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ISOLATION AND SCREENING OF L - APARAGINASE PRODUCING BACTERIA FROM MARINE SOURCES

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ABSTRACT

The marine environment is a potential source of novel bacteria which are a potent source for number of antibiotics and novel bioactive compounds .L-asparaginase is responsible for the hydrolysis of L-asparagine into L-aspartic acid and ammonia. L-asparaginase is used as an effective therapeutic agent against lymphocytic leukaemia. It also finds applications in food industry. Though much has been unravelled about LA, it may appears today that it is at the tip of iceberg and there is tremendous scope in screening of novel LA sources and studying their properties and applications. In this study 4 bacterial isolates were collected from Chirala, Coastal area of Andhra Pradesh and studied effect of physical properties on their growth.

INTRODUCTION

Marine ecosystem is the great sink of microbial biodiversity of life and marine microbes represent a potential source for commercially important bio-active compounds [1]. The secondary metabolite produced by marine microbes has great impact in human society as they are machineries for pharmaceutically active molecule with biological moieties. The number of potential compounds isolated from marine habitat has virtually soared and this number exceeds 10,000 with hundreds of new compounds still being discovered every year[2]. Many of them are endowed with pharmacodynamics properties.Now a days research have been showing interest towards marine microorganisms as sustainable sources, because active compounds in marine animals and plants as also limitation of bio resource supply. Secondary metabolites produced by marine microorganisms have more novel and unique structures owing to the complex living circumstance. Diversity of species and bioactivities are much stronger in marine microorganisms compared to Terrestrial organisms[3]. Studies on marine microbes as a source of anticancer, antidiabetic, antibiotic compound are very few because of limitation of bio resource supply[4].ASNase (EC 3.5.1.1) is an important enzyme used in the pharmaceutical, biosensor and food industries1 and has anticarcinogenic potential for the treatment of acute lymphoblastic leukaemia, lymphomas and other cancers .ASN ase selectively targets the metabolism of cancer cells by exploiting deficiencies in metabolic pathways and catalyzing the degradation of L-asparagine into L-aspartic acid and ammonia, causing nutrient starvation of cancer cells and bringing about their demise. Thus, there is a need for novel and robust ASNases from new microorganisms that exhibit improved stability, lower glutaminase activity, high substrate affinity, and low Km values for use as therapeutics. Two types of commercial ASNase are currently in clinical use for chemotherapy, enzymes from Erwinia chrysanthemi and E. coli. However, these enzymes have drawbacks in that they exhibit low substrate specificity and high glutaminase

activity[5] , the latter of which can cause liver dysfunction, pancreatitis, leukopenia, neurological seizures, and coagulation abnormalities that can lead to intracranial thrombosis or haemorrhages. Therefore, it is important to identify sources and methods of producing greater amounts of glutaminase free ASNases and exhibit high substrate affinity and therapeutic activity[6,7] . A wide range of microbes have been shown to be valuable sources of enzymes, such as Pichia pastoris, Saccharomyces cerevisiae, E. coli, Aerobacter, Pseudomonas, Bacillus, Xanthomonas, Serratia, and Streptomyces[8] . Recently, ASNases from E. coli and Erwinia carotovora produced by submerged fermentation were approved for use in medical applications bythe United States Food and Drug Administration .investigated with respect to its harbouring microbes with biopharmaceutical and biotechnological potential[10].Marine microbe-derivedASNases may be more effective and have fewer side effects as therapeutic agents for acute lymphoblastic leukaemia treatment than traditional sources of these enzymes[11]. The enhanced production of novel glutaminase free ASNase from *Pectobacteriumcarotovorum*[12]and *Erwinia carotovora*[13]was achieved by submerged fermentation in batch and fed batch bioreactors. Cell immobilization is considered to be a promisingapproach for enhancing fermentation processes for ASNase production[14]Thus the goal of present work is aim to study isolation, selection and screening of L-Asparaginase producing marine bacteria inhabited to mangrove ecosystem and molecular identification of marine bacteria, their diversity in production of L-Asparaginase and Bio-processing of L-Asparaginase by submerged and solid state fermentation and molecular weight determination of purified L-Asparaginase of selected marine bacteria.

MATERIALS AND METHODS:

Chemicals and glassware: Borosil made glass ware were sterilized with soap solution and soaked in acid water for 4-6 hrs then rinsed with distilled water for two to three times, dried and sterilized in autoclave at 15lbs pressure/121^{0}C for 30 mins before use. The analytical reagent (AR) grade chemicals for Fischer, CDH, Himedia and Merck are used for the research work.

Sample Collection: Marine soil samples (10 cm Depth) were collected from Chirala, coastal area of Andhra Pradesh in sterile zip lock covers and brought to the lab bench.

Isolation of bacteria:

Isolation of marine bacteria was done by serial dilution method by diluting 1gm of marine soil sample in 10ml sterile water (Mother suspension) followed by serial dilution till 10^{-8} and 0.1ml of last three dilutions were plated on Zobelle's Agar medium and incubate the plates at room temperature for 24-72 hrs. After incubation morphological distinct colonies were identified and purified by quadrant streaking method and transferred on agar slants for further research studies.

Screening of L-Asparaginase producing Bacteria:

Primary screening by rapid plate technique:

L-asparaginase producing bacteria were screened by inoculating the purified culture from isolation step on Modified Czapek Dox (MCD) agar plates supplemented with 0.3 ml of 2.5 % phenol red indicator (pH-6.8) and 0.1% L-Asparagine substrate. After inoculation plates were incubated for 48 - 72 hrs at room temperature and observed for the formation of pink color zone around the colony. Colonies with pink color zonewere further analyzed by Nesslerization method[15].

Step: 2 Secondary screening by *Nesslerization method:*

Step: 1- 0.1 ml of 72 hrs culture was inoculated into MCD broth and inoculated. After 72 hrs inoculation the broth was centrifuged at 10,000 rpm for 15 mins at 4℃ and supernatant collected was used as crude enzyme [16].

Step: 2- 0.5 ml of crude enzyme was added to 1.5 ml of reaction mixture **(R_m)** [0.5 ml of 0.5 M Tris-HCl buffer (pH 8.6), 0.5 ml of 0.04 M asparagine and 0.5 ml of distilled water] and incubated at room temperature. Reaction mixture without crude enzyme was

used as control. After 30 mins of incubation 0.5 ml of 15% TCA was added to reaction mixture and centrifuged at 10,000 rpm for 10 mins at 4^0C.

Step:3- 0.1 ml of supernatant **(S)** was added to 4.9 ml of Nesslerization mixture (3.7 ml of distilled water ,1 ml of 2N NaOH and 0.2ml of Nessler's reagent) and incubated for 20 mins at room temperature. Development of orange color is positive test for ammonia production. O.D was measured at 450nm. Standard graph was plotted with different concentrations of ammonium sulphate ranging from 10µg/mlto
100 µg/ml for calibration. Ammonia (mole) liberated was calculated. Enzyme activity was calculated by the formula

Enzymes activity = NH_3 liberated X R_m (IU /ml)
S X Incubation period

[One international unit (IU) represents number of moles of enzyme required to liberated 1 µmol of ammonia in 1 min at 37°C]

Identification ofBacteria

Morphological identification by Cellshape [17]and Gramstaining[18], Physiological identification by Amylase Test[19], Protease Test[20], Lipase production, GelatinaseTest, Hydrogen Sulfide (H_2S)Production UreaseTest,[21] Nitrate Reduction[22], LitmusTest[23], Biochemical identification by Indole production[21], Methyl red and Vogues ProskaeurTest[21], Citrate Utilizationtest[21], Catalasetest[19], Oxidasetest[22]. Molecular identification of Bacterial isolates includes Isolation of genomic DNA from Bacterial isolates PCR Amplification Phylogeneticanalysis[24].

Colony Forming Unit (CFU)

Carefully clean and dry the chamber slide and coverslip. Dilute the fully grown broth cultures (e.g., > 1 x 10^9 cells per mL) the specimen 1:10 or 1:50 with particulate-free diluent. 10 µl of your cell suspension (or 1 drop from a transfer pipette) to the v-shaped groove on each side of the Petroff -Hausser chamber by using a micropipette, quickly and smoothly without interruption, If the slide is clean, the suspension should move quickly under the cover slip over the entire reflective surface of the Petroff -Hausser chamber and allowed the liquid under the cover slip to stabilize for a minute or two before counting. The bacterial cells in the above shown slide are visualized and counted 400-500X magnification. The microbial population in the sample is calculated by considering the chamber's volume and sample dilutions. 25 squares covering an area of 1 mm^2, therefore, the total number of bacteria present in 1 mm^2 of the chamber is equal to number/square × 25 squares . The depth of the chamber is 0.02 mm andtherefore,

Bacteria/mm^3 = (bacteria/square) × (25 squares) × (50)

Number of bacteria per cm^3 is 10^3 times this value.

Optimization studies for Bio processing includes Effect of Physical parameters onLAP like temperature[25]**,** pH[25],Incubationperiod[26],Effect of Inducers on Bio-processing like Carbonsources[27], Nitrogensource[27], Amino acidssources[28], Phosphatesources[29], Metal ions /Mineral salts sources Solid StateFermentation Solid StateFermentationSix different agro industrial waste substrates Coconut oil cake, Coffee, Green tea, Groundnut oil cake, Red gram and Sesame oil cake were used as substrate in SSF[29].

Bio-processing under solid state fermentation:

Purification ofL-asparaginase [30, 31]:

The enzyme L–asparaginase was purified from the GPSK isolates using ammonium sulphate precipitation, Dialysis. After each step, the L-asparaginase activity and total protein content weredetermined.

Mass cultivation of L-Asparaginase:

L-asparaginase EnzymeExtraction, Ammonium sulphateprecipitation, DialysisMolecular weight characterization by SDS-PAGEanalysis for study of Enzyme Kinetics[32]: Effect of Substrate Concentration onEnzyme, Effect of Temperature, Effect of IncubationTime, Effect of pH[31].

Hemolytic activity of enzymes[33]:

Hemolytic activity of enzymes was evaluated following the protocol as 0.5 ml of blood was centrifuged at 15000 rpm for

30 minutes and supernatant containing plasma was discarded. Pellet containing red blood cells was washed with 0.75% saline by centrifugation at 1500 rpm for 5 minutes and cells were suspended in normal saline. 0.5 ml of cell suspension was than mixed with 0.5 ml of the sample (enzyme) and incubated for 30 minutes at 37º C followed by centrifugation at 1500 rpm for 10 minutes. After centrifugation free hemoglobin in supernatant was measured using spectrophotometer at 540 nm by takingdistilledwater and phosphate buffer saline as minimal and maximal hemolytic controls. The% of hemolytic activity was calculated using the following formulae:

% hemolytic activity = (At- An)/ (Ac- An) X 100

Where A_t is absorbance of sample

A_n is absorbance of control

A_c absorbance of control

Development of electrochemical Biosensor for detection of L-asparagine by using agar [34]and agarose [34]

Isolation of Marine Bacteria:

A total of 12 morphologically distinct bacterial colonieswere isolated from collected marine soil samples and coded as Chirala Marine Bacteria (CMB).

Screening of L-Asparaginase producers:

Out of 12 CMB isolates, 4 isolates show positive response to L-Asparaginase activity and renamed as GPSK-1, GPSK -2, GPSK -3, GPSK -4. Table 1 gives different isolates of CMB.

Quantitative analysis of L-Asparaginase activity of GPSKisolates:

L-Asparaginase activity of selected 4 GPKS isolates was determined quantitatively in MCD broth. Values are represented with ± SE values (n=3) .Values are statistically analyzed by One way ANOVA. 9 GPSK isolates showed L- Asparaginase activity ranging from 12.32 IU to 33.26 IU. Based on the values recorded isolates are classified into three groups

Maximum activity >25 IU

- Moderate activity > 15 IU < 25 IU

Minimum activity < 15 IU

GPSK-3, showed maximum L-Asparaginase activity, GPSK-1, GPSK-4, GPSK-2 showed minimum activity, results were shown in Table 2

L-Asparaginase producing Marine Bacteria

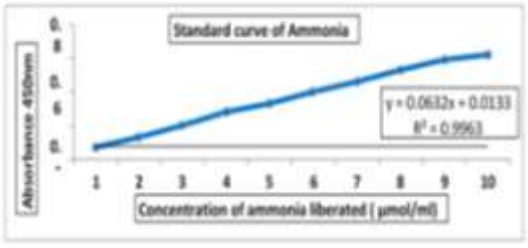
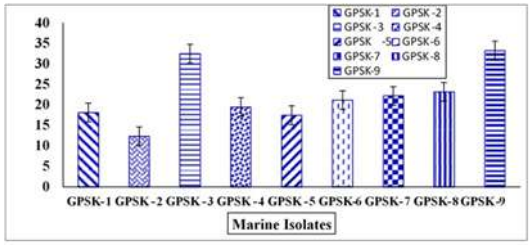
Table:1 Different isolates of CMB isolates.

No of isolates	L-Asparaginase Positive isolates	L-Asparaginase Negative isolates
4	CMB -1,CMB -3,CMB -4,	CMB -2,

Table 2:Quantitative estimation of L-Asparaginase activity of GPSKisolate

Isolates	GPSK-1	GPSK -2	GPSK -3	GPSK -4
L-Asparaginase activity (IU)	18.16 ± 0.00	12.32 ± 0.01	32.54 ± 0.02	19.45 ± 0.01

Values are mean of triplicates with ± S.E (F- 9.55, P < 0.01).

Standard graph of Ammonia by Nesslerization	Quantitative analysis of L-Asparaginase activity of selected GPSK isolates
	

Identification of Marine bacteria:

Colony and strain characteristics of GPSK bacterial isolates:

Table: 3 Physical Properties of Isolates

S.NO	Isolate Name	Shape	Colony characters on agar media	Strain	Motility
1	GPSK 1	Rod	Creamy white	G +ve	Motile
2	GPSK 2	Rod	Creamy white	G +ve	Motile
3	GPSK 3	Rod	Gelatinous	G –ve	Non-Motile
4	GPSK 4	Rod	Gelatinous	G - ve	Non-Motile

Table: 4 Tentative identification of GPSK isolates to Genus level

Cluster	Isolate Name	Genus Name
1	GPSK 1,GPSK2,	*Bacillus sp*
2	GPSK 4	*Brucella sp*
3	GPSK3	*Pseudomonas sp*

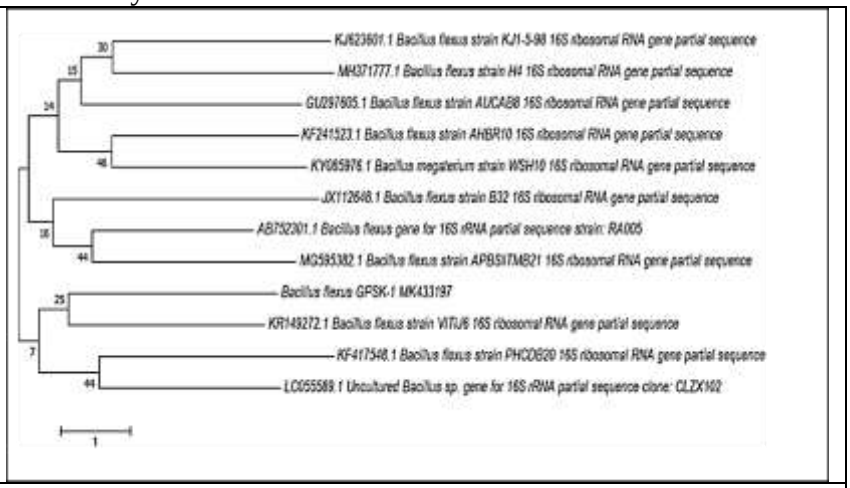
Molecular Characterization:

Molecular identification of GPSK isolates were standardized by 16 S rRNA partial gene sequencing. Isolates were identified on the basis of sequence similarity criteria of selection parameters: (a) Similarity of the query sequence and the reference sequence is 99% or above the unknown isolate would be assigned to reference species; (b) Similarity is between 95 –99 %, the unknown isolate would be assigned to the corresponding genus; (c) Similarity is less than 95 %, the unknown isolate would be assigned to a family. (Bosshard*et al.*,2006)

Identification of GPSK1

981 bp of 16S rRNA gene sequence was amplified from GPSK 1 and blast in ex-taxon for pair wise sequence similarity search 16S rRNA gene sequence of GPSK 1 had 98% similarity to 16S rRNA partial sequence of *Bacillus flexus* strain KJ1-5-98 (Accession number : KJ623601) and 98 % similarity to 16S rRNA partial sequence of *Bacillus flexus* strain AHBR10 (Accession number: KF241523). Phylogenetic analysis based on 16S rRNA partial gene sequence revealed that GSPK 1 was closely related to *Bacillus flexus* (**Figure 5**) .Based on consistence results of sequence analysis of the 16S rRNA partial gene, GPSK 1 was identified as *Bacillus flexus*. The sequence was deposited in NCBI as *Bacillus flexus* GSPK 1 with accession number **MK433197** GEN BANK has given the taxonomic position as follows:

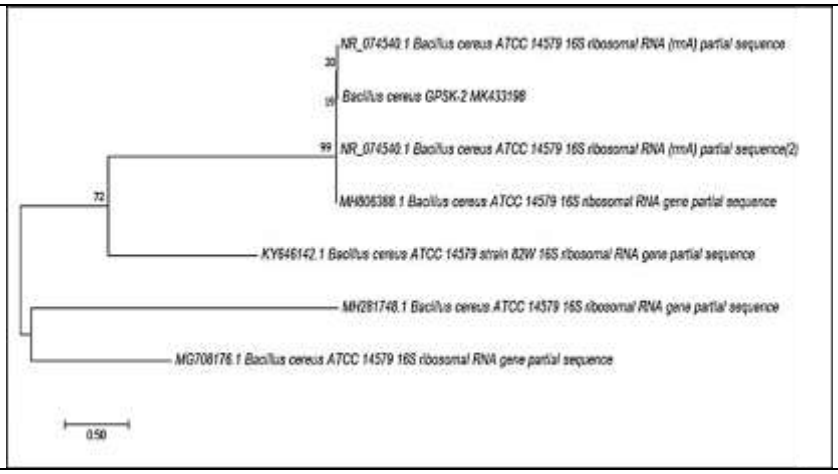
Domain: Bacteria
Division: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Bacillaceae
Genus: Bacillus
Species: flexus
Strain: GPSK 1



Phylogenetic analysis and divergent linkage of *Bacillus flexus* GPSK 1 (MK433197) from cluster of *Bacillus flexus*

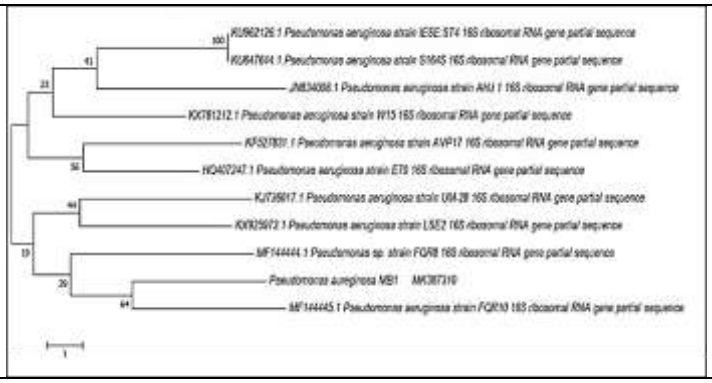
Identification of GPSK2

1512 bp of 16S rRNA gene sequence was amplified from GPSK 2. GPSK 2 strain blast in ex-taxon showed pair wise sequence similarity search showed that 16S rRNA gene sequence of GPSK 2 had 99 % similarity to 16S rRNA partial sequence of *Bacillus cereus* strain Xpp-15 (Accession number : MK184559), 98 % similarity to 16S rRNA partial sequence of *Bacillus cereus* strain FORC60 (Accession number: CP020383). Phylogenetic analysis based on 16S rRNA partial gene sequence revealed that GPSK-2 was closely related to *Bacillus cereus***(Figure:6)** .Based on consistence results of sequence analysis of the 16S rRNA partial gene, GPSK 2 was identified as *Bacillus cereus*. The sequence was deposited in NCBI as *Bacillus cereus* GPSK 2 with accession number **MK433198** GEN BANK has the taxonomic position as follows:

Domain: Bacteria Division: Firmicutes Class: Bacilli Order: Bacillales Family: Bacillaceae Genus: Bacillus Species: cereus Strain: GPSK 2	
	Phylogenetic analysis and divergent linkage of *Bacillus cereus* GPSK 2(**MK433198**) from cluster of *Bacillus cereus*

Identification of GSPK3

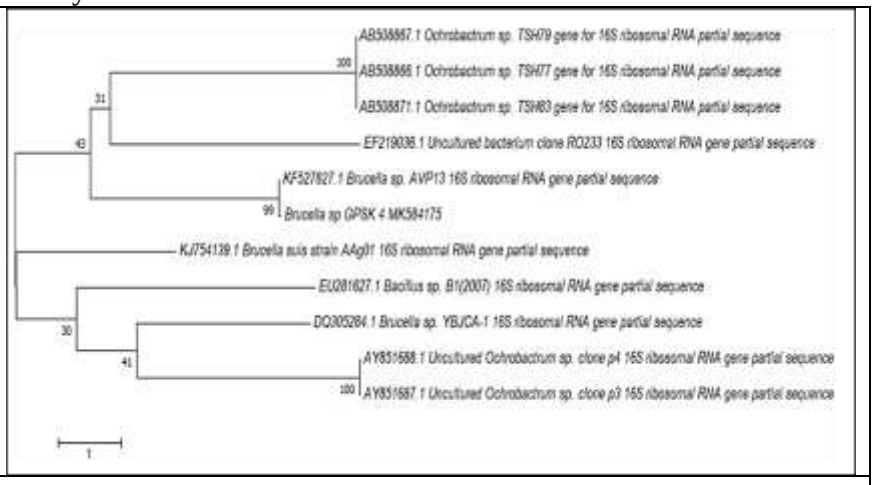
787 bp of 16S rRNA gene sequence was amplified from MB 1. MB 1 strain blast in ex-taxon showed pair wise sequence similarity search showed that 16S rRNA gene sequence of MB 1 had 99 % similarity to 16S rRNA partial sequence of *Pseudomonas aeruginosa* FQR 10 (Accession number : MF144445). Phylogenetic analysis based on 16S rRNA partial gene sequence revealed that MB 1 was closely related to *Pseudomonas aeruginosa* (**Figure:7**) .Based on consistence results of sequence analysis of the 16S rRNA partial gene, GPSK 3was identified as *Pseudomonas aeruginosa*. The sequence was deposited in NCBI as *Pseudomonas aeruginosa* MB 1 with accession number **MK387310** GEN BANK has the taxonomic position as follows:

Domain: Bacteria Phylum: Proteobacteria Class: Gamma proteobacteria Order: Pseudomonadales Family: Pseudomonadaceae Genus: Pseudomonas Species: aureginosa Strain: MB 1	
	Phylogenetic analysis and divergent linkage of Pseudomonas aeruginosa **MB1 (MK387310)** from cluster of Pseudomonas aeruginosa

Identification of GPSK4

994 bp of 16S rRNA gene sequence was amplified from GPSK 3. GPSK **3** strain blast in ex-taxon showed pair wise sequence similarity search showed that 16S rRNA gene sequence of GPSK 3 had 99 % similarity to 16S rRNA partial sequence of *Brucella sp*AVP 13 (Accession number : KF527827). Phylogenetic analysis based on 16S rRNA partial gene sequence revealed that GPSK 3 was closely related to *Brucella sp*strain **(Figure:8)** .Based on consistence results of sequence analysis of the 16S rRNA partial gene, GPSK 3 was identified as *Brucella sp*. The sequence was deposited in NCBI as *Brucella sp*GPSK 3 with accession number **MK584175** GEN BANK has the taxonomic position asfollows:

Domain: Bacteria
Division: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Bacillaceae
Genus: Brucella sp
Strain: GPSK 3



Phylogenetic analysis and divergent linkage of Pseudomonas aeruginosa **MB1 (MK387310**) from cluster of Pseudomonas aeruginosa

Growth analysis of GPSKisolates:

Determination of GrowthCurve:

Code	Log Phase	Stationary Phase
GPSK 1	7 hrs of Log phase (from 160 to 610 mins)	2 hrs of Stationary phase 1 ½ hrs from (670 to 730 mins)
GPSK 2	10 hrs of Log phase (from 90 to 670 mins)	2 hrs of Stationary phase from (640 to 730 mins)
GPSK 3	10 ½ hrs of Log phase (from 90 to 670 mins)	1 hrs of stationary phase from (640 to 730 mins)
GPSK 4	8 ½ hrs of Log phase (120 to 610 mins)	2 hrs of Stationary phase from (640 to 730 mins)

Colony Forming Unit

After Optimization CFU of individual isolate was measured and illustrated all the isolates initiated log phase at 7th hour and were in exponential growth from 7th hr to 11th hr. During this stage there was an increase in the optical density 600nm. The stationary phase begins at the 8thh of growth. GPSK 2, GPSK 3 and GPSK 7 were found to be more active and sustainable in all 3 seasons. All 9 Isolates showed variation in growth pattern.

A decline in optical density was observed at the 12th from the time of incubation

Bio-Processing:

Effect of physical parameters on LAP

Growth optimization of physical and chemical parameters were carried out in selected bacteria individually at different Incubation Temperature, Incubation Period, pH, Carbon source, Nitrogen source, phosphate sources, metal elements and Amino acids for

optimization of L-Asparaginase activity for submerged fermentation.

Effect of Incubation Temperatures on LAP

All 4 GPSK isolates were grown in Modified M9 broth at three different temperatures 35^0C, 37^0 C, 40^0C and LAP wasanalysed. All 4 GPSK isolates showed maximum LAP at 37^0C **.**At 37^0C, LAP was reported high in GPSK-4 (32.54 IU) followed by GPSK-3 (19.45 IU), *Bacillus flexus* GPSK-1 (18.16 IU) and GPSK-2 (12.32 IU). At 37^0c variation of significance was observed in all GPSK isolates (F-5.14, P < 0.03) with confidence level 95%. Hence 37^0C is selected as optimized temperature.

Effect of Incubation period on LAP

All 4 GPSK isolates were grown in M9 broth at four different incubation periods 24 Hrs, 48 Hrs, 72 Hrs and 96 Hrs and analysed LAP.Out of 9 GPSK isolates showed maximum LAP at 72 HrsLAP was reported high in GPSK-3 (67.64 IU) followed by GPSK-4 (62.13 IU), *Bacillus flexus* GPSK-1 (57.72 IU), GPSK-2 (41.54 IU), shows 40.66 IU at 72 Hrs incubation period. Variation of significance was observed in all GPSK isolates (F-4.06, P < 0.03) with confidence level 95%.

Effect of pH onLAP

All 4 GPSK isolates were grown in M9 broth at different pH ranging from 5- 17. LAP was analysed.4 GPSK isolates showed variation in LAP in different pH**.**LAP was reported high in GPSK-3 (32.54 IU) followed by *Bacillus flexus* GPSK-1 (18.38 IU) at pH-7. At pH-10 maximum LAP was observed in GPSK-4 (29.3 IU), and GPSK-2 (19.85 IU).

In summary GPSK-1, GPSK-3, showed maximum LAP at pH -7, GPSK-2, GPSK-4, showed maximum LAP at pH-10. Variation of significance was observed in all GPSK isolates (F-2.84, P < 0.03) with confidence level 95%.

Effect of Additives and Inducers onLAP

Bio-processing of LAP in presence of additives was analysed in Smf by supplementing different Carbon sources, Nitrogen Sources, Phosphate sources, Amino acids and Metal ions in the productionmedium.

Effect of Carbon sources onLAP

Optimization production of LAP was studied with 16 different carbon sources at standardized pH and Temperature **.**

In *Bacillus flexus* GPSK-1, maximum LAP activity was reported in Galactose (71.23 IU). LAP was increased by 292.23 % in Galactose followed by D-Ribose (264.59 %), Xylose (258.59 %), Dextrose (214.42 %), Rhamnose (203.63 %), Fructose (206.35 %), Sorbitol (156.27 %), Maltose (148.45 %), D-Trehalose (77.09%), Glycerol (76.98 %), Lactose (76.26 %), Starch (45.87 %), Sucrose (22.95 %), Mannitol (21.69 %), compared to control **.**In GPSK-2, maximum LAP activity was reported in Fructose (89.23 IU). LAP was increased by 624.26 % in Fructose followed by Galactose (461.93%), Glycerol (429.30%), Dulcitol(356.25%),Dextrose(347.72%),Xylose (344.72%),Sorbitol (267.12 %), Rhamnose (259.25 %), D-Ribose (258.11 %), Starch (208.27 %),Mannitol (161.03 %), D-Trehalose (161.03 %), D-Arabinose (82.95%) compared to control

In GPSK-3, maximum LAP activity was reported in Xylose (87.69 IU)LAP was increased by 169.48 % in Xylose followed by Fructose (82.69 %), Galactose (72.74 %),D-Ribose (66.59 %), Rhamnose (38.66 %) compared to control **.**

In GPSK-4, maximum LAP activity was reported in Rhamnose (67.45 IU). LAP was increased by 246.78 % in Rhamnose followed by Sorbitol (178.81 %), Xylose (139.28%), Starch (132.44 %), Dulcitol (126.83 %), Glycerol (65.14%),Fructose(65.03%),D-Arabinose(36.19%),Dextrose(19.02%),D-Ribose(18.87%), Maltose (13.62 %) compared to control **.**

Effect of Nitrogen sources onLAP:

Optimization production of LAP was studied with twelve different nitrogen sources at standard pH and Temperature.

In GPSK-1, maximum LAP activity was reported in Urea (66 IU). LAP was increased by 263.43 % in urea followed by Ammonium sulphate (149.06%), Beef (145.3 %), sodium nitrate (97.96 %), Tryptone (92.45%), ammonium borate (72.13%), Potassium nitrate (46.09%), Yeast extract(27.31%), Peptone(15.63%), ammonium oxalate

(17.29%), Casein (11.56%), ammonium chloride (4.35%) compared to control **.**

In GPSK-2 ,Maximum LAP activity was reported in Creatinine (66 IU).LAP was increased by 435.71 % in Creatinine followed by ammonium borate (315.82 %), ammonium oxalate (302.27 %),Casein (186.20 %), sodium nitrate (267.12%), Ammonium sulphate (153.73%), Urea (169.96%), Peptone (99.35%),Yeast extract (81.16%), ammonium chloride (72.32%), Beef (58.76%) compared to control**.**

In GPSK-3 maximum LAP activity was reported in ammonium oxalate (78.56 IU). LAP was increased by 141.42 % in ammonium oxalate followed by Creatinine (100.46%), Urea (70.92%), Beef (21.57%), Casein (66.59%), Peptone (54.21%),ammonium chloride (2.21%) compared to control **.** In GPSK-4 maximum LAP activity was reported in ammonium oxalate (74.21 IU). LAP was increased by 281.54 % in ammonium oxalate followed by Urea(252.64%),Beef (225.09 %),Casein (132.54 %) Ammonium sulphate (103.39%), Peptone(103.18%),Tryptone(30.17 %) Creatinine(19.79 %),sodium nitrate (9.51%) compared to control

Effect of Amino acids onLAP. Optimization production of LAP was studied with 16 different amino acids at standardizes pH and Temperature **.**

In *Bacillus flexus* GPSK-1 LAP was reported more in Arginine (87 IU). LAP was increased by 379.07% in Arginine followed by Alanine (313.00%), Leucine (241.41%), Glutamine (208.37 %), Methionine (92.73%), DL-Alanine (43.17%), Proline (37.67 %), DL-phenyl alanine (32.16 %), DL-aspartic acid (21.15 %), Tryptophan (4.63 %) compared to control**.**

In GPSK-2 maximum LAP was reported in DL-aspartic acid (98 IU). LAP was increased by 695.45% in DL-aspartic acid followed by Histidine (427.60 %),DL- Alanine (403.25 %) , Glycine (354.55 %), Tyrosine (338.31 %), Alanine (265.26 %), Glutamine (159.74 %), Tryptophan (113.80%), Leucine (102.92%), L-phenyl alanine (102.92 %), Arginine (70.45%), DL-phenyl alanine (70.45%), Proline (21.75%), Methionine (42.37%) compared to control**.**

In GPSK-3 maximum LAP was reported in Tryptophan (59.24 IU). LAP was increased by 82.05% increase Tryptophan followed by Histidine (75.17%), Glutamine (50.58%), DL-phenyl alanine (38.94%), DL-Alanine (38.29%), Alanine (16.78%), Cysteine (12.20 %), Leucine (2.21%), Glycine (0.09%) compared to control**.**

In GPSK-4 maximum LAP was reported in Arginine (95 IU). LAP was increased by 388.43% in Arginine followed by Tryptophan (301.03 %), Tyrosine (234.19%), Glycine (229.05 %), Alanine(213.62%), L-glutamic acid (213.62%),Leucine (151.93%), Glutamine (115.94%), , Methionine (79.95 %), DL-Alanine (64.52%),Cysteine (49.10%), Histidine (43.96 %), L-phenyl alanine (33.68%),Proline (28.53%), DL-phenyl alanine (28.53 %) ,DL-aspartic acid(7.97%) compared to control**.**

Effect of Metal salts on LAP

Optimization production of LAP was studied with 47 different metal salts at standardizes pH -7 and Temperature **.**

In *Bacillus flexus* GPSK-1 LAP was reported to be high FeCl3 (44.39 IU). LAP was increased by 144.43 % in FeCl3 followed by NaCl (126.87 %), ferric citrate (121.03%),KCl(120.20%),$AgNO_3$ (104.40%),$MgCl_2$(101.65%),$MgSO_4$(88.93 %),Zn(88.10%),$BaCl_2$(87.22%),copper-phosphate(84.80%),$MnSO_4$(83.03%), Se (82.59 %), $HgCl_2$ (75.05 %), lead acetate (72.08 %), cupric nitrate trihydrate (70.70%),AlNO3 (70.20 %), $CuSO_4$ (65.19%), aluminum sulphate (25.49 %), barium bromide (59.14 %), FeSO4 (45.53 %), potassium carbonate (37.39%), Sodium tungstate (32.27 %), ammonium molybdate (32 %), $CaCO_3$ (34 %), Potassium thiocyanate(30.19%),Sodiumthiosulphate(25 .19%),Potassiumferricyanide(22.08 %), Potassium acetate (21.16 %), sodium acetate (20.17 %), ammonium ferrous sulphate (20 %), $LiSO_4$ (19.98 %), zinc sulphate (14.69 %), Ammonium cericsulphate (11.94 %), NaOH (10.99 %) compared to control**.**

In GPSK-2 maximum LAP was reported in FeSO4 (42.06 IU). LAP was increased by 241.39 % in FeSO4 followed by Sodium thiosulphate (235.87 %), $BaCl_2$ (232.46 %),

ammonium thiocyanate (225.97 %), barium bromide (224.67 %), $MnSO_4$ (216.55 %), Potassium thiocyanate (207.30 %), lead acetate (197.56 %), KNO_3 (193.66 %), Sodium .hydrogen carbonate (187.5 %),$CuSO_4$ (188.23 %),$LiCl_2$ (185.30 %), Zn (187.74 %), Potassium acetate (184.74 %),Potassium ferricyanide (176.05 %), FeCl3 (175.56 %), Se (170.69 %), ammonium molybdate (168.83 %), $AgNO_3$ (160.71 %), $MgSO_4$ (151.46 %), $HgCl_2$ (117.69 %), copper-phosphate (177.35 %), $ZnCl_2$ (141.47 %), ferric citrate (125.24 %), potassium carbonate (123.13 %), Trisodium citrate (122.64 %), $LiSO_4$(103.81 %), sodium acetate (96.59%), ammonium ferrous sulphate (97.40 %),Sodium tungstate (88.55 %), NaOH (87.66 %), $CaCO_3$ (85.22 %),ammonium ceric sulphate (63.71 %), zinc sulphate (45.61 %), aluminum sulphate (42.24 %), $MgCl_2$ (40%), $NaCl_2$ (15.66%), KCl (13.63 %) compared to control**.** In GPSK- 3 maximum LAP was reported in copper-phosphate (49.08 IU). LAP has increased by 50.82% in copper-phosphate followed by NaCl (49.96 %) , $HgCl_2$(40.25%),Potassiumferricyanide(35.83%),Potassiumthiocyanate(29.65%), $MgSO_4$(20.43%),calciumoxalate(20.46%),$CaCO_3$(20.40%),$LiCl_2$(19.85%), FeSO4 (16.10 %), $MgCl_2$ (14.01 %), Sodium thiosulphate (12.47 %),$MnSO_4$ (11.18 %), AlNO3 (8.38 %), $AgNO_3$ (8.14 %), Sodium tungstate (6.51 %),lead acetate (2.15%), ferric citrate (1.72 %), Se (1.72 %) compared to control**.** In GPSK- 4 maximum LAP was recorded in AlNO3 (49.81 IU). LAP was increased by 156.09 % in $AlNO_3$ followed by aluminum sulphate (138.45 %), $MnSO_4$ (137.585 %), $CuSO_4$ (128.89 %), $BaCl_2$(125.03 %), Potassium acetate (119.94%),cupric nitrate trihydrate (117.99 %), ferric citrate (117.37 %), Sodium thiosulphate (114.60 %), barium bromide (106.58 %), NaCl (103.08 %),lead acetate (99.12 %), FeSO4 (96.76 %), Se (91.87 %), ammonium molybdate (91.51 %),cobaltous acetate (86.06 %), Zn (88.27 %), Potassium thiocyanate (83.44 %),$AgNO_3$ (79.84 %),$MgSO_4$(76.34 %),cupric sulphate (75.73%), $MgCl_2$ (77.06%), copper-phosphate (65.80%), $LiCl_2$ (65.24%), KNO_3 (62.41%),Potassium ferricyanide (58.25%),$HgCl_2$ (55.78%), FeCl3 (54.24%), $LiSO_4$ (46.01%),Sodium tungstate (44.57%),ammonium carbonate (42.46%), Trisodium citrate (36.19%),$ZnCl_2$ (29.61%),potassium carbonate (27.76%), NaOH (24.26%), ammonium ferrous sulphate (23.90%), $CaCO_3$ (21.64%), $(NH_4)_2SO_4$ (9.30%), ammonium ceric sulphate (4.47%), sodium acetate (8.94%),zinc sulphate (2.82%),Sodium hydrogen carbonate (**0.61**%) compared to control **.**

Solid state fermentation (SSF)

Bioprocessing of LAP and TPC was analysed by solid state fermentation using Sesame oil cake (SOC), Groundnut oil cake (GOC), Coconut oil Cake (COC), Spent Coffee waste (COF), Green Tea (GT) and Red Gram Husk (RGH) as solid substrate was carried by using Single substrate and results are illustrated Variation of Significance was observed in all 8 GPSK isolates with (F-2.84, $P < 0.03$) except GPSK 11 (F-3.86, $P < 0.03$) with confidence level 95%.

Coconut oil Cake (COC): GPSK isolates showed variation in L-Asparaginase activity in COC fermentation and values are compared with Control. Increasing L-Asparaginase was 189.54 % in GPSK-1, 180.44 % in GPSK-2, 138.15 % in GPSK-4.

Spent Coffee waste(COF): GPSK isolates showed variation in L-Asparaginase activity in COF fermentation and values are compared with Control. Increasing L-Asparaginase was 252.11 % in GPSK-2, 35.218% in GPSK-3, 12.956 % in GPSK-4.

Green Tea(GT) GPSK isolates showed variation in L-Asparaginase activity in GT fermentation and values are compared with Control. Increasing L-Asparaginase was 101.87%inGPSK-1, 4.38%inGPSK-2, 1.78%inGPSK-4.

Sesame oil cake (SOC)

GPSK isolates showed variation in L-Asparaginase activity in SOC fermentation and values are compared with Control. Increasing L-Asparaginase was 33.04% in GPSK-1, 204.38 % in GPSK-2, 12.969% in GPSK-3, 400.87% in GPSK-4.

Groundnut oil cake(GOC)

GPSK isolates showed variation in L-Asparaginase activity in GOC fermentation and values are compared with Control. Increasing L-Asparaginase was 265.58% in GPSK- 1, 222.24 % in GPSK-2, 60.257% in GPSK-4.

Red Gram Husk(RGH)

GPSK isolates showed variation in L-Asparaginase activity in RGH fermentation and values are compared with Control. Increasing L-Asparaginase was 154.24 % in GPSK-1, 136..93 % in GPSK-2, 98.832% in GPSK-3, 20.051% in GPSK-4.

Purification ofL-Asparaginase

Enzyme was purified by ammonium sulphate precipitation and DEAE cellulose column from culture filtrate of nine GPSK isolates. L-Asparaginase was partially purified using 20-80% ammonium sulphate for precipitation. Precipitation recovered at 80% ammonium sulphate showed maximum L-Asparaginase activity. 80% ammonium precipitation was further used forpurification.

In GPSK-1 compared the crude enzyme showed 3.75 IU/ml specific activity , whereas ammonium sulphate precipitation has increased specific activity to

4.79 IU/ml with 1.27 fold purification and 57.49% protein recovery. After Dialysis specific activity was increased to 8.75 IU/ml with 2.33 fold purification and 38.49% recovery of protein.

In GPSK-2 compared the crude enzyme showed 3.285 IU/ml specific activity, whereas ammonium sulphate precipitation has increased specific activity to 5.27 IU/ml with 1.60 fold purification and 96.37% protein recovery. After Dialysis specific activity was increased to 8.59 IU/ml with 2.61 fold purification and 74.63% recovery of protein.

In GPSK-3 compared the crude enzyme showed 2.9 IU/ml specific activity , whereas ammonium sulphate precipitation has increased specific activity to 3.53 IU/ml with 1.21 fold purification and 84.59% protein recovery. After Dialysis specific activity was increased to 7.67 IU/ml with 2.64 fold purification and 66.09% recovery of protein **.**

In GPSK-4 compared the crude enzyme showed 4.61 IU/ml specific activity , whereas ammonium sulphate precipitation has increased specific activity to6.62 IU/ml with 1.43 fold purification and 54.63% protein recovery. After Dialysis specific activity was increased to 10.9 IU/ml with 2.36 fold purification and 43.29% recovery of protein.**Molecular weight ofL-asparaginase**

SDS PAGE Analysis was carried out to determine the molecular weight of L-Asparaginase of individual GPSK isolates .Molecular weight of protein subunits was calculated approximately based on standard ladder (250 KD -2 KD) used in the experiment **.**

In GPSK-1 purified enzyme is resolved into 3 protein subunits at 90 KD, 60 KDand20KDindicatingthattheapproximately molecularweightofenzymein170 K.D.In GPSK-2 purified enzyme is resolved into 1 protein band at 90 KD indicating that the approximately molecular weight of enzyme in 90 K.D. In GPSK-3 purified enzyme is resolved into 3 protein subunits at 90 KD, 20 KD and 15 KD indicating that the approximately molecular weight of enzyme in 125K.D. In GPSK-4 purified enzyme is resolved into 4 protein subunits at 90 KD, 20 KD, 37 KD and 15 KD indicating that the approximately molecular weight of enzyme in 162 K.D.

Enzymekinetics

Enzyme kinetics of L-Asparaginase in terms of Km and Vmax wasanalysed at three different (24hrs, 48hrs and 72 hrs) incubation periods by using different concentrationsofsubstraterangingfrom0.01M to1M(0.01,0.03,0.05,0.07,0.09,0.1, 0.3, 0.5, 0.7, 0.9, 1) at different incubation times (24hrs 48hrs and 72 hrs) and Km and Vmax of L-asparaginase of 4 GPSK isolates were illustrated.

At24hrsincubationperiodtheKmandVmaxwas reportedinGPSK-1(Km-2.293 mmol/lit, Vmax-0.03ms^{-1}), GPSK- 2 (Km-1.965 mmol/lit, Vmax-0.025 ms^{-1}, GPSK- 3 (Km-15.625,mmol/lit, Vmax-0.074 ms^{-1}), GPSK-4 (Km-3.64 mmol/lit , Vmax-0.046 ms^{-1}).

At 48 hrs incubation period the Km and Vmax was reported in GPSK-1 (Km-1.328 mmol/lit, Vmax-0.02 ms^{-1}), GPSK- 2 (Km-1.485 mmol/lit, Vmax-0.022 ms^{-1}, GPSK- 3

(Km-2.008, mmol/lit, Vmax-0.025 ms^{-1}), GPSK-4 (Km-1.677mmol/lit, Vmax-0.019 ms^{-1}).

At 72 hrs incubation period the Km and Vmax was reported in in GPSK-1 (Km-1.426 mmol/lit, Vmax-0.017 ms^{-1}), GPSK- 2 (Km-1.945 mmol/lit, Vmax-0.02 ms^{-1}, GPSK- 3 (Km-1.802, mmol/lit, Vmax-0.024 ms^{-1}), GPSK-4 (Km-0.908mmol/lit, Vmax-0.011 ms^{-1}). Various reaction time periods (10mins,20mins,30mins,40 mins and 50min), Temperature (27^0C,37^0C,47^0C,57^0C,67^0C), pH (2.8,4.8, 6.8,8.8,10.8), based on the result all 4 GPSK isolates activity was high at 20mins rection time , 37^0C temperature and pH 6.8 **.** All GPSK enzymes are non haemolyticVariation of Significance was observed in all 9 GPSK isolates with (F-2.21, P < 0.03) with confidence level 95%.

Biosensor formulation:

Biosensor formulation was done by using agar, agarose , Gelatin method. In agar based biosensor formulation 9 GPSK isolates were used and the concentration of substrate .i.e. L-Aspargine is 10^{-2} to 10^{-10}. Based on the results the time taken for color change of the cakes is less in Agar compared to agarose as shown in Table5

Table 5: Colony Formed Units of GPSK isolates under Optimized condition

Isolate	**CFU**
GPSK 1	2.25×10^7
GPSK 2	5.0×10^7
GPSK 3	8.5×10^7
GPSK 4	6.5×10^7

Table 6:Effect of Incubation Temperatures on LAP of 4 GPSK isolates:

Isolate	**Temp (35**°C)	**Temp (37**°C)	**Temp (40**°C)
GPSK 1	15.13 ± 0.02	18.16 ± 0.00	12.16±0.02
GPSK 2	8.45±0.02	12.32±0.01	8.1±0.02
GPSK 3	11.76±0.02	32.54 ± 0.02	10.3±0.02
GPSK 4	8.61±0.02	19.45 ± 0.01	7.35±0.02

Values are mean of triplicates with ± S.E

Table 7: Effect of Incubation period onLAP

Incub. Period	GPSK 1	GPSK 2	GPSK 3	GPSK 4
24hrs	18.16 ± 0.00	12.32 ± 0.01	32.54 ± 0.02	19.45 ± 0.01
48 hrs	40.66±0.02	29.41±0.01	52.57±0.02	35.29±0.01
72 hrs	57.72±0.02	41.54±0.02	67.64±0.02	62.13±0.02
96 hrs	45.26±0.02	31.27±0.02	57.26±0.02	59.13±0.02

Values are mean of triplicates with ± S.E

Table 8: **Effect of pH on LAP of 4 GPSKisolates**

pH	**GPSK 1**	**GPSK 2**	**GPSK 3**	**GPSK 4**
5	6.65±0.01	6.42±0.01	8.23±0.01	10.23±0.01
6	15.23±0.01	7.58±0.01	12.56±0.01	15.64±0.01
7	18.16 ± 0.00	12.32 ± 0.01	32.54 ± 0.02	19.45 ± 0.01
9	14.54±0.01	17.42±0.01	23.5±0.01	22.46±0.01
10	12.03±0.01	19.85±0.01	20±0.01	29.3±0.01
12	--	16.42±0.01	17.09±0.01	24.12±0.01
14	--	--	8.66±0.01	16.72±0.01

Table 9: Effect of Carbon sources on L-Asparaginase activity (IU/ml) of *Bacillus flexus*

Effect of Carbon sources on L-Asparaginase activity (IU/ml) of *Bacillus flexus*

Carbon sources	GPSK 1	GPSK 2	GPSK 3	GPSK 4
Control	18.16 ± 0.00	12.32 ± 0.01	32.54 ± 0.02	19.45 ± 0.01
Sucrose	22.51±0.01	8.56±0.01	21.32±0.01	13.26±0.01
Lactose	32.01±0.01	2.31±0.01	32.46±0.01	11.23±0.01
Maltose	45.12±0.01	65.21±0.01	24.61±0.01	22.1±0.01
Mannitol	22.1±0.01	32.16±0.01	11.23±0.01	16.51±0.01
Fructose	55.64±0.01	89.23±0.01	59.45±0.01	32.1±0.01
Dulcitol	11.23±0.01	56.21±0.01	8.21±0.01	44.12±0.01
Sorbitol	46.54±0.01	45.23±0.01	22.45±0.01	54.23±0.01
D-Trehalose	32.16±0.01	32.16±0.01	13.26±0.01	12.02±0.01
D-Arabinose	31.26±0.01	22.54±0.01	24.15±0.01	26.49±0.01
Galactose	71.23±0.01	69.23±0.01	56.21±0.01	16.54±0.01
Xylose	65.12±0.01	54.79±0.01	87.69±0.01	46.54±0.01
Rhamnose	55.14±0.01	44.26±0.01	45.12±0.01	67.45±0.01
Starch	26.49±0.01	37.98±0.01	20±0.01	45.21±0.01
Glycerol	32.14±0.01	65.21±0.01	21.56±0.01	32.12±0.01
Dextrose	57.1±0.01	55.16±0.01	26.31±0.01	23.15±0.01
D-Ribose	66.21±0.01	44.12±0.01	54.21±0.01	23.12±0.01

Table 10:Effect of Nitrogen sources onLAP

Sources	GPSK 1	GPSK 2	GPSK 3	GPSK 4
Control	18.16 ±0.00	12.32 ±0.01	32.54 ±0.02	19.45 ±0.01
Yeast extract	23.21±0.02	22.32±0.02	21.62±0.02	15.26±0.01
Sodium nitrate	35.95±0.01	45.23±0.03	12.3±0.01	21.3±0.00
Ammonium sulphate	45.23±0.00	31.26±0.04	29.56±0.03	39.56±0.03
Urea	66±0.03	33.26±0.02	55.62±0.0	68.59±0.01
ammonium oxalate	21.3±0.04	49.56±0.01	78.56±0.02	74.21±0.02
Ammonium borate	31.26±0.02	51.23±0.00	23.23±0.01	10.32±0.01
Creatinine	12.3±0.01	66±0.01	65.23±0.02	23.3±0.00
ammonium chloride	18.95±0.00	21.23±0.00	33.26±0.00	15.02±0.03
Potassium nitrate	26.53±0.03	12.3±0.02	12.26±0.01	18.65±0.02
Tryptone	34.95±0.01	10±0.03	21.03±0..05	25.32±0.01
Beef	44.56±0.00	19.56±0.01	39.56±0.00	63.23±0.03
Peptone	21±0.04	24.56±0.02	54.21±0.01	39.52±0.00
Casein	20.26±0.03	35.26±0.03	10±0.03	45.23±0.01

Values are mean of triplicates with ± S.E

Table: 10 Effect of Amino acids on L-Asparaginase activity(IU/ml) of 4 GPSK isolates

Amino acids	GPSK 1	GPSK 2	GPSK 3	GPSK 4
Control	18.16 ±0.00	12.32 ±0.01	32.54 ± 0.02	19.45 ± 0.01
Proline	25 ± 0.00	15± 0.02	21.32± 0.02	25± 0.02
Cysteine	12± 0.03	10 ± 0.00	36.51± 0.03	29± 0.02
Methionine	35± 0.03	17.54±0.03	23.14 ± 0.00	35± 0.03
Leucine	62± 0.02	25± 0.03	33.26± 0.03	49± 0.03
Tryptophan	19± 0.02	26.34±0.03	59.24± 0.02	78± 0.03
Tyrosine	14± 0.03	54± 0.02	21.06± 0.02	65± 0.03
Arginine	87± 0.03	21± 0.03	22.94± 0.03	95± 0.03
Glycine	15± 0.03	56± 0.03	32.57± 0.03	64± 0.03

DL-phenyl Alanine	24± 0.03	21± 0.03	45.21± 0.03	25± 0.03
L-glutamic acid	17± 0.03±0.03	10± 0.03±0.03	32±0.03±0.03	61± 0.03±0.03
DL-Alanine	26± 0.03	62± 0.03	45 ± 0.00	32± 0.03
DL-aspartic acid	22± 0.03	98± 0.03	21.03± 0.03	21± 0.03
L-phenylalanine	13± 0.03	25± 0.03	26 ± 0.00	26± 0.03
Histidine	18± 0.03	65± 0.03	57± 0.03	28± 0.03
Glutamine	56± 0.03	32± 0.03	49± 0.02	42± 0.02
Alanine	75± 0.03	45± 0.03	38± 0.03	61± 0.03

Table 11: Effect of Phosphate sources on L-Asparaginase activity (IU/ml) of 4 GPSK isolates

Phosphate sources	**GPSK 1**	**GPSK 2**	**GPSK 3**	**GPSK 4**
Control	18.16 ± 0.00	12.32 ± .01	32.54 ± 0.02	19.45 ± 0.01
Disodium hydrogen phosphate	16.23±0.02	10.23±0.00	21.03±0.03	17± 0.03
Dipotassium hydrogen phosphate	26.23±0.02	12.02±0.03	18.26±0.02	16.5±0.00
Sodium dihydrogen phosphate	32.06±0.02	23.23±0.02	20.13±0.03	18± 0.03

Agar method time response:

Bacterial isolates	**Response time(Seconds)**	**Conc.ofL-Asparagine**
GPSK-1	7sec	10^{-8}
GPSK-2	7sec	10^{-2}
GPSK-3	30 sec	10^{-10}
GPSK-4	10 sec	10^{-4}

Agarose method:

Bacterial isolates	**Response time (Minutes)**	**Concentration of L-Asparagine**
GPSK-1	1.42	10^{-4}
GPSK-2	2	10^{-6}
GPSK-3	2	10^{-8}
GPSK-4	3	10^{-2}

CONCLUSION:

All isolates belong to *Bacillus* sp. showed variation in growth optimization. Smf with formulated M9 medium supplemented with additives and inducers showed significant enhancement in L-Asparaginase production during Bio-processing assumed that this marine enzyme from marine origin is highly labile to the stringent experimental

conditions. Individual isolates are highly specific to additives and inducers during Bio- processing by smf. Results summarized that each isolate has specific set of inducers and and additives. Apart from, these marine isolates showed variation in Km and Vmax of L-Asparaginase and diversity in their protein characterization and molecular weight. These findings revealed that these marine bacteria are highly diversified organism and exclusive in physiological behavior and expression of enzyme proteins. However, smf is not a cost effective process. SSF in presence of domestic and agri- waste solid substrates revealed that protein waste such as (RGH) is highly effective in the production of L-Asparaginase compared to oil cakes assumed that extracellular enzymes of the marine isolates are not promising in hydrolysis of fatty acids, a positive marker for using the enzyme as a human friendly drug. Application of solid state fermentation with selective substrates under stringent cultural conditions should be a boom and promising cost effective, ecofriendly technology to pharmaceutical industries. This investigation is first report on production of L-Asparaginase from Non-recombinant, native bacterial strains of marine origin isolated from mangrove ecosystem.However, there are no reports so far on characterization of L-Asparaginase in*Brucella sp.* In present research, characterization of L-Asparaginase of *Brucella* sp is the first report and one of the highlights.Our study proves that we can increase production level of asparaginase by adding inducer, L-asparagine in industrial level.The effect of Solid state fermentation on L-asparaginase activity produced was studied here using agri waste substrates. This work would be precious for the judicious selection of different process parameter levels. Easy and time limited diagnosis bybiosensor.

REFERENCES:

1. Faulkner, W. "Marine microbial biodiversity and drug discovery. Abstract in: - Natural products from marine microorganisms". An international symposium held under the auspices of the European society for marine biotechnology., Greifswald, 2002 Germany.
2. Proksch, P., Edrada, R. A., Ebel, R. "Drugs from status and microbiological implications".Biotechnol., 59(2002)125-134.
3. Bultel, P., Berge, V.Debitus, J.P., Nicolas, C. "Metabolites from the sponge- associated bacterium *Pseudomonas* species". Mar. Biotechnol., 1999, 1384- 390.
4. Sarma, M. V. R. K., Saharan, K., Prakash, "Application of Fluorescent Pseudomonads Inoculant Formulations on *Vigna mungo*". International Journal of Biological and Life Sciences., 2009, 5:1
5. Duval, M. et al. Comparison of Escherichia coli–asparaginase with Erwinia-asparaginase in the treatment of childhood lymphoid malignancies: results of a randomized European Organisation for Research and Treatment of Cancer-Children's Leukemia Group phase 3 trial. Blood 99 (2009) 2734–2739.
6. Sushma, C., Anand, A. P. &Veeranki, V. D. Enhanced production of glutaminase free L-asparaginase II by Bacillus subtilis WB800N through media optimization. Korean J. Chem. Eng. 34(2017), 2901–2915.
7. Farag, A. M., Hassan, S. W., Beltagy, E. A. & El-Shenawy, M. A. Optimization of production of anti-tumor L-asparaginase by free and

immobilized marine Aspergillus terreus. Egypt Aqu. Res. 41(2015) 295–302 .

8. Trilokchandran, B., Agrawal, P. & Krishna, V. Screening studies of microbial species for L-asparaginase production. J. Env. Res. Dev. 10(2016) 712–716.
9. Badoei-Dalfard, A. L-asparaginase production in the pseudomonas pseudoalcaligenes strain JHS-71 isolated from JooshanHotspring. Mol. Biol. Res. Commun. 5(2016) 1–10.
10. Nielsen, J. et al. Building a bio-based industry in the Middle East through harnessing the potential of the Red Sea biodiversity. Appl. Microbiol. Biotech. 101(2017), 4837–4851.
11. Lakshmi, T. V., Mallika, D. S., Amos, S. J. & Kasturi, K. Marine L-asparaginase: A novel microbial therapeutic approach for cancer. Int. J. Pharm. Sci. Rev. Res. 33(2015), 228–234 .
12. Kumar, S., Prabhu, A. A., Dasu, V. V. &Pakshirajan, K. Batch and fed-batch bioreactor studies for the enhanced production of glutaminase-free L-asparaginase from Pectobacteriumcarotovorum MTCC 1428. J. Prep. Biochem. Biotechnol. 47(2017), 74–80 .
13. Goswami, R., Veeranki, V. D. & Hegde, K. Enhanced production of novel glutaminase free recombinant L-asparaginase II of Erwinia Carotovora Subsp. AtrosepticaScri 1043 in Escherichia coli BL21 (DE3) in a batch and fed-batch culture. Austin. J. Biotechnol. Bioeng. 2, www.austinpublishinggroup.com (2015).
14. Vimal, A. & Kumar, A. Biotechnological production and practical application of L-asparaginase enzyme. Biotech. Gen. Eng. Rev. 33(2017), 40–61 .
15. Krishna Raju Patro and NibhaGupta, Extraction, purification an characterization of L-Asparaginase from *Penicillium* sp.
16. Imada, A., Igarasi, S., Nakahama, K. and Isono, M..Journal of General Microbiology.,76(1973),85-99.
17. Aneja, K. R. "Experiments in Microbiology Plant Pathology and Biotechnology".4th edition, New Age International Publishers, New Delhi, India,2003.
18. Hucker,G.J.andConn,H.J."MethodsofGramstaining".Tech.Bull .N.Y. Agric. Exp. Sta. no. 93,1923.
19. Aneja, K. R., "Experiments in Microbiology Plant Pathology and Biotechnology".4th edition, 2003, New Age International Publishers, New Delhi, India.
20. Salle, A. J.**,** "Laboratory Manual on Fundamental Principles of Bacteriology".Mc Graw-Hill Book Co. 1948,3rdedition.
21. Seeley, H.W. and VanDemark, P.J., "Microbes in Action: A Laboratory Manual of Microbiology". 3rd Ed, 1981, W.H.Freeman and Company,USA.
22. Cappuccino, J.G., Sherman, N. "Techniques for isolation of pure culture In Microbiology: a laboratory manual.(VI Ed.)". Pearson Education, Inc. Singapore. Collee,JG, Duguid, JP, Frasee, AG and Marmion, BP 1989, published by Pearson Education (Singapore)., Pte.Ltd.63-66; 2002, 149-185;428-431.
23. MacFaddin J. F.**,** "Biochemical tests for

Identification of Medical Bacteria". 3rd Ed., Lippincott, Williams and Wilkins, 2000, Baltimore.

24. Sambrook, J. and Russell, D.W. **,**"Molecular cloning: A laboratory manual". 1(3): Cold Spring HarbourPress, 2001, Nova.York.
25. Hosamani, R. and Kalival, B. B.,"L-asparaginase - an anti-tumour agent production by *Fusarium equiseti*using solid-state fermentation". International Journal of Drug Discovery.,3(2), (2011)88-99.
26. Thaer, T.A., Ellaiah, P. **2013**. "L-asparaginase production by *Streptomycete*and optimization of production parameters". J Pharmaceut Biomed Sci., 29(2013) 859-869.
27. Radhika Tippani and GirishamSivadevuni.,"Nutritional factors effecting the production of L-asparaginase by the *Fusarium* sp".African Journal of Biotechnology.,11(15), (2012), 3692-3696.
28. Krishna Raju Patro and Nibha Gupta.,Extraction, purification an characterization of L-Asparaginase from *Penicillium* sp.
29. Mukherjee, J., Majumdar, S., Scheper, T., "Studies on nutritional and oxygen requirements for production of Lasparaginase by *Enterobacter aerogenes*". Appl Microbiol Biotechnol., 53(2), (2000)180-184.
30. Bertini, I., Gray, H. B., Stiefel, E. I and Valentine, J. S."Biological inorganic chemistry". University Science Books, 2006, Sausalito.
31. Siddalingeshwara, K.G., "Production of tumour inhibitory L- asparaginase through solid state fermentation from *Aspergillus terreus*".Ph.D Thesis, GulbargaUniversity., 2005, Gulbarga.
32. Laemmli, U.K., "Cleavage of structural proteins during the assembly of the head of bacteriophage T4".Nature.,227,(1970)680-685.
33. Seeley, H.W. and VanDemark, P.J.,"Microbes in Action: A Laboratory Manual of Microbiology". 3rd Ed, W.H.Freeman and Company, 1981,USA.
34. Prakasham, R.S., Rao, C.S., Rao, R.S., Lakshmi, G.S. and Sarma, P.N., "L-asparaginase production by isolated *Staphylococcus* sp.-6A: design of experiment considering interaction effect for process parameter optimization". Journal of Applied Microbiology.,102(2007),1382-1391.

by submerged fermentation. International Journal for I