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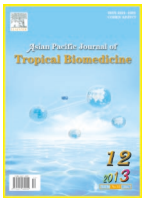

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Screening and characterization of extracelluar L-asparaginase producing *Bacillus subtilis strain hswx*88, isolated from Taptapani hotspring of Odisha, India

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PEER REVIEW

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Comments

This is a valuable research work in which the authors characterized the L-asparaginase, a clinically acceptable anti-cancer agent. This characterization was performed by morphological, biochemical and physiological tests. The results obtained allowed the identification of *Bacillus strain hswx88* (JQ237656.1) with a extracellular enzyme yielding 1.7 and 14.5 times higher than the reference organism *Pectobacterium carotovorum* MTCC 1428.
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ABSTRACT

Objective: To screen and isolate an eco-friendly, a thermophilic and potent L-asparaginase producing bacterium, with novel immunological properties that may obviates hypersensitivity reactions.

Methods: In the present study bacterial strain isolated for extracellular L-asparaginase production from hotspring, identified by morphological, biochemical and physiological tests followed by 16S rDNA technology and the L-asparaginase production ability was tested by both semi quantitative and quantitative enzymatic assay.

Result: The bacterial strain was identified as *Bacillus subtilis strain hswx*88 (GenBank Accession Number: JQ237656.1). The extracellular enzyme yielding capacity isolate *Bacillus subtilis strain hswx*88 (23.8 IU/mL) was found to be 1.7 and 14.5 times higher than the reference organism *Pectobacterium carotovorum* MTCC 1428 (14.2 IU/mL) and *Bacillus* sp. BCCS 034 (1.64 IU/mL).

Conclusion: The isolate is eco-friendly and useful to produce bulk quantity of extracellular, thermophilic L-asparaginase for the treatment of various tumor cases and for preparation of acrylamide free fry food preparation.

KEYWORDS

L-asparaginase, Thermophilic bacteria, *Bacillus subtilis strain hswx*88, 16S rDNA.

1. Introduction

L-asparaginase (L-asparagine amidohydrolase, E.C.3.5.1.1) (LAse) is a clinically acceptable anti-cancer agent, for the effective treatment of certain lymphomas and leukemias in both experimental animals and humans and has been used in combination with other agents in the treatment of acute lymphoblastic leukemia (mainly in children), reticle sarcoma, Hodgkin disease, acute myelocytic leukemia, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymphosarcoma and melanosarcoma chemotherapy[1,2]. The enzyme inhibited the growth of the two human cell lines including hepatocellular carcinoma (Hep-G2) and colon carcinoma (Hct-116) with IC_{50} value of 8.38 μg/mL and 4.67 μg/mL, respectively[3]. It is also used for the treatment of pancreatic carcinoma.

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Treatment with LAse, polyphenoloxidase and low frequency laser decreased adhesion of uropathogenic *Escherichia coli* to human erythrocytes[4]. LAse also known commercially as Oncaspar, Colaspase, Crasnitin, Kidrolase, Erwinase and Elspar[5]. It is also used in food industry for the production of acrylamide (a potent carcinogen and a neurotoxic compound) free starchy fry food[6].

Preventase and acrylaway produced from *Aspergillus niger* and *Aspergillus oryzae* respectively are commercially available LAse currently used in food industry[7]. LAse is a model enzyme for the development of new drug delivery system[8], L-asparagine biosensor for leukemia[9]. LAse like glutaminase and urease also plays an important role in the biogeocycling of carbon and nitrogen in natural waters and sediments[10]. LAse production by using microbial systems has attracted considerable attention, owing to the cost-effective and eco-friendly nature.

LAse is an amidohydrolase that catalyzes the hydrolysis of the amino acid asparagine to aspartic acid and ammonia. The tumor cells have a compromised ability to generate L-asparagine endogenously, either due to low expression levels of asparagine synthetase[11] or insufficient amount of its substrates, aspartate or glutamine[12]. Because of their dependence on exogenous L-asparagine, the cancerous acute lymphoblastic leukemia cells, but not normal cells, can be starved and eliminated by LAse treatment which depletes the levels of L-asparagine in circulating pools[13]. So the commonest therapeutic practice is to inject the enzyme intravenously.

Microbial LAse production is reported from *Escherichia*[14], *Aerobacter*, *Erwinia*, *Serratia*[15], *Xanthomonas*, *Pectobacterium*, *Photobacterium*[1], *Streptomyces* and *Aspergillus* species[15]. However the purified enzyme from *E. coli*, *Erwinia sp*. and *Serratia marcescens*[15] has been used as anti-tumor and anti-leukemia agent.

LAse produced extracellularly is advantageous and preferred over intracellular type because of higher accumulation of protein, easy extraction and downstream processing[16]. The extracellular compartment in bacteria is protease deficient and the liberated protein exported to the medium is mostly soluble, biologically active and has an authentic N-terminus, relatively free from endotoxins those results in minimization of adverse effects. Secretion also facilitates proper folding of proteins specially that requiring disulfide bridge formation, as it passes through a more favorable redox potential in the periplasmic space.

The medical utilization of LAse from the reported sources suffer the limitations of eliciting immunological responses leading to hypersensitivity in the long-term usage, allergic reactions, anaphylaxis and instance of spontaneous resistance of the tumor cells[17]. So the present investigation was under taken with an aim and objective to search for a novel bacterium from an ecologically unexplored and extreme habitat for the production of cost effective, eco-friendly, thermostable and a potent LAse with new antigenic properties. Thermozymes extracted from extreme environment might produce a serologically different LAse with novel immunological properties to that of proteins extracted at normal conditions.

2. Materials and methods

2.1. Isolation of LAse producing bacteria

Sediment samples were collected from Taptapani, a hotspring of Ganjam district of Odisha, India in sterile screw capped tubes and processed immediately for further microbiological studies. Suspensions were prepared by mixing 10 g of sediment samples into conical flasks containing 100 mL of sterile phosphate buffer, rotated on rotary shaker at 50 r/min for 30 min, kept aside to sediment the suspending matter and the clear supernatant was decanted and serially diluted. Further, 100 μL of the suspension from different dilution tubes were aseptically spread plated on to sterile plates containing modified M9 medium[2]. The composition of the medium (g/L): $Na_2HPO_4.2H_2O$, 6; KH_2PO_4, 3; NaCl 0.5; L-asparagine, 5; 1 mol/L $MgSO_4.7H_2O$, 2 mL; 0.1 mol/L $CaCl_2$ $2H_2O$, 1 mL; 20% glucose stock solution 10 mL, agar, 20 and pH 7.0 in distilled water to 1 L with phenol red (2.5%): 0.04-0.36 mL indicator. The plates were then incubated at 37 °C for 24 h, to obtain colonies with pink zones around them[2]. The colonies obtained were further purified by streaking techniques on nutrient agar plates. The purified culture was further maintained at 4 °C for further use.

2.2. Secondary screening for detection of LAse positive cultures

2.2.1. Rapid plate semi quantitative assay

Modified M9 medium was supplemented with different concentrations of the phenol red dye. A 2.5% stock of the dye was prepared in ethanol and the pH was adjusted to 7.0 using 1 mol/L NaOH. The stock solution of the dye, ranging from 0.04 mL to 0.3 mL, was added to 100 mL of modified M9 medium, giving final dye concentrations of 0.001-0.009% respectively. The media were autoclaved and plates were prepared. Control plates were of modified M9 medium (i) without dye and (ii) without asparagine (instead containing $NaNO_3$ as nitrogen source). The plates were inoculated with a 24-h culture of *Pectobacterium carotovorum* MTCC 1428 as a reference organism. The dye concentration was optimized and then the zone and colony diameters of the selected isolates and reference organism were measured by the plate assay after 24 h incubation at 37 °C[2]. The selected isolates and reference organism were then subjected to LAse enzymatic assay.

2.2.2. Enzyme solution preparation

The isolated colonies which exhibited highest zone diameter were transferred to 250 mL Erlen-meyer flasks with 50 mL modified basal broth medium containing (g/L): L-asparagine, 3.0; glucose, 2.0; $Na_2HPO_4.2H_2O$, 6.0; KH_2PO_4, 3.0; NaCl, 0.5; $MgSO_4.7H_2O$, 0.5; $CaCl_2.2H_2O$, 0.015; yeast extract, 1.0; peptone, 1.0 and initial pH was maintained at 6.5 and incubated in a shaker incubator (150 r/min, 37 °C) for 36 h[9]. After incubation, the cells were removed by centrifugation at 6000×g for 5 min. The supernatant was used to assay

extracellular LAse activity.

2.2.3. Enzymatic assay

LAse activity was measured by direct Nesslerization of ammonia. The activity of LAse was measured employing the modified method of Wriston[18]. The LAse catalyzes L-asparagine to Laspartic acid and ammonia and the latter react with the Nessler's reagent to produce an orange colored product. The enzyme assay mixture consisted of 100 μL of freshly prepared L-asparagine (189 mmol/L) in Tris-HCl buffer (pH 8.6) and 100 μL of crude extract of the enzyme. The reaction mixture was incubated at 37 °C for 30 min and the reaction was stopped by adding 100 μL of 15% trichloroacetic acid (TCA). The reaction mixture was centrifuged at 6000×g for 5 min at 4 °C to remove the precipitates. The ammonia released in the supernatant was determined using colorimetric technique by adding 500 μL Nessler's reagent into the sample containing 200 μL supernatant and 4.3 mL distilled water. The contents in the sample were vortexed and incubated at room temperature for 10 min, scanned for $ë_{max}$. OD was measured at $ë_{396}$ nm against the blanks that received TCA before the addition of crude enzyme. The ammonia produced in the reaction was determined based on the standard curve obtained with ammonium sulfate. One International Unit (IU) of LAse activity was defined as the amount of the enzyme that liberates 1 μmol/L of ammonia/ min at 37 °C.

2.3. Identification of the bacterial isolate of TPS-12

The bacterial isolate TPS-12 showing highest enzyme activity was subjected to different morphological, biochemical, physiological characteristics as well as 16S r-DNA technique for strain level identification. The results of the morphological, biochemical and physiological tests were put into Bergey's Manual of Determinative Bacteriology[19] and bacterial identification software's like PIBwin version 2.0 and ABIS online for accurate identification. The isolate TPS-12 was exposed to temperature range of 40-100°C, pH range of 3-12 and sodium chloride concentrations range of 2%-10% for evaluation of physiological characteristics. The antibiogram pattern of the isolate TPS-12 was tested for sensitivity against different antibiotic discs (Himedia, Mumbai) *viz.*, erythromycin (15 μg), streptomycin (25 μg), ciprofloxacine (30 μg), ampicillin (10 μg)+solbactum (10 μg), ceftriaxone (30 μg)+tazobactum (10 μg), cotrimoxazole (25 μg), amikacin (30 μg), gentamicin (30 μg), cefotaxime (30 μg), ofloxacin (2 μg), chloramphenicol (50 μg), nalidixic acid (30 μg) and polymyxin (300 U).

2.4. 16S rDNA gene sequencing of TPS-12

Further the genomic DNA was isolated from the culture by using GeneiUltrapure™, bacterial genomic DNA purification kit KT162. Using consensus primers, about 1.5 kb 16S rDNA fragment was amplified using *Taq* DNA Polymerase. The PCR product was bi-directionally sequenced using the forward, reverse and an internal primer. Sequence data was aligned and analyzed for finding the closest homologous microbes. PCR was performed in a final volume of 50 μL in glass distilled water containing Genomic DNA: about 20 ng, dNTP mix (2.5 mmol/L each): 1.0 μL, forward primer: 100 ng, reverse primer: 100 ng, *Taq* buffer A (10X): 1X, *Taq* polymerase enzyme: 3U. PCR amplification conditions were initial denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 30 seconds, 55 °C for 30 seconds and 72 °C for 1.3 min with a final extension of 72 °C for 10 min. The PCR products were loaded on 1.0% agarose gel along with StepUp™ 500 bp DNA ladder.

2.5. Phylogenetic analysis of the strain

The 16S rDNA gene sequence of the strain TPS-12 was used as a query to search for homologous sequence in the nucleotide sequence databases by running BLASTN program. The high scoring similar to 16S rDNA gene sequences were identified from the results and retrieved from GenBank database. The identified sequences were aligned using CLUSTAL-W algorithm in MEGA 4.0 software. Phylogenetic trees were inferred by using the neighbor-joining bootstrap analysis with the help of MEGA 4.0 software package based on 1000 re-samplings.

3. Results

3.1. Isolation of bacterial strains

A total of 34 bacterial colonies were isolated from all the samples. Out of 34 isolates, 19 were selected based on their macroscopic cultural characters, eliminating those that appeared close to each other. They were examined for LAse production. Among these 19, isolates showed intense pink zone around the colonies on modified M9 medium, containing phenol red as indicator, indicating the increase in pH due to ammonia accumulation in the medium. The dye indicator is yellow at acidic condition and turns to pink at alkaline condition. Studies with different concentrations of the dye revealed that as the concentration of the dye increased, the clarity and visibility of the pink zone increased and the optimum concentration of the dye was found to be 0.007% (0.28 mL of 2.5% solution of the Phenol Red), which supported the growth of the test bacteria.

Among 19 selected bacterial isolates tested by rapid plate semiquantitative assay, the 6 isolates designated as TPS-4, TPS-5, TPS-8, TPS-11, TPS-12, TPS-16 gave prominate pink zone diameter ranging from 2.5-3.6 cm after 24 h incubation. TPS-12 is one of the good LAse producer that exhibited 3.2 cm pink zone, which was more in compared to the reference organism (1.9 cm). Enzymatic assay of TPS-12 shown good enzymatic activity *i.e.* 23.8 IU/mL and that is more in compared to reference organism *Pectobacterium carotovorum* MTCC 1428 (14.2 IU/mL), indicating existence of a direct correlation between zone diameter and enzyme activities in broth. TPS- 12 was identified as *Bacillus* subtilis by the help of Bergy's manual of determinative bacteriology, PIBwin version 2.0 and ABIS online.

3.2. Characterization of TPS-12

The morphological and cultural characteristics of the isolate TPS-12 are appended herewith. The test colony was found to be medium sized that exhibited circular or irregular configuration with entire and smooth margin, flat elevation, dry and granular surface, non-pigmented and opaque. The isolate was found to be Gram positive rods of average size 2.3 μm in length, having single or short chain arrangement. It was found to be very motile having spore(s). The photomicrographs of Gram's reaction and scanning electron microscopy (SEM) of TPS-12 is given at Figure 1 and Figure 2 respectively. TPS-12 isolate developed prominent pink colur zone when inoculated in modified M9 medium (Figure 3). The isolate TPS-12 when exposed to temperature range of 40-100 °C, showed growth in the temperature range of 150-70 °C, while the growth was restricted above and below the above specified range. The isolate TPS-12 when exposed to different pH range (3-12) and tolerance to different concentrations of sodium chloride (2%-10%), exhibited growth at pH range of 5-10 and tolerated sodium chloride concentrations 2%-7% respectively. The isolate showed no growth at pH 3 and 10 % sodium chloride. The isolate TPS-12 subjected to various biochemical tests and the results therein is shown at Table 1.

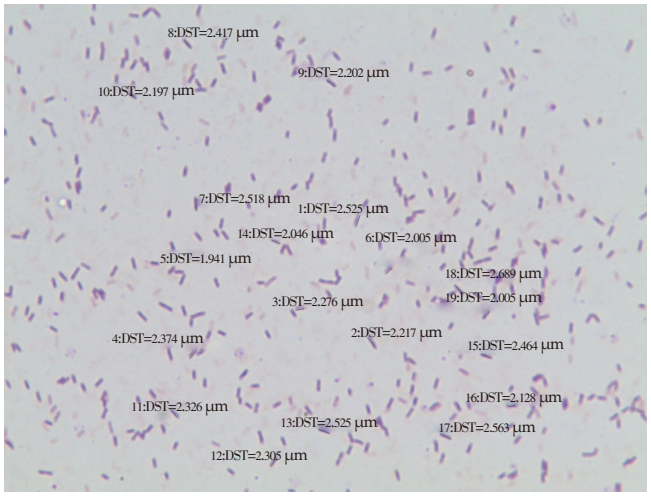

Figure 1. Gram staining photomicrograph of TPS-5.

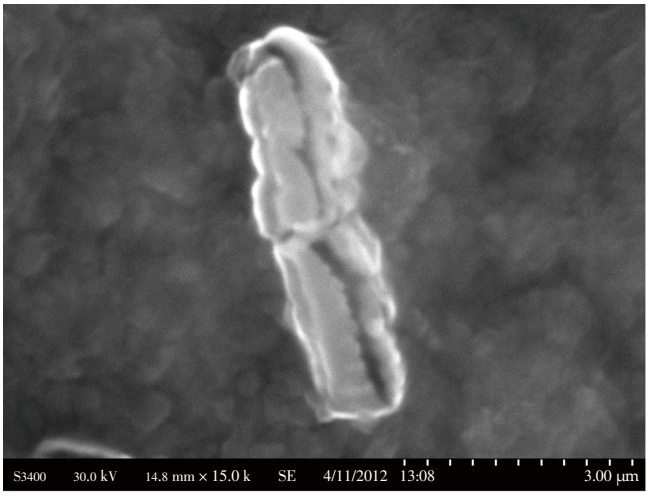

Figure 2. Scanning electron microscopy (SEM) of TPS-5.

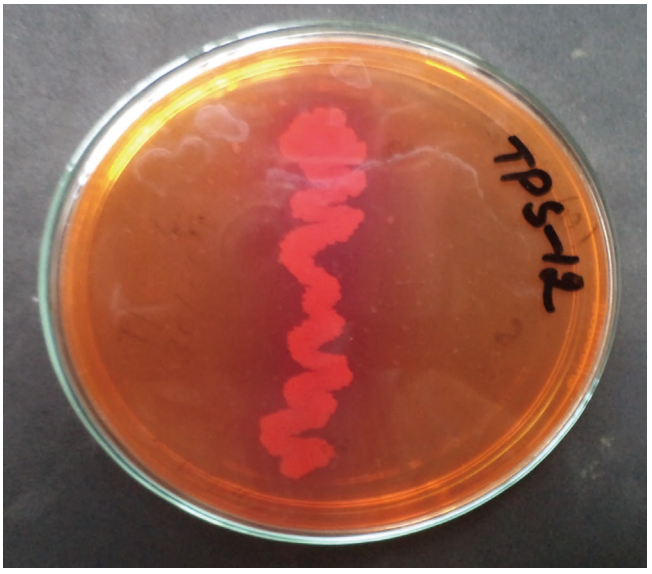

Figure 3. Developed prominent pink colur surrounding the microorganism growing in modified M9 medium.

The results of the antibiogram pattern revealed that the isolate TPS-12 was very sensitive to all the tested antibiotics.

Table 1
Biochemical tests of the isolate TPS-12.

Tests	Result	Tests	Result	Tests	Result
Production:		Phenyl Alanine Deamination	-	Lactose	-
Indole test	-	Lysine decarboxylase	-	Maltose	-
Voges Proskuer test	-	Hydrolysis Tests:		Mannitol	-
Methyl red test	+	Aesculin hydrolysis	+	Raffinose	-
Citrate utilization	-	Gelatin hydrolysis	+	Rhamnose	-
Koser Citrate	-	Starch hydrolysis	+	Salicin	-
Nitrate reduction	+	Urea hydrolysis	-	Nitrogen Source Utilization:	
H2S production	-	Sugar utilization test:		Phenylalanine, Serine and Histidine	-
Oxidase test	-	Glucose	-	L-asparagine, Methionine, Hydroxy proline,	
Arginine dihydrolase	+	Adonitol	-	Valine, Threonine, Cysteine HCl and Arginine	+
Ornithine decarboxylase	+	Arabinose	-		

(+): Indicates positive result, (-): Indicates negative result

3.3. 16S rDNA gene sequence analysis of TPS-12

Based on the morphological, physiological and biochemical characterization, the isolated strain TPS-12 was identified as *Bacillus* subtilis. In order to evaluate the same and to further confirm the sub-species level, the genomic DNA of this strain was amplified and analyzed for molecular-based identification. The amplicon was purified and used to determine the 16S rDNA gene. The PCR amplification of 16S rDNA gene revealed efficient amplification; a single band of amplified DNA product of ~1.5 kb was recorded. The result of the PCR was blasted with other sequenced bacteria in NCBI GenBank and RDP database showed similarity to the 16S small subunit rDNA of other bacteria. Edited sequences were used as queries in BLASTN searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi), to determine the nearest identifiable match present in the complete GenBank nucleotide database. Blast analysis revealed that the sequence of TPS-12 to be 99% similarity to *Bacillus subtilis strain hswx*89 having Gene Bank Accession Number : JQ237657.1). Phylogenetic tree was constructed by taking the sequences obtained in the blast search. The phylogentic tree revealed the isolate TPS-12 to be *Bacillus subtilis strain hswx*88 (GenBank Accession Number: JQ237656.1) is given in Figure 4(Sample green represents TPS-12).

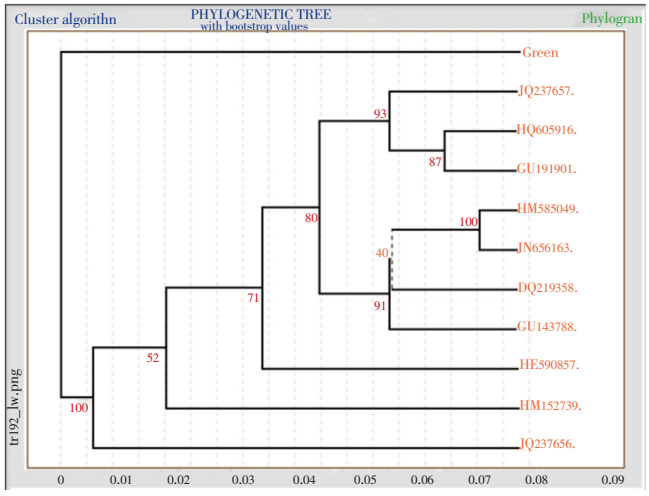

Figure 4. Phylogenetic tree of TPS-5 using neighbour joining method.

4. Discussion

Taptapani a natural hot spring (Dist-Ganjam, Odisha), well known for its natural beauty, curative powers and religious significance is a popular tourist destination. The limits of temperature, pressure, pH, salinity and water activity at which extremophilic environment with greater microbial diversity might claim the production of thermozymes. The microbial flora mainly includes prokaryotic and eukaryotic microorganisms. Thermophiles thrive under conditions that would kill most other creatures and cannot survive in the normal anthropogenic conditions including high temperature environments (45 to>100 °C). The extremozymes or thermozymes from thermophilic organisms are preferred owing to their guaranteed properties of stability in adverse conditions and different industrial process at which they operate[20].

TPS-12 produced LAse extracellularly, that is advantageous and preferred over intracellular type because of higher accumulation of mostly soluble biologically active protein, easy extraction, downstream processing[16]and relatively free from endotoxins those results in minimization of adverse effects. The isolate TPS-12 is growing in the temperature range of 150-70°C, which confirm its thermophilic characteristic. TPS-12 was very sensitive to all the tested antibiotics which indicate it as a safe and ecofriendly microorganism.

LAse is an antineoplastic agent to treat Acute Lymphoblastic Leukemia. However the limitations of relatively short half-life and instability in the processes of production and treatment requires repeated administration leading to more serious toxic effects on patients. Thermophilic environment may be the ideal microbiological niche to explore LAse production and further thermostability of the enzyme produced may be studied extensively to combat adverse effects of the existing enzyme.

Based on different morphological, cultural, physiological characteristics, nucleotide homology and phylogenetic analysis the isolate TPS-12 was found to be *Bacillus subtilis strain hswx*88 (GenBank Accession Number: JQ237656.1.)

The extracellular enzyme yielding capacity of our isolate *Bacillus subtilis strain hswx*88 (23.8 IU/mL) was found to be 1.7 and 14.5 times higher than the reference organism *Pectobacterium carotovorum* MTCC 1428 (14.2 IU/mL) and *Bacillus* sp. BCCS 034 (1.64 IU/mL) in the supernatant as reported by Kumar and Ebrahiminezhad with their associates[9,16].

The isolated strain *Bacillus subtilis strain hswx*88 is an ideal producer of extracellular LAse. Future investigations are aimed at optimization and purification studies of LAse and evaluation of antitumor activity.

Hot spring environments are active environments inhabitated by a vast variety of microbial communities. These environments contain novel microbial strains with novel properties of biotechnological interest. The findings of the present work revealed that the bacterium isolated from Taptapani hotspring of Odisha, India, was identified as *Bacillus subtilis strain hswx*88 (GenBank Accession Number: JQ237656.1) with a potential for extracellular LAse production that warrants futuristic approach for the purification studies and evaluation of its antitumor activity.

Conflict of interest statement

We declare that we have no conflict of interest.

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Education, New Delhi, India through "Research Promotion Scheme" (AICTE Letter- F.No. 8023/RID/RPS-17/(POLICYIV)/(GOVT.)/2011-12, Dt:09/07/2012, Vch. No.: 340) is gratefully acknowledged.

Comments

Background

L-asparginase is a clinically acceptable anti-cancer agent, being important the characterization of this enzyme from microorganisms like *Bacillus subtilis*.

Research frontiers

The present work study the bacterial strain isolated for extracellular L-asparginase producing from hotspring, identified by morphological, biochemical and physiological tests followed by 16S rDNA technology and the LAse production ability was tested by both semi quantitative and quantitative enzymatic assay.

Related reports

The enzyme inhibited the growth of the two human cell lines including hepatocellular carcinoma (Hep-G2) and colon carcinoma (Hct-116) and its used for the treatment of pancreatic carcinoma. In this work is missing reference to previous works that characterize this enzyme.

Innovations and breakthroughs

The literature reported the activity anti-cancer of this enzyme. In this scientific work LAse was characterized.

Applications

This enzyme may be purified and tested in the future with respect to its antitumor activity.

Peer review

This is a valuable research work in which the authors characterized the LAse, a clinically acceptable anti-cancer agent. This characterization was performed by morphological, biochemical and physiological tests. The results obtained allowed the identification of *Bacillus strain hswx88* (JQ237656.1) with a extracellular enzyme yielding 1.7 and 14.5 times higher than the reference organism Pectobacterium carotovorum MTCC 1428.

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