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Isolation of L-asparginase from marine bacterium *Bacillus subtilis* and its characterization

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Microbial L-asparginases has wide range of applications as therapeutic agents and in industries. In the present study, 57 bacterial isolates from Konark beach, Bhubaneshwar were screened for L-asparginase production and KBI-13 isolate was found to be potential producer strain. KBI-13 was identified as *Bacillus subtilis* at molecular levels. During production optimization, pH (8.0), temperature (40 ºC), carbon and nitrogen sources (dextrose- 0.5 %; yeast extract 1 %), aeration conditions, metal salts ($FeSO_4$) and NaCl (4 %) were found to be optimum. The enzyme was produced under optimized conditions and was purified by sephadex G-50 column and the purification was obtained upto 61.54 fold. The activity of enzyme was increased upto pH 8.0 and temperature 40 ºC and its stability was observed upto 16 hrs at 40 ºC temperature and pH 8.0. Pretreatment of 0.5 mM $CaCl_2$ increased the enzyme activity upto 20 % while, 250 mM concentration of L-aspargine was suitable for optimum activity of enzyme which was further confirmed by values of Vmax (1.25 µM/min) and Km (0.05 mM). The reaction end products did not show any significant change in enzyme activity.

[**Keywords**: *Bacillus subtilis*, KBI-13, L-asparginase, Marine isolate]

Introduction

L-asparginases (E.C.3.5.1.1) are the enzymes that are involved in catalyzation during hydrolysis of aspargine to aspartic acid and ammonia[1]. It is widely used as an antitumour and anti-neoplastic agent to treat the cancers and some tumor forming cell diseases in human. The antitumor activity was reported by Lee *et al.*[2] by L-asparginase from *Erwinia carotovora*. Since, L-asparginase is eco-friendly, harmless and can be easily delivered to the location directly it acts as an ideal agent for the treatment of such diseases. L-asparginase is also used as technological adjuvant in food making to prevent the formation of acrylamide in over cooked and stove baked foods in particular in potato chips. The acrylamide formation takes place after reaction between aspargine and reducing sugars, deaminization of aspargine avoids this reaction and acryamide formation is prevented[3].

L-asparginase is widely distributed in nature and can be isolated from serum of certain rodents, many tissues, bacteria and plants[4,5]. Generally L-asparginase is isolated from microorganisms due to ease of large scale production, easy recovery and high yield. But L-asparginase production in submerged conditions has reportedly shown lower yields as compared to solid state fermentations[6]. Hence, to improvise this problem, production optimization can act as best remedy. The studies for the isolation of L-asparginase is carried for bacteria *Erwinia carotovora*[2,7] and *Bacillus* sp.[8], fungi (*Mucor* sp.[9], and *Penicillium* sp.[10]), yeasts (*Candida utilis*[11]) and actinomycetes (*Streptomyces karnatakensis*[12], *Streptomyces venezuelae*[13]). A key drawback in using L-asparginase in treatment is that it can cause hypersensitivity reaction, coagulopathy (decline in protein synthesis), can cause bleeding of thrombotic actions like stroke due to accumulation of coagulation or anticoagulating factors and an instance of spontaneous response to tumor cells[14].

Keeping in view all above aspects, a study was designed to produce L-asparginase from an isolate of marine environment which can be harmless to humans because saline state of human blood are much related to sea environments.

Materials and Methods

Isolation of marine bacteria

The marine water samples were collected from Konark beach, Bhubaneshwar at a depth of 15 cm in pre-sterilized polyethene bottles using composite random sampling method and stored at 4 ºC till further processing. 10 ml of water was transferred in an Erlenmeyer flask containing 90 ml of Zobell

marine broth prepared for pre-enrichment of samples. The flask was incubated at 37 ^{o}C upto seven days under shaking conditions at 120 rpm in an orbital incubator shaker. A loop full of the enriched broth was streaked onto Zobell marine agar (ZMA) plates and the plates were incubated at 37 ^{o}C for 48-72 hrs. Well isolated discrete colonies were selected and used for further analysis.

Screening of isolates for L-asparginase (LAse) activity

The isolates to be tested were spot inoculated on sterile modified M9 medium plates containing Glucose -15 g/l, $MgSO_4$ - 4 g/l, $(NH_4)_2SO_4$ - 3.5 g/l, K_2HPO_4 - 30 g/l, KH_2PO_4 - 15 g/l, Citric acid - 2 g/l, NaCl - 0.5 g/l, and trace element solution - 1.5 ml/l prepared in 50 % artificial sea water supplemented with 1 % aspargine. The incubation was carried out at 37 ^{o}C for 48-72 hrs, colonies showing pink zones around them[15] were selected as LAse producers. The purified cultures were maintained at 4 ^{o}C on ZMA slants for further use. The colonies such obtained were further purified by making repeated subcultures on ZMA plates.

Quantitative assay

The selected isolates were inoculated in M9 broth and upto 48 hrs in an incubator shaker (120 rpm at 37 ºC). After incubation, cell separation was done by centrifugation for 5 min at 8,000 rpm at 4 ^{o}C. The supernatant was collected and used to measure LAse activity.

Enzyme assay

Direct Nesslerization of ammonia was used for enzyme activity measurement as described by Wriston[16]. The assay was carried by mixing 500 µl of supernatant with 500 µl of L-asparagine (189 mM/l) in Tris-HCl buffer (0.05 M, pH 8.6). The reaction mixture was incubated at 37 ^{o}C for 30 min and 500 µl of trichloroacetic acid (15 %) was used to stop the reaction. Precipitate formed during the reaction was removed by centrifugation at 5000 rpm at 4 ^{o}C for 5 min to eliminate precipitated proteins and supernatant was used for determining the release of ammonia using spectrophotometric method. The method involves addition of 500 µl Nesslers reagent into 500 µl of supernatant and diluting the mixture with distilled water to 5 ml. Optical density was read at 425 nm after 10 min of incubation at room temperature against the blank prepared with distilled water in place of enzyme. The concentration of ammonia released during the reaction was determined from standard curve drawn by using with ammonium sulphate. One international unit of LAse was defined as the amount of enzyme that liberates 1 µM/l of ammonia/ min at 37 ^{o}C. Concentration of protein was determined by Bradfords test using bovine serum albumin as a standard[17].

Identification of LAse producing bacterium

Identification of the selected LAse producing bacteria was carried out at molecular level by 16S rRNA sequencing. DNA from KBI-13 cells was isolated by phenol chloroform extraction method and used as a template for PCR analysis as primers 8F: 5' AGATTTGATCCTGGCTCAG3' and 907R: 5'CCGT CAATTCMTTTRAGTTT3'.

The conditions used for amplification were 94 ºC for 3 min, 52 ºC for 45 sec, 72 ºC for 1 min and final extension step of 72 ºC for 3 min. The product purification after PCR was done by PEG-NaCl method and sequenced by using ABI 3730XL sequencing machine. The assemblage of both forward and reverse sequences was carried out through DNA Baser V35.0 software and alignment was done with other closely related taxa which were retrieved from GenBank database. 16S rRNA gene sequence of KBI-13 was compared with other known bacterial sequences by using NCBI BLASTn and 772 bp sequence obtained was submitted in NCBI GenBank and its Accession number was obtained. The reference sequences required for comparison were downloaded from http://ncbi.nim.nih.gov/ Genbank. CLUSTAL W was used for the sequence alignment which is a multiple sequence alignment program, gaps were removed manually and pair wise evolutionary distances were computed using the Kimura 2-parameter model. Boot strap analysis was carried out using 1000 replicates of original data set and was used for construction of phylogenetic tree in MEGA 5.2.2 using Neighbour joining method.

Enhancement of LAse production using one factor at a time approach

Effect of carbon and nitrogen sources

Six different carbon sources (10 g/l) namely dextrose, lactose, fructose, maltose, sucrose and mannitol and 6 nitrogen sources (5 g/l) including ammonium sulphate, tryptophan, potassium nitrate, yeast extract, meat extract and peptone were added to M9 broth[3]. Active culture of KBI-13 was inoculated to all the flasks separately. The flasks were incubated

upto 5 days on a rotary shaking incubator and after incubation activity of LAse was evaluated as mentioned earlier.

Effect of incubation period

KBI-13 was inoculated to M9 broth containing 1 % aspargine and was incubated upto 7 days. After every 24 hrs interval enzyme activity was determined as stated earlier.

Effect of temperature and initial pH

M9 broth was inoculated with active culture of KBI-13 and incubated at different temperatures *viz*. 20, 30, 40, 50 and 60 ^{o}C upto five days. Effect of pH was studied by adjusting the pH of the M9 broth to 4, 5, 6, 7, 8, 9 and 10 with 0.1 M NaOH or HCl. All the flasks were inoculated with KBI-13 and incubated at 40 ^{o}C for five days keeping other parameters constant. After 5 days of incubation, enzyme activity of broth was determined.

Effect of NaCl and metal salts

Different concentrations of NaCl (1-10 %) and metal salts ($ZnSO_4$, $MgSO_4$, $MnSO_4$, $NiCl_2$ and $FeSO_4$; 25 and 50 mM) were added to M9 broth. The flasks were inoculated with KBI-13; afterwards flasks were incubated at 40 ^{o}C for five days under shaking conditions. The flasks were subjected for enzyme activity determination after 5 days.

Effect of aeration

To study the effect of aeration, one flask of inoculated M9 broth was incubated at static conditions while one flask was kept on shaking incubator at 120 rpm for five days. After incubation, the flasks were subjected for enzyme activity determination.

Purification of LAse

Ammonium sulphate precipitation was used for coagulting the enzyme by saturating the crude enzyme upto 40-80 % with ammonium sulphate by keeping it at 4 ^{o}C overnight. The contents were then centrifuged at 10,000 rpm for 5 min at 4 ^{o}C. Precipitates were collected separately and were further purified by dialysis. 0.1 M phosphate buffer was used for dissolution of precipitates and dialyzed overnight in pre-activated dialysis tubes against phosphate buffer (pH 7, 0.1 M). The dialyzed sample was used for determining enzyme activity and protein concentration. The dialyzed fraction was dispersed in 0.1 M phosphate buffer (pH 7.2) to be loaded onto sephadex G-50 (25 × 2 cm) column[3]. The column was pre-standardized with phosphate buffer (0.1 M, pH 7.3) and was eluted with the same buffer containing 0.25 M NaCl. For fraction collection, the flow rate was maintained as 0.5 ml/min and subjected for analysis of enzyme activity and determining protein concentration.

Enzyme characterization

The purified LAse activity was tested at different pH, temperature, substrate concentrations and incubation period and in presence of salts of metal ions. Purified enzyme was incubated with 0.05 M buffers of pH 3-10 as per assay conditions and the amount of ammonia liberated was determined. The buffers used were potassium phosphate (pH 3-7), Tris-HCl (pH 8-9), and glycine-NaOH (pH 10). To determine pH stability of LAse, LAse was pre-incubated with buffer of pH 8 for 1000 min and then used for the residual activity determination at different time intervals. Optimum temperature for LAse activity was determined by the incubation of assay mixture at temperature ranging from 20 to 80 ºC. To determine thermal stability of LAse the enzyme was pre-incubated at different temperatures for 1000 min. To study the effect of the incubation time on LAse activity, incubation time of assay mixture was varied in the range of 5 to 45 min[18]. All the experiments for enzyme characterization were performed in triplicates.

Effect of metal ions on purified L-asparaginase activity

The enzyme was incubated with various salt solutions of metal ions (Mg^{2+}, Co^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, Ca^{2+}, Cd^{2+}, Hg^{2+}) including magnesium sulphate, cobaltous chloride, ferrous chloride, copper chloride, zinc chloride, calcium chloride, cadmium chloride and mercuric chloride at 0.5 mM, 1 mM and 2 mM concentration for 30 min at 37 ºC. After incubation, enzyme assay was performed to determine the enzyme activity which was expressed as the percentage of the activity observed without additions of metal ions.

Effect of substrate concentrations on LAse activity

To study the effect of various substrate concentrations, aspargine concentrations in assay mixture was varied from 25-500 mM and assay was performed to determine the enzyme units. The Km and Vmax values were calculated from LB plot.

Effect of reaction end products on L-asparaginase activity

To study end product effects (aspartic acid and ammonia), the enzyme activity was determined by

including 5 mM, 10 mM and 25 mM L-aspartic acid and ammonium chloride in assay mixture and activity was determined as stated earlier.

Results

Isolation of LAse producing marine bacteria

Total 57 bacterial colonies were isolated from water samples collected from Konark beach of Bhubaneswar. Plate assay method was used for screening of the isolates for LAse production potential. From the 57 isolates, 36 showed pink zone formation around colonies on modified M9 medium. However, only 17 isolates showed higher intensity of pink coloration with zone diameter ranging from 0.7 to 4.2 mm. The isolate KBI-13 showing maximum zone diameter of 4.2 cm with 71.42 % (Table 1) LAse hydrolysis efficiency was selected for further studies. The quantitative assay performed to determine LAse activity from KBI-13 showed liberation of 19.6 µM/ml/min ammonia under assay conditions.

KBI-13 was identified as *Bacillus subtilis* based on morphological characterization and molecular analysis performed by 16S rRNA sequencing (Fig. 1). Phylogenetic study indicated that isolate showed 99 % similarity with *B. subtilis* strain DSM 10. These results revealed that the strain KBI-13 as a new strain of *Bacillus subtilis*.

Production of LAse by KBI-13

Effect of incubation period on LAse production

Production of LAse by KBI-13 was initiated after 10-12 hrs of bacterial growth. The enzyme activity was found to be increasing with the increase in the incubation time. Maximum activity was observed after 120 hrs of incubation from the day of culture inoculation and decreased thereafter with further incubation beyond 120 hrs (Fig. 2a).

Effect of initial pH and temperature

Effects of initial pH of the medium and different incubation temperatures on LAse production are

Table 1 — Screening of isolates for LAse activity

Sr. No	Isolate Name	Zone of Dimeter (mm)	Colony Diameter (mm)	Efficiency (%)
1	KBI2	1.0	0.8	20
2	KBI 13	4.2	1.2	71.42
3	KBI 16	1.6	0.9	43.75
4	KBI 17	0.7	0.4	42.85
5	KBI 20	3.2	2.2	31.25
6	KBI 23	2.3	1.9	0.21
7	KBI 25	1.2	0.5	17.39
8	KBI28	2.5	1.2	1.08
9	KBI29	2.1	0.6	52
10	KBI30	0.8	0.6	25

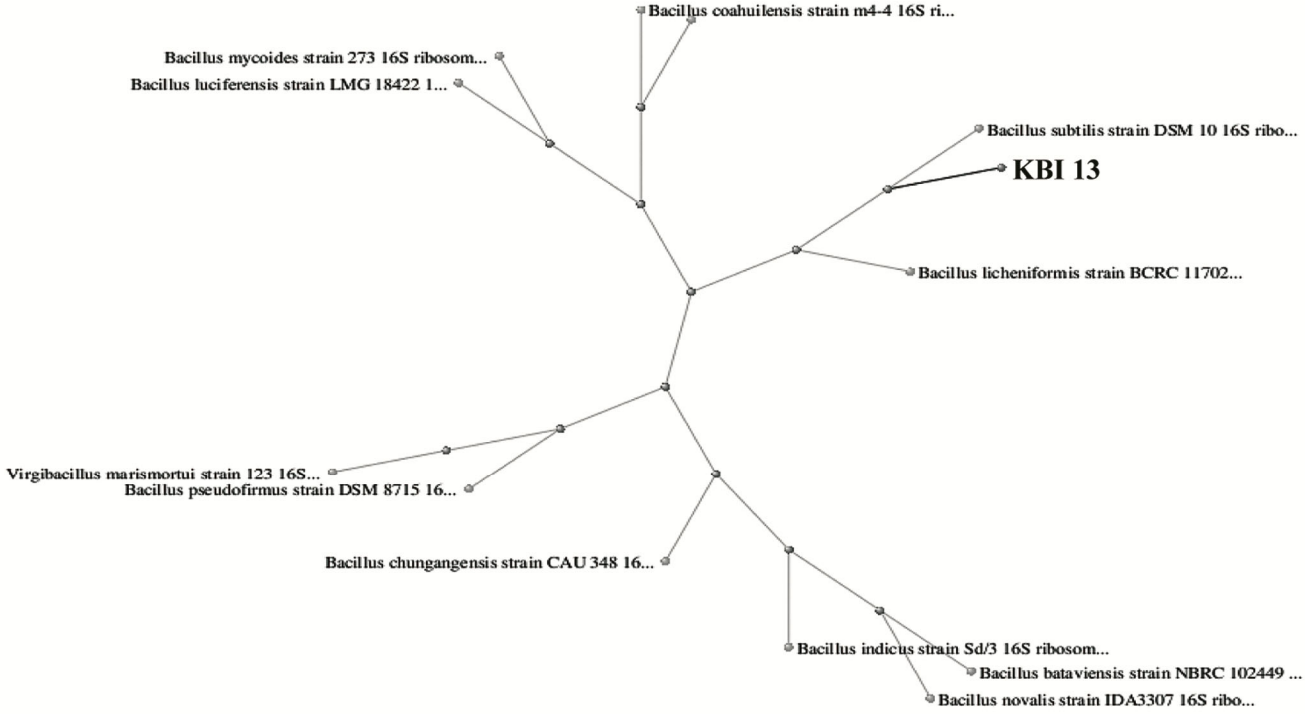

Fig. 1 — Neighbor joining tree based on 16S gene sequencing and showing phylogenetic relationship between *B. subtilis* KBI-13 and related members of the genus *Bacillus*

shown in Figures 2(b) & (c). The enzyme activity was highest at pH 6 (128.73 µM/min) for all incubation periods. The effect of incubation temperatures on LAse production was observed at a temperature range of 20-80 ºC where optimal enzyme production was observed at 40 ºC and afterwards it was found to be reducing gradually with the increase in temperature. However, at the two extremes of temperature *i.e.* 20 ºC and 80 ºC the enzyme production was completely absent.

Effect of carbon sources

The M9 broth was supplemented individually with different carbon sources (1 %) such as maltose, sucrose, mannitol, dextrose, lactose & fructose to determine their impact on LAse production. The enzyme production was recorded to be high in the presence of dextrose followed by mannitol while in presence of lactose the enzyme production was observed to be lowest (Fig. 2d).

Effect of nitrogen sources

To study the effect of nitrogen sources on LAse production, different nitrogen sources were supplemented to modified M9 broth containing 1 % dextrose. Yeast extract was found as the most fitting nitrogen source for optimal LAse production by KBI-13 (Fig. 2e). The comparable enzyme production was observed in the presence of ammonim sulpahte, peptone, meat extract and potassium nitrate. However, interestingly tryptophan did not support well for LAse production.

Effect of aeration

The effect of shaking and stationary conditions on LAse production is depicted in Figure 2(f). The

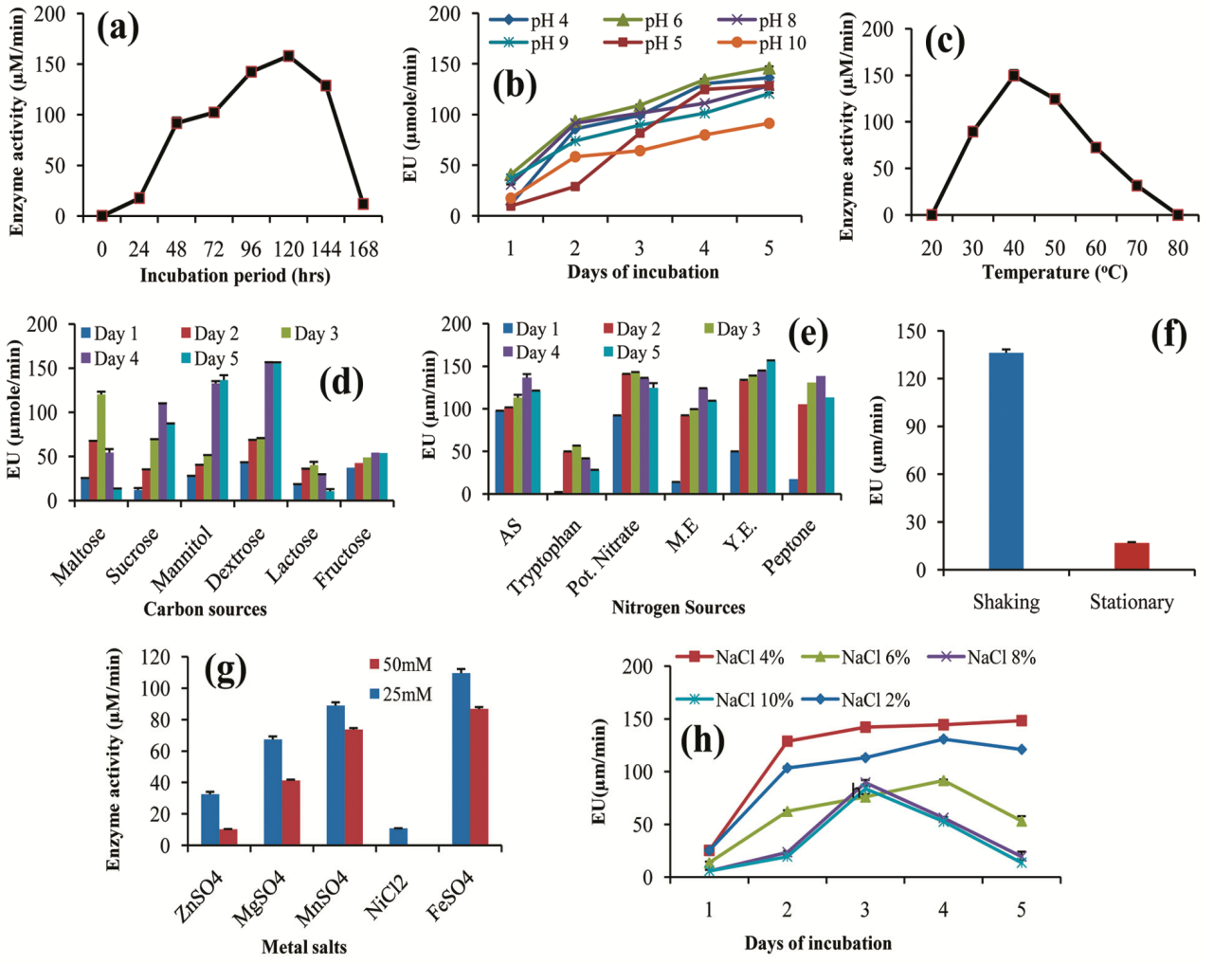

Fig. 2 — Effect of: a) incubation period at pH 7.0, b) pH, c) temperature, d) carbon sources, e) nitrogen sources, f) aeration condition, g) metal salts, and h) NaCl concentration; on LAse production by KBI-13

drastic reduction in enzyme activity was observed when the culture was grown under static conditions as compared to the culture incubated on a rotary shaker at 120 rpm.

Effect of metal salts

Different metal salts including $ZnSO_4$, $MgSO_4$, $MnSO_4$, $NiCl_2$ and $FeSO_4$ were incorporated separately in modified M9 broth at 25 mM and 50 mM concentration to determine their effects on LAse production by KBI-13. The maximum activity of enzyme was noted when 25 mM $FeSO_4$ was present transited by $MnSO_4$ at same conc. However, the activity was decreased when concentration was increased to 50 mM (Fig. 2g).

Effect of salts (NaCl)

The effect of varying concentration of NaCl (2-10 %) on LAse production by KBI-13 was studied over 5 days of incubation period. The maximum enzyme production was observed in the presence of 4 % NaCl (Fig. 2h). The activity was increased from 20-135 EU/ml during 48 hrs of incubation and thereafter remained almost constant till fifth day of incubation. At higher concentration of NaCl (8-10 %) the enzyme production was very low as compared to the enzyme production at 2-4 % of NaCl.

Purification of LAse

The results of KBI-13 produced LAse purification performed by salt precipitation, dialysis and gel chromatography (Fig. 3) are noted in Table 2. Ammonium sulphate 2 mM/ml and dialyzed sample showed a specific activity of 2.14 and 3.85 EU/mg, respectively whereas the specific activity in final purification step in sephadex G-50 was 76.93 EU/mg. The enzyme purity was increased successively from ammonium sulphate to sephadex G-50 purification over the crude supernatant and was ranged from 1.71 to 61.54 fold.

Enzyme kinetics

Effect of temperature and thermostability

Optimal incubation temperature for maximum enzyme activities was decided by incubating the enzyme with the substrate L-aspargine for 30 min at different incubation temperature (20-80 ºC with 10 ºC interval). The LAse activity was increased gradually from 20-40 ºC followed by a gradual decrease in activity beyond 40 ºC was studied further by pre-incubation of the enzyme at 40 ºC for various time intervals (1000 min/16 hrs). Enzyme was relatively stable upto 16 hrs at 40 ºC where almost 50 % of enzyme activity was restored (Figs. 4a & a1).

Effect of pH

Figure 4(b) depicts the effect of pH buffer on LAse activity of KBI-13. The results show that the LAse was more active at pH 8 in comparison to buffers of other pH values used. The activity was gradually increased from pH 4 to 8 indicating broad pH tolerance by the enzyme. The pH stability studies (Fig. 4b1) showed the enzyme was stable at pH 8 for about 16 h where 20 % of residual enzyme activity was noticed.

Effect of metals

The effects of different metal salts on LAse activity are shown in Figure 4(c). $CaCl_2$ at 0.5 mM concentration showed activation of enzyme where around 20 % increase in enzyme activity was observed over control. On the other hand, with the increase in $CaCl_2$ concentration the enzyme activity was decreased gradually.

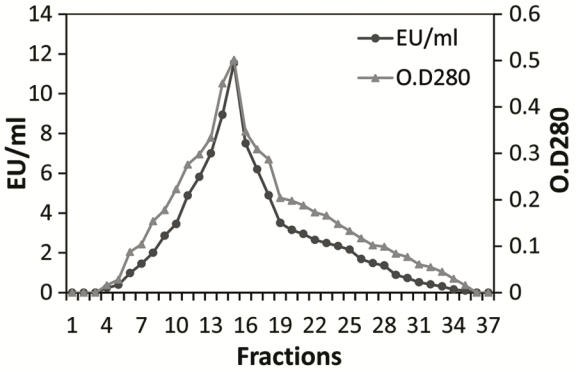

Fig. 3 — Purification of LAse from *B. subtilis* in terms of enzyme activity and protein concentration of fractions collected by gel filtration chromatography.

Table 2 — Purification of LAse produced by *Bacillus subtilis* KBI-13

	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Fold purification	Yield (%)
Crude extract	685.96	858.4	1.25	1	100
A.S. precipitation (40-80%)	298.31	639.18	2.14	1.71	74.46
Dialysis	119	458.50	3.85	3.08	53.41
Sephadex G-50	3.59	276.2	76.93	61.54	32.17

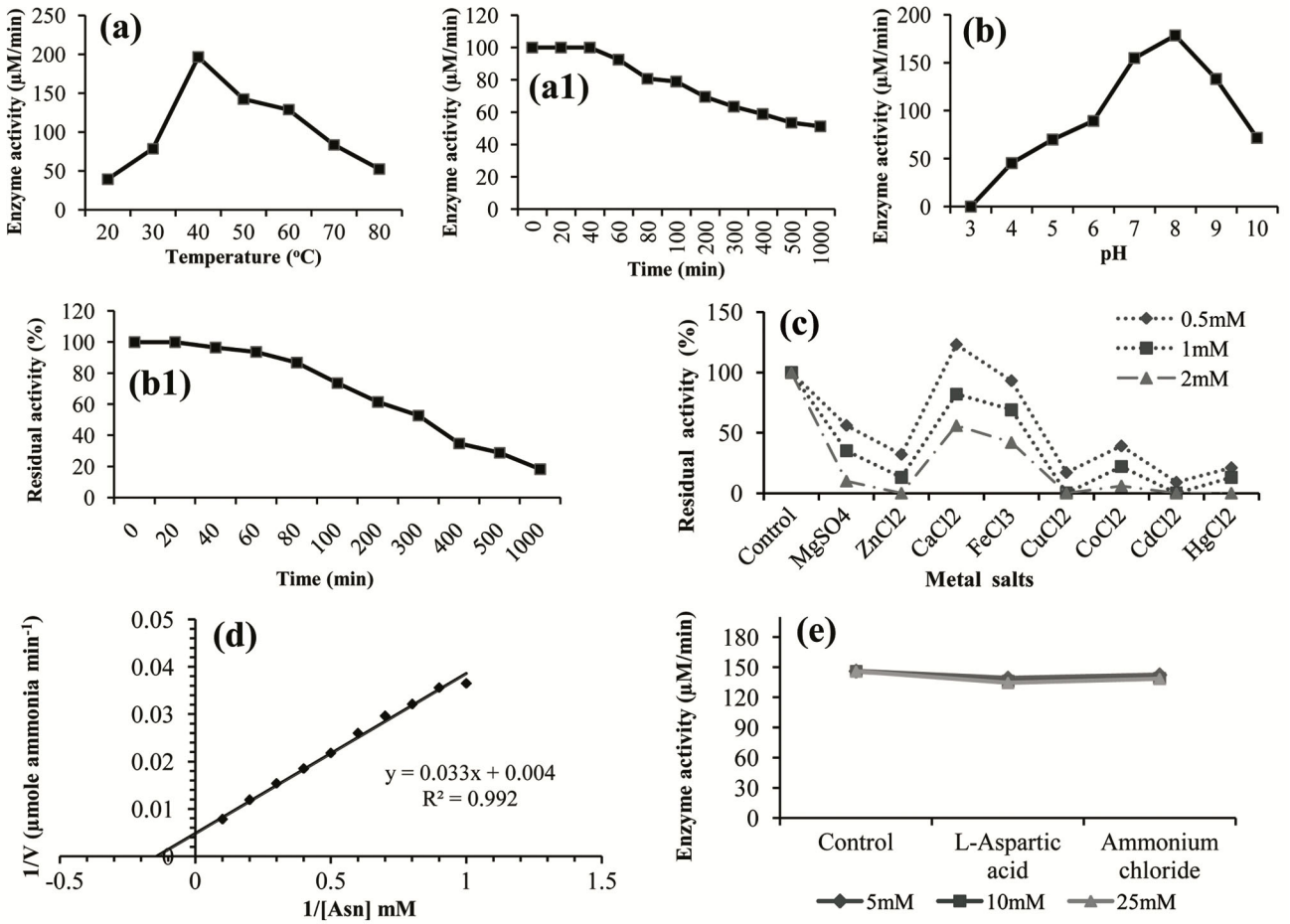

Fig. 4 — Effect of: a) temperature, a1) its stability at 40ºC, b) pH, b1) its stability at pH 8.0, c) different metal salts, d) different substrate concentration, e) different concentrations of reaction end product; on LAse activity

Effect of substrate concentration

The optimum L-aspargine concentration for high enzyme activity was found to be 250 mM (186.98 EU/ml) as shown in Figure 4(d). The enzyme kinetics study revealed the Vmax and Km values of 125 µM/min and 0.055 mM, respectively.

Effect of reaction end products

LAse activity was moderately affected by the presence of reaction end products at all three concentrations of L-aspartic acid and ammonium chloride as compared to control (Fig. 4e).

Discussion

L-asparginase is an enzyme of high remedial value owing to its antilymphomic, anti-leukemic and antitumor activities[2]. Its uses for the treatment of blood cancer, Hodgkin disease, acute myelocytic leukemia, chronic lymphocytic leukemia are well documented[17]. The enzyme is also used to avoid acrylamide formation in fried and overcooked foods[3]. The enzyme has been reported to be produced by *Escherichia coli*, *Erwinia cartovora*, *Aspergillus tamari*, *Pseudomonas aeroginosa*[18] *and Serratia mercens*[19]. In recent years, the pace of isolation of novel and biologically active metabolites from marine microorganisms is increasing day by day. The bacterial enzymes have proved their significance in many industrial and clinical applications due to their uniqueness.

In the present study an effort has been made to isolate and characterize LAse produced by marine *Bacillus subtilis* isolated from marine waters of Konark beach, Bhubaneshwar. In the present study, the isolate KBI-13 was selected among 57 bacterial strains as a potent LAse producer and was identified as *Bacillus subtilis* by rRNA sequencing. It showed highest LAse activity of 19.6 EU/ml. Different researchers have reported different LAse activities from different marine microorganisms. The reported LAse activity of KBI-13 was higher than that found in

marine *Streptomyces* sp.[20], bacterial strains of Persian gulf[20], *Pseudonocardia endophytica* VUK-10[(ref. 21)], *Streptomyces* PDK 7 and PDK 2[(ref. 22)] and *Pectobacterium carotovorum* MTCC 1428[(ref. 23)].

The production of enzyme by a typical bacterial cell is not a fixed property but it changes according to the varying conditions of nutrition and cultivation. Hence, complete knowledge of optimal conditions required for enhanced enzyme production by bacterial strains is required. The optimal production of microbial enzymes depends on various parameters used during fermentation process. Hence, the effects of different nutritional and growth parameters on LAse production were also studied.

Initial pH of medium and temperature are the very important parameters for thriving production of enzymes, as it governs the growth and metabolic activities of bacterial cells. Hence, any changes in pH optima and temperature may influence the bacterial growth and enzyme production. In the presented work, highest activity of LAse was found at pH 6 and at temperature 40 ºC. Most of the studies have reported pH 7 as the optimum pH for LAse production[24] by *Erwinia cartovora*[25], *Serratia marcenscens*[26] and *Aspergillus terreus* SM07[(ref. 27)].

The changes in incubation temperature significantly affected the LAse production by *B. subtilis* KBI-13. The maximum production was observed at 40 ºC which is followed by a continuous decrease in production till 80 ºC. This is true with *Bacillus* sp. grown optimally at 37 ºC.

The increase in LAse production with increasing incubation time was till 120 hrs after which the production was decreased. This is obvious because during the initial phases of growth, the nutrients are available in optimal concentration. As the bacterial growth increases, the nutrients became limited with accumulation of produced metabolites.

The carbon and nitrogen sources used in the media formulation affected significantly to the LAse production by *B. subtilis* KBI-13. Dextrose and yeast extract were found suitable for enhanced LAse production. Different carbon and nitrogen sources have been reported appropriate for optimal LAse production from groups of bacteria including lactose, maltose, glucose, starch and sucrose. Media supplemented with yeast extract exhibited highest LAse activity as these outcomes are in agreement with previous studies by Narayana *et al*.[28] and Khamna *et al*.[29].

Trace elements also play a critical role in LAse production[14]. Augmentation of enzyme activity in the presence of metal ions such as Ca^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+} could be based on its stability to interact with negatively charged amino acid residues such as aspartic acid and glutamic acid[30].

Aeration and agitation conditions were also found to influence the bacterial growth and metabolism significantly as they provide the nutrients in uniform dissolution and maintain uniform distribution of nutrients in the medium allowing easy access to bacteria.

The optimum temperature and pH are important parameters which affects the enzymatic reaction rates beyond which decline in enzyme activity is observed. Temperature profile shows that the enzyme activity was enhanced from 20-40 ºC; parallel results were observed by Messas *et al.*[31] and Jia *et al.*[32] for LAse from *Coryneabacterium glutamicum* and *Bacillus subtilis* BI1-06, respectively. Similarly the enzyme is highly active at pH 8. Similar results have been reported by Ahmed *et al*.[26] for LAse from *Erwinia cartovora* and for *Streptomyces ginsengisoli*[33].

The high sensitivity of LAse towards metal ions indicates the dependence of LAse activity on sulfhydryl functional groups[20]. The activity of LAse was enhanced in the presence of $CaCl_2$ though inhibited in the presence of $MgSO_4$, $CuCl_2$, $CdCl_2$ and $HgCl_2$. While Basha *et al.*[6] and Moorthy *et al.*[8] reported the enhancement in LAse activity in presence of Mg^{2+}.

The attraction of LAse to its substrate is related to its degree of effectiveness against tumours[34]. Linearity of LB plot suggests that the isolated LAse follows the M M kinetics. Km values indicating the substrate affinity were reported for LAse from *Proteus vulgaricus* and *Erwinia aroideae* were $2.6x10^{-5}$ and $3x10^{-5}$ M, respectively[35,36].

Conclusion

Marine environments are ideal for isolation of microbes producing enzymes and active metabolites with unique characteristics of biotechnological interest. Present investigation, has revealed that bacteria KBI-13 isolated from marine waters of Konark beach, Bhubaneshwar, India identified as *Bacillus subtilis* and found to have potential for extracellular L-asparginase production. Its production

was optimized and the optimum conditions for enzyme production are simple and easy to manage. The enzyme was purified and characterized. All the characteristics proved that the enzyme is highly potential for its future applications in industrial and therapeutic sectors. In future this same enzyme can be explored for various applications in different industries.

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Conflict of Interest

There is no conflict of interest among authors.

Author Contributions

HJB has designed the project and guided during the research work, SZU has contributed in carrying out this research work and TAK has helped in draft designing.

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