (SOCS) proteins are a family of proteins that inhibit receptor kinase activity. This kinase interferes with receptor substrates of insulin IRS 1 and IRS 2 binding to insulin receptor. So cytokines induces these SOCS proteins. This induction also promotes IRS degradation [18, 19]. In both immune and non-immune cells cytokines increases the inflammation and inhibits the action of insulin by activating and inhibiting the enzymes of inflammatory signalling pathways i.e., c-Jun N-

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terminal kinase (JNK) and inhibitor of kappa beta kinase (IKK-beta) [20, 21]. Diabetes is the result of increased inflammation in adipocytes.

Signalling And Inflammation In Adipocytes : In adipocytes, the signalling system is disrupted during obesity. This disruption results in many immunological and metabolic disorders. Adipokines modulates the metabolic signalling and immune function [22, 23]. Proteins of adipocytes modulate inflammation. Energy homeostasis and metabolism of glucose and lipids are influenced by proteins. Chemerin and vaspin are regulators of insulin sensitivity [24]. Adiponectin, display anti-inflammatory properties [25]. Resistin boosts inflammatory responses by interfering with insulin and glucose homeostasis [26]. Deviation in adipokines and cytokines release by adipocytes during obesity influence metabolic as well as immune signalling pathways. Hence adipocytes are linked with diabetes and obesity [27].

Handling Obesity And Diabetes : Physical exercise and diet control are the important factors influencing obesity. In mice suppression of inflammation during type 2 diabetes, has improved the glucose intolerance. Exercise and diet are the two effective factors that can attenuate obesity [28].

Obesity Causes Cancers: Being overweight or obese is clearly linked with an increased risk of many types of cancer, including cancers of the: Breast, Colon and rectum. Several studies have explored why being overweight or obese may increase cancer risk and growth. The possible reasons that obesity is linked with cancer include: Increased levels of insulin and insulin growth factor-1 (IGF-1), which may help some cancers develop. Several factors can cause women with ovarian cancer to gain weight. Deposits of cancerous cells 2 centimetres or more are often found in the abdomen during stage 3C of ovarian cancer. Constipation is another cause of weight gain. Most people with cancer will lose weight at some point. When you lose weight for no known reason, it's called an unexplained weight loss. This happens most often with cancers of the pancreas, stomach, oesophagus, or lung. Half of all cancer patients suffer from a wasting syndrome called cachexia. Affected patients lose weight, including muscle, no matter how much they eat. The wasting is the immediate cause of about a third of all cancer deaths [29, 30]. The raising links between obesity-cancer is biggest thread for the United States and worldwide. Numerous cohort studies, summarized in systematic reviews, have shown a link between obesity and cancer incidence overall and for selected cancer sites. Data showing reduced cancer incidence and mortality among individuals who lose weight and maintain the loss strengthen confidence in the obesity–cancer link, and also provide a note of hope that weight loss in obese individuals may help them prevent cancer. Research on the biological mechanisms underlying the obesity–cancer link is still in early stages, but may lead to a better understanding of the process of carcinogenesis in obesity-related cancers as well as potential treatments and preventive agents [31, 32].

Obesity Leads Gall Bladder Diseases And Gall Stones : Digestive system has multifaceted origin having diseases which is called as Gallstone disease (GSD) and it is a worldwide disease [33]. Most of the reported prevalence of GSD in Western counties ranged from 10–15%. Research shows it affects 10%-15% adult population, meaning 20–25 million adults in America are afflicted with GSD, with medical cost for the treatment of almost 6.2 billion annually. In 1989, it was reported that the prevalence of gallstones in China was 6.29% among 100,000 people [34, 35]. Although the mortality rate for GSD is low at 0.6%, an increased overall mortality, particularly from cancer and cardiovascular disease in GSD patients, resulting in a heavy burden on the economy and public health services [36].

The term gallbladder disease is used for several types of conditions that can affect the gallbladder. The gallbladder is a small pear-shaped

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sac located underneath of liver. Gallbladder's main function is to store the bile produced by liver and pass it along through a duct that empties into the small intestine. Bile helps digest fats in small intestine. Inflammation causes the majority of gallbladder diseases due to irritation of the gallbladder walls, which is known as cholecystitis [37, 38]. This inflammation is often due to gallstones blocking the ducts leading to the small intestine and causing bile to build up. It may eventually lead to necrosis (tissue destruction) or gangrene [37, 39]. Although there are many risk factors which would increase the likelihood of developing gallbladder disease, two of the major causes are obesity and rapid weight-loss; therefore, gallbladder disease is an important issue for a patient with obesity. Being overweight or having obesity may make more likely to develop gallstones, especially if you are a woman. Researchers have found that people who have obesity may have higher levels of cholesterol in their bile, which can cause gallstones. The gallbladder could be not emptying fully (biliary dyskinesia), and lack of bile causes improper fat digestion. Constipation and weight gain can also be symptoms of gallbladder problems, though these are not usually as relatable to fat intake. Gallbladder disease and gallstones are more common if you are overweight [40-42].

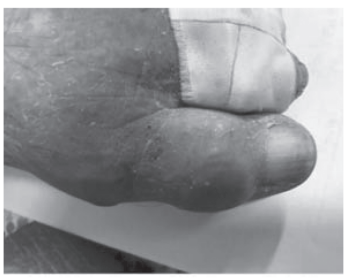
Obesity Causes gout : Gout is a disease that affects the joints. Uric acid in the blood is the indication of Gout. The extra uric acid can form crystals that deposit in the joints. Gout is more common in overweight people. The more weight gain, the more likely the persons are to get gout. Over the short term, sudden weight changes may lead to a flare-up of gout [43-45](Figure 2).

Obesity causes Breathing Problems and Asthma : The prevalence of obesity has increased dramatically over recent decades, and obesity is recognized as an important risk factor for a diagnosis of asthma. Furthermore, evidence from recent years suggests a probable shared genetic basis for obesity and asthma. More recently, obesity has also been recognized as a potential risk factor for more severe asthma. Obesity may therefore be a potentially modifiable risk factor for asthma, possibly not least with regard to the more severe asthma. If excess weight not only leads to the development of asthma, but also has a negative impact on the clinical manifestations of asthma, including symptoms and response to therapy, weight loss may be expected to have the opposite effect or at least to improve the clinical status of individuals suffering from asthma [46-50].

Overall asthma control, in contrast to, For instance, symptom control, is the aim of asthma management. Asthma control is assessed on the basis of a combination of the following items: exacerbations, frequency and severity of day-time and night-time symptoms, use of rescue medication, limitations and level of daytime activities, variability of lung function i.e., forced expiratory volume in 1 second (FEV1), and diurnal variability in peak flow [51-53].

Sleep apnea, a condition that leads to difficulty breathing and decreased oxygen levels at night, is also common among obese people, and it is associated with asthma-like symptoms such as wheezing and shortness of breath as well [54, 55]. Obesity could be a risk factor for asthma, but asthma could also promote obesity due to decreased activity. Obesity can also increase the risk for gastrointestinal reflux disease, which has been linked to worsening asthma. Relationships have also been proposed between genetic factors and inflammation. Obesity itself can cause many of these symptoms due to the mechanical changes

Fig.2. Chronic tophaceous gout (left greater toe).



caused by having more abdominal fat on the lungs. Many of the reasons why obese asthmatics feel that their asthma is under poor control may not be from an increase in airway inflammation or lung function [56, 57]. Sleep apnea is a breathing condition that's linked to being overweight. Sleep apnea can cause a person to snore heavily and to briefly stop breathing during sleep. Sleep apnea may cause daytime sleepiness and make heart disease and stroke more likely. Exercise and activity is good for most people with asthma, as well as helping you lose weight. Researchers have found that when people who are overweight or obese lose weight with a combination of diet and exercise, they lose on average 20 per cent more weight than if they only diet. Obesity can cause shortness of breath as extra weight in the chest and abdomen increases the work the muscles that control breathing must do [58-60].

Alcohol And Gut Related Inflammation : Alcohol-related medical conditions result in chronic inflammation. Alcohol induced-inflammation act as etiological factor for many other diseases. Pro and anti-inflammatory cytokines are involved in alcohol liver diseases [61]. In mice, tumorigenesis is observed due to activation of inflammatory signalling through alcohol [61]. Neurodegeneration in mouse brain is due to increased TNF- á, a pro-inflammatory cytokine by induction of alcohol [62]. Knock out gene for receptor of TNF-á in mice is resistant to liver injury caused by alcohol [62]. Alcohol influences a number of factors. First it is involved in impairing gut barrier function by enhancing LPS translocation across the gut barrier. Second, LPS dissemination via lymphatics is stimulated by alcoholics. Third, alcohol affects the liver's ability to detoxify LPS as well as its ability to synthesize interleukin (IL)-10. Lastly, neuroendocrine effector synthesis which can regulate systemic inflammation is also reduced [63].

In alcoholic liver diseases, the circulating LPS are increased, which causes acute inflammation [64]. There are a number of events occurs in LPS translocation and host response. Firstly, LPS enters circulation by two routes upon translocation across the gut barrier. These are through the portal vein or lymphatic vessels. In portal vein, LPS directly entered in to the liver where mostly it is detoxified and also excreted. In lymphatic vessels, through the thoracic duct openings [65, 66]. LPS are released into the circulation. Among these two routes the dominant one is portal dissemination. Lymphatic dissemination is found during fatty diet and ischemic injury. Secondly, liver also plays a role in detoxification of LPS in the portal vein and systemic circulation. It also maintains a balance for contained inflammation and limited damage. Thirdly, LPS plays a role in multi organ damage as they enterin circulation. Last but not least, the LPS and other immune mediators activate neuroendocrine response. This further activates the hypothalamo-pituitary-adrenal (HPA) axis, which synthesises the cortisol. The produced cortisol down-regulates the inflammatory responses in the periphery [67, 68].

Innate immune system mediates the inflammation, whenever "danger" signals tissue injury or infection occurred [69]. As a result, pro-inflammatory cytokines and chemokine are released by macrophages a type of innate immune cells. These stimulate neutrophil recruitment at injury site and increase vascular permeability. Reactive oxygen species and anti-microbial peptides are released by neutrophils and there by phagocytose dead and foreign material. Proteases and opsonins are produced by plasma proteins in order to combat infection. C-reactive protein, mannose binding protein, complements factors, ferritin, serum amyloid A and P, and surfactant proteins are the acute phase reactants that are generated by liver [70, 71].

A microbe or an endogenous productbinds to pattern recognition receptors (PRRs) including toll-like receptors (TLRs) and results in an inflammatory response. This binding leads to activation of PRRs intracellular signalling. This leads to expression of genes encoding pro-inflammatory cytokines and other effectors. Anti-inflammatory effectors are also synthesised to regulate the inflammatory response. In alcoholic

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liver chronic inflammation persistent expression and recruitment of pro-inflammatory cytokines and monocytes, neutrophils are observed that leads to damage of host tissue in contrast to acute inflammation [72, 73].

Gram-negative bacteria cell wall contains lipopolysaccharide (LPS). In chronic obstructive pulmonary disease (COPD) the infection is due to bacteria. The acute inflammatory response shows the release of inflammatory cytokines. Lipopolysaccharides reach the liver via blood and activate the macrophages. As a result, interleukin 8 (IL-8, CXCL8, CXC ligand 8) and other cytokines are released and cause acute inflammatory response [74]. Lipopolysaccharide (LPS) of gut microflora and alcohol damaged cells are the two main sources for inducing alcohol related inflammation. Reactive oxygen species produced by alcohol metabolism stimulates a nuclear transcription factor êB (NF-êB) [75]. Alcohol metabolism also results in hypoxia condition which in turn induces inflammatory response [75].

TLR signalling is activated through adaptor proteins due to inflammatory response. The signal transduction pathways NF-êB and AP-1 are also activated and this induces the gene expression [76, 77]. The bacterial LPS are used to study the inflammatory models. This is because it mimics TNF-a, IL-1 b or IL-6 of cytokines. Toll like cellular receptor 4 (TLR4) is identified as the LPS signal transducer [77-79]. LPS binding to TLR4 leads to activation of NF-B, MyD88, IL-1R kinase (IRAK), TNFR associated factor 6 (TRAF-6), and NADPH oxidase (Nox) [80, 81]. NF-êB plays important role in inflammatory response and innate immunity by regulating the gene expression. In lungs and monocytes, reactive oxygen species (ROS) controls the activation of NF-êB [82-85].

Inflammation Through Activated G Protein-Coupled Receptors In Bile Acid : Bile acids are important in nutrient absorption and secretion of biliary lipids, xenobiotics and toxic metabolites. Bile acids regulate hepatic lipid, glucose, and energy homeostasis through the activation of nuclear receptors and G protein-coupled receptor (GPCR) signalling. Bile acid toxicity leads to inflammation, apoptosis, and cell death. Bile acid-activated nuclear and GPCR signalling protects against inflammation in liver, intestine, and macrophages. In human's chronic liver diseases, obesity, and diabetes are treated by bile acids derivatives.

In macrophages the proinflammatory cytokine production is inhibited due to the activation of TGR5 [86]. TGR5 signalling protects against inflammatory diseases such as fatty liver disease, inflammatory bowel diseases, atherosclerosis, and diabetes. In enteroendocrine cell line STC-1, TGR 5 stimulates the GLP-1 secretion. GLP-1 regulates appetite, insulin and glucagon secretion in pancreas, glucose homeostasis, and diabetes. Knockdown of TGR5 expression suppress the GLP-1 secretion [87]. 6á-ethyl-23(S)-methyl-CA (6EMCA, INT-777) is an agonist of TGR5, activates TGR5, hence enteroendocrine L cells produces GLP-1. As a result the calcium mobilization in the cell increases; this improves the insulin sensitivity [88].

In liver, NF-êB-mediated inflammation is a negatively modulated by TGR5. NF-êB is activated rapidly during lipopolysacchride (LPS) or pro-inflammatory cytokines exposure and these are detected in acute infections and inflammations [89, 90]. The mechanism behind the suppression of NF-êB-mediated liver inflammation by the activation of TGR5 is due to inhibition of phosphorylation of IêBa and nuclear translocation of p65 [91]. Thus in gastric inflammation, TGR5 acts as a potential target for therapeutic intervention by antagonizing NF-êB signalling [92].

Smooth Muscle Inflammation In Atherosclerosis : Atherosclerosis is one of the major causes of cardiovascular diseases. The chronic progressive inflammation of atherosclerosis includes leukocyte recruitment, transmigration and also accumulation of lipid and ûbrous factors in the large arteries [93, 94]. Vascular smooth muscle cells (VSMCs) play an important role in early stages of atherosclerosis. In later stages platelets, endothelial cells (ES),

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Table 1: An infectious and Non-infectious factor leads to Inflammation.

Non-infectious factors	Infectious factors
Physical : burn, frostbite, physical injury, foreign bodies, trauma, lionizing radiation Chemical: glucose, fatty acids, toxins, alcohol, chemical irritantsBiological : damaged cellsPsychological: excitement	Bacterial, virus and other microorganisms

Table 2 : Obesity-Related Conditions

Cardiovascular	Endocrine	Gastrointestinal	Genitourinary
Atrial fibrillation Cardiomyopathy Dyslipidemia Hypertension Thromboembolism	Hypoandrogenism Infertility Metabolic syndrome Type 2 diabetes mellitus Polycystic ovary syndrome	Colon cancer Esophageal Cancer Gastroesophageal refluxHiatal hernia Irritable bowel syndrome Nonalcoholic fatty liver disease	Breast cancer Ovarian cancer Prostate cancer Renal Cell cancer Uterine cancer

VSMCs, and inûammatory cells are also involved. These release inflammatory mediators like cytokines and growth factors that can induce various effects [95]. The cytokine-inducible immunoglobulin gene superfamily members, vascular cell adhesion molecule-1 VCAM-1 and intracellular cell adhesion molecule-1 ICAM-1 bind to leukocyte integrin.

VCAM-1 expression is involved in lesion and lesion-predisposed region where as ICAM-1 expression involved in lesion protected regions [96]. Monocytic and macrophagic inûammatory cells adheres with VSMCs and further progresses the disease. This adhesion is mediated by vascular cell adhesion molecule-1 (VCAM-1), and its expression is reported in atherosclerotic lesions [97]. TNF-β is an inûammatory cytokines also expresses the VCAM-1[98]. This expression of VCAM-1 by TNF-β induces release of several chemokines and enhances the adhesive nature to arterial walls [99]. The cell adhesion molecules expressed cells in response to TNF-β, regulates the MAP kinases and plays a role in signalling transduction pathways [100]. The transcriptional activation of muscle cell adhesion molecules requires the activation of NFêB transcription factor by TNF-β [101]. The inhibitors MAP kinase and NF-êB reduces the expression of cell adhesion molecule. Therapeutic drugs inhibit the vascular inûammation of atherosclerosis [102]. Quinic acid (QA) is a cyclohexane carboxylic acid inhibits the TNF-á-mediated VCAM-1 expression by inhibiting MAP kinase and NF-αB signalling pathways [94].

Summary

Overweight and obesity together make up one of the leading preventable causes of death in worldwide. Obesity is a chronic disease that can seriously affect our health. In many ways, obesity is a puzzling disease. But the risk factors that determine obesity can be complex. They are usually a combination of your genes, socioeconomic factors, metabolism, and lifestyle choices. Some endocrine disorders, diseases, and

medicines may also affect a person's weight. Overweight and obesity are preventable diseases. To prevent them, we need to choose healthier, lower-energy foods and be more physically active. Everyone can and should seek changes to their lifestyle to help them stay a healthy weight.

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NEWS ITEM

Jawaharlal Nehru University (JNU) researchers worked out new way to eliminate dormant Tuberculosis bacteria present in stem cells

Delhi-based researchers have found that inhibiting lipid synthesis inside stem cells that produce bone cells (mesenchymal stem cells) can help in killing TB bacteria that are found inside the stem cells in a dormant state and safely shielded from the host immune system and TB drugs. While TB bacteria inside the macrophages actively divide, microbes inside stem cells lie dormant and also make the stem cells less likely to replicate thus surviving for an extended period of time. Ex vivo studies with human stem cells and work on mice showed that the two cells are programmed very differently to support active and dormant TB bacteria infection.A team led by Gobardhan Das from the Special Centre for Molecular Medicine at the Jawaharlal Nehru University (JNU) found that TB bacteria are free in the intracellular fluid (cytosol) of the mesenchymal stem cells while they are surrounded by the macrophage cell membrane on being engulfed. This allows the bacteria to promote rapid synthesis of lipids inside the stem cells and hide within the lipid droplets so created. The results were published in Journal of Clinical Investigation. Studies using human mesenchymal stem cells and macrophages and mice model studies helped us understand how TB bacteria hijack the cellular mechanism to stop the stem cells from replicating and turn themselves dormant, says Prof. Das. The bacteria instruct the stem cells to synthesise lipids and hide inside them. The stem cells don't kill microbes that are inside lipid droplets. There was sustained expression of genes controlling dormancy in the bacteria isolated from stem cells while genes that promote replication were expressed in bacteria isolated from macrophages. Mouse mesenchymal stem cells and macrophages too showed similar behaviour. In vitro studies using human stem cells showed the bacteria inhibiting stem cell replication.Inhibiting autophagy is one of the ways by which TB bacteria survive inside host cells. The researchers treated human macrophages and stem cells infected with TB bacteria with an anti-TB drug (isoniazid) and/or rapamycin. While isoniazid eliminated replicating bacteria found in macrophages, rapamycin induced autophagy in stem cells to kill the microbes. Similar results were obtained in mouse models too.

Researchers from IIIT-Hyderabad constructed Indian Brain Atlas

The average Indian brain is smaller in height, width, and volume as compared to the western and eastern population like the Chinese and Korean according to the first-ever 'Indian Brain Atlas' created by researchers of the International Institute of Information Technology-Hyderabad (IIITH). These differences are found even at the structure level like the volume of hippocampus and so on. But overall, the 'IBA 100 is more' comparable to the Chinese and Korean atlases than the distant Caucasian one, according to the research team led by professor from the Centre for Visual Information Technology Jayanthi Sivaswamy. Construction of the Indian human brain atlas was done in collaboration with the Department of Imaging Sciences and Interventional Radiology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. IIITH team made a maiden effort at creating an Indian-specific brain atlas involving 50 subjects selected across genders. MRI scans of these subjects' brains were taken at three different hospitals across three different scanners to rule out variations found in scanning machines. After a successful pilot study, the team recruited 100 willing participants in construction of Indian Brain Atlas or 'IBA 100'.

Researchers found that Rewiring and Repairing of Brain done by Immune cells during sleep

Researchers have found that immune cells called microglia, which play an important role in reorganising the connections between nerve cells,

fighting infections, and repairing damage, are also primarily active while we sleep. Microglia serve as the brain's first responders, patrolling the brain and spinal cord and springing into action to stamp out infections or gobble up debris from dead cell tissue. This research shows that the signals in our brain that modulate the sleep and awake state also act as a switch that turns the immune system off and on,"said study lead author Ania Majewska, Professor at University of Rochester in the US. In previous studies, Majewska's lab has shown how microglia interact with synapses, the juncture where the axons of one neuron connects and communicates with its neighbours.The microglia help maintain the health and function of the synapses and prune connections between nerve cells when they are no longer necessary for brain function.The current study points to the role of norepinephrine, a neurotransmitter that signals arousal and stress in the central nervous system.This chemical is present in low levels in the brain while we sleep, but when production ramps up it arouses our nerve cells, causing us to wake up and become alert.The study showed that norepinephrine also acts on a specific receptor, the beta2 adrenergic receptor, which is expressed at high levels in microglia. "This work suggests that the enhanced remodeling of neural circuits and repair of lesions during sleep may be mediated in part by the ability of microglia to dynamically interact with the brain," said study first author Rianne Stowell. The study was published in the journal Nature Neuroscience.

WHO reported the eradication of wild poliovirus strain

Wild poliovirus type 3 has been eradicated, the World Health Organisation said on Thursday, hailing the development as an "historic achievement for humanity" that leaves only one strain of the virus in transmission. The last confirmed case of WPV3 was recorded in northern Nigeria in 2012.Wild poliovirus type 3 has been eradicated, the World Health Organisation said on Thursday, hailing the development as an "historic achievement for humanity" that leaves only one strain of the virus in transmission.All three types of wildpolio can cause paralysis and death, but WHO categorises them separately in terms of eradication because of certain virological differences.An independent panel, chaired by WHO chief Tedros Adhanom Ghebreyesus, concluded that the required criteria have been met to "verify that this strain is truly gone", the United Nations health agency said in a statement.

Study reported that malfunctioning of Microprotein may cause major infections

Researchers have discovered that a microprotein called PIGBOS found in the powerhouse of the cells – mitochondria – contributes to mitigating stress happening within the cells – an advance that may lead to better understanding of disease conditions like cancer. The researchers, including those from the Salk Institute in the U.S., said that while an average protein molecule present in the human body has around 300 chemical units called amino acids, the microproteins had fewer than 100 of the building blocks. The study, published in the journal Nature Communications, noted that PIGBOS was made of 54 amino acid molecules, and indicated that the microprotein could be a target for cell stress based human diseases like cancer and neurodegeneration. Usually to track and find the functions of proteins, researchers attach a jelly fish derived probe called the green fluorescent protein (GFP) to them, which glows and indicates the protein's presence in cells, the study noted. However, the researchers of the current study ran into a roadblock when they tried to mark PIGBOS with GFP as the microprotein was too small relative to the size of the fluorescent tag.They solved the problem using a less common approach called split GFP where they fused just a small part of GFP, called a beta strand, to PIGBOS.PIGBOS interacted with a protein called CLCC1, which, the researchers said, is part of a cell organelle called the endoplasmic reticulum (ER)."PIGBOS is like a connection to link mitochondria and ER together," said study co-author Qian Chu from the Salk Institute.According to Chu and his team, it was unusual to see a mitochondrial protein playing a role in the unfolded protein response.Chu said that the ER is more

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stressed in cancer patients than in a normal person, and added that ER stress regulation could be a good target to tackle the disease.

IIT Bombay Researchers discovered novel quantum materials for technology of clean energy

Researchers from IIT Bombay have discovered special properties in a class of materials called "semi-Dirac metals" that have been recently talked about in the scientific literature. Examples of semi-Dirac metals are systems such as TiO2/V2O3 nanostructures. Through calculations, the researchers have shown that such materials would be transparent to light of a given frequency and polarisation when it is incident along a particular direction. The material would be opaque to the same light when it falls on it from a different direction. There are many known applications for transparent conducting films – the common example being touch screens used in mobiles. These results were published in Physical Review B. "Our results in this paper show a very high optical conductivity of semi-Dirac materials for electromagnetic waves [light waves] of a specific frequency and specific polarisation" says Alestin Mawrie, a post-doctoral fellow at Department of Electrical Engineering, and the first author of the paper. Optical conductivity is a measure of the opacity offered by the material to the passage of light through it. Semi-Dirac metals behave like Dirac metals in one direction and like normal metals in the perpendicular directions (since their microscopic structure is different along the two directions).In this paper, the authors have shown theoretically that the direction-dependence of the microscopical properties gives the material special optical properties.This technology is used in efficient cars, where it is used to keep lights on and to warm seats. Spacecrafts like Voyager which are too far from the sun to use solar energy can make use of thermoelectricity.

NOBEL PRIZE AWARDS: 2019

Physiology and Medicine

Awarded to William G. Kaelin Jr, Sir Peter J. Ratcliffe and Gregg L. Semenza for their discoveries of how cells sense and adapt to oxygen availability.

Physics

Awarded to James Peebles, Michel Mayor and Didier Queloz for contributions to our understanding of the evolution of the universe and Earth's place in the cosmos.

Chemistry

John B. Goodenough, M. Stanley Whittingham and Akira Yoshino for the development of lithium-ion batteries.

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