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Full Length Research Paper

Isolation and classical identification of potent extracellular alkaline protease producing alkalophilic *Bacillus* sp from coastal regions of Tamil Nadu

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Microbial proteases are vastly demanded industrial enzyme with broad commercial application in various sectors. Naturally, the bacterial strains are well known for their ability to excrete enzymes into the environment. The current work was undertaken to isolate and characterize an effective extracellular alkaline protease producing *Bacillus* sp. A total of twenty nine bacterial strains were isolated from coastal regions of rhizosphere soil around the Cuddalore District Tamil Nadu, India. Out of twenty nine isolates, seven strains namely, IAS01, IAS02, IAS03, IAS04, IAS05, IAS06 and IAS07 show 45% clearance of hydrolyzing zone on skim milk agar plate. The positive isolates were further assay on small scale laboratory fermentation media for their protease productivity when compared with standard culture, *Bacillus subtilis* (MTCC-1789). As a result, the highest enzyme productivity that appeared in the strain IAS01 was found to be 332.13±1.31 U/ml at alkaline condition. Therefore, the selected isolate IAS01 was a promising strain for alkaline protease producer and identified as *B. subtilis* based on its morphological, physiological, biochemical characters and analysis of the 16S rRNA gene sequencing study. The partial 16S rRNA sequence was submitted to Genbank, with the accession number KF761633.

Key words: *Bacillus subtilis*, alkaline protease production, SPSS statistic analysis.

INTRODUCTION

Enzymes are well known as biocatalysts and are used for various commercial purposes in industries. Generally, bacterial proteases are the most preferred group of industrial enzymes as compared to plant, animal and fungal proteases, because of their ability to grow in simple culture medium with minimum space requirement and to obtain higher yield within short period. The *Bacillus* sp. produce various type of proteases, interestingly the alkaline proteases have extensive applications like laundry detergents, pharmaceutical, food

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industry, leather processing and waste bioremediation (Bayoudh et al., 2000; Jellouli et al., 2009). There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial process (Gupta et al., 2005; Habib et al., 2012). Proteases represents one of the three largest groups of industrial enzymes and have traditionally held the predominant share of the industrial enzyme market accounting for about 60% of total sale of enzymes (Beg et al., 2003; Deng et al., 2010)

Protease production is an inherent capacity of some microorganisms and large numbers of bacterial species are known to produce alkaline proteases. Considering the richness of microbial diversity, there is always a chance of searching new organisms to produce enzyme with better properties and suitability for commercial exploitation. Microbial proteases are extracellular in nature and are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases obtained from plants and animals. Mukhtar and Ikram-ul-Haq (2008) reported the production of alkaline protease by *Bacillus subtilis* and its application as a depilating agent. The aim of the present study was to isolate and characterize an effective alkaline protease producing *Bacillus* sp. from rhizosphere soil which might have useful industrial applications.

MATERIALS AND METHODS

Collection of soil sample and isolation of microorganisms

Samples of rhizosphere soil were collected from five different locations in the coastal regions of Cuddalore District, Tamil Nadu. Plant roots with adherent soil were taken from healthy plants, placed in sterile bags and transported to the laboratory within 1 h. Before isolation, the roots were gently shaken to remove excess soil and vortexed for 10 min in sterile distilled water (1 g per 10 ml). Samples were serially diluted with sterile distilled water from 10^{-1} to 10^{-6} dilutions and 100 µl of each dilution was plated onto nutrient agar at pH 9.0. After incubation for 24 h at 37°C, 27 colonies were picked based on divergence in morphology, size and color from dilution plates and maintained as pure cultures in nutrient agar slants with periodic transfers to fresh medium for further study.

Screening of potent protease producer

Screening assay performed for all the isolates on standard nutrient agar medium maintained at different pH (5.0-11.0) with supplemented 1% of skim milk was used as a substrate in the media for all the ingredients except skim milk autoclaved at 121°C for 20 min at 15 lbs pressure; skim milk was autoclaved for separately for 6 min to prevent the coagulation of milk. After cooling at 45°C, the skimmed milk was mixed with media and poured in the plates and allowed to solidify. After incubation at 37°C for 24 h (to remove moisture and check sterility), the plates were streaked with bacterial cultures using sterile needles. After incubation, seven isolates namely IAS1, IAS2, IAS3, IAS4, IAS5, IAS6 and IAS7 exhibited the prominent zones of clearance on skim milk agar plate at pH 9.0. Therefore, they were collected for further quantitative estimation of protease production when compared with standard culture *B. subtilis* (MTCC-1789).

Inoculum preparation for quantitative estimation of protease production

A volume 25 ml of nutrient broth taken in a 100 ml Erlenmeyer flask was inoculated with a loop full of pure culture from 24 h old plate and kept at 37°C in a rotary shaker. After 24 h of incubation, 1 ml of this nutrient broth culture was used as the inoculums found to be 3×10^6 CFU/ml and was added to the protease production medium containing: Glucose 6%, Soybean meal 2%, $CaCl_2$ 0.04% and $MgCl_2$ 0.02%. Media were autoclaved at 120°C for 20 min and later kept at 45°C in water bath to add 1 ml of inoculum for incubation at different culture condition namely pH (5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11), temperature (30, 35, 37, 40, 45, 50 and 55°C), incubation period (24, 36, 48, 60, 72, 84 and 96) and agitation (80, 100, 120, 140, 160, 180 and 200 rpm). After incubation, the culture was centrifuged at 1000 x*g* for 30 min and the supernatants were used for quantitative estimation of proteolytic activity.

Determination of proteolytic activity and protein content

Protease activity was determined using casein as a substrate. The reaction mixture containing 2 ml of 1.0% casein solution in 0.05 M Glycine-NaOH buffer having pH 11 and 1 ml of an enzyme solution were incubated at 60°C for 15 min. The reaction was then stopped with 3 ml of 10% tri-chloroacetic acid and the reaction mixture was allowed to stand for 10 min. The reaction mixture was then centrifuged at 9000 x*g* for 10 min at 4°C to get the clear solution. Absorbance of the liberated tyrosine in the solution was measured at 660 nm against blank. Amount of tyrosine released by the action of protease was determined from the tyrosine standard factor, according to the following relationship. Sample (µg/ml) = Absorbance of sample x Average standard factor, Where average standard factor was determined from the concentration/absorbance ratios of all points of the standard curve. One proteolytic unit was defined as the amount of the enzyme that released 1 µg of tyrosine per ml per min under the assay conditions (Kembhavi et al., 1993; Yang and Haung 1994). The protein content in the samples was estimated by following the method of Lowry et al. (1951). The amount of protein present in the sample was calculated from the standard curve.

Determination of cell biomass

The cell biomass was determined from a known amount (100 ml) of sample centrifuged at 10000 xg for 15 min at 4°C and the cell pellet was collected and washed with sterilized normal saline three times to remove the suspended particles. The washed cell pellet was transferred to a dried pre-weighed filter paper and then kept in oven at 105°C till the constant weight was achieved. The dry cell biomass was calculated by X = Weight of dry filter paper + cell biomass (g) - Weight of dry filter paper (g).

Identification of the bacterial strain for biochemical and 16s rRNA analysis

The highest enzyme producing isolate was identified as based up on the morphological and biochemical test carried out using basic method.

Morphological study

SEM was used to investigate the morphology of isolated strain. The sample for SEM was prepared by transferring the microbial strain to a clean Eppendorf tube containing approximately 1.5 ml of 3.5% glutaraldehyde solution. Then, culture was incubated for 4 h at room temperature followed by wash with phosphate buffer (100 mM, pH 7.2). The culture was then dehydrated using alcohol gradient from 10 to 100%. The dehydrated sample was then air dried and fixed on the stubs using double adhesive tape. A thin layer of gold was coated over the sample using HUS-5GB Hitachi vacuum evaporator for 90 s. These samples were then observed under scanning electron microscopy (Annamalai University, Tamil Nadu) at various magnifications at acceleration voltage of 10.0 KV.

Biochemical study

The selected isolate was identified according to the methods recommended in Bergey's Manual of Systematic Bacteriology (Grimont and Grimont, 1984; Holt et al., 1994) and Diagnostic Microbiology (Betty et al., 2002). The classical biochemical properties were identified by using diagnostic test kit TREK (The Sensititre GPID plate is an *in vitro* diagnostic product for the automated identification of Gram positive bacteria). Molecular identified was carried by 16S rRNA gene sequencing. The genomic DNA was extracted as per the protocol (Babu et al., 2009 and Manufacturer guide) and using microbial DNA extraction kit (insta-gene TM Matrix, Bio –Rad). The amplification of 16S rRNA gene was carried out by using Thermal cycler (PTC-225) in 100 µl reaction mixture containing 2.5 mM each of four dNTP, 10X PCR buffer, 3 U of Taq DNA polymerase, 10 ng template DNA and 400 ng each of primer 27F 5' AgAgTTTgATCMTGGCTCAg-3' primer 1492R 5'-TACggYTACCTTgTTACgACTT-3', were used to amplify 16s rRNA gene. The programs was set as initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 2 min and a final extension at 72°C for 5 min. The sequencing was performed by using Big Dye Terminator Cycle Sequencing Kit with AmpliTag DNA polymerase (FS enzyme) (Applied Biosystems). The sequence producing product were resolved on an Applied bio-systems model 3730 XL automated DNA sequencing system (USA). The purified 16s rRNA gene sequence was compared with the Gen Bank nucleotide database (NCBI) using BLAST and BLASTX algorithms. The sequence alignments and the phylogenetic tree were constructed by using MEGA software version 5.2 (Altschul et al., 1990). The phylogenetic tree was constructed using a neighbor joining method and assessed with 1000 bootstrap replication.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 (SPSS, Inc., Chicago) statistical package. Data were expressed as men standard deviation (SD). One way analysis of variance (ANOVA) followed by Duncan multiple comparison method was used to correlate the difference between the variables. Data was considered statistically significant if $p \leq 0$ 0.05.

RESULTS AND DISCUSSION

Qualitative screening of protease producer

In the present study, a total of 27 morphologically distinct bacterial colonies were obtained from coastal regions of rhizosphere soil screened for the potent alkaline protease producing strain on skim milk plate assay. Seven strains were identified as protease producer by zone of hydrolysis around the colonies at pH 9.0 (Figure 1).

Optimization of process parameter for protease production

The culture condition determines the rate of bioprocess like pH and temperature. In this study, the highest enzyme production was found to be 235.05±2.91 U/ml at pH 9.0, this variation is shown in Table 3. Similar researcher (Sankareswaran et al., 2014) reported that the pH 9.0 is suitable for protease production. Therefore, this may be attributed to the microbial growth and protease activity inactivated at higher and lower level of hydrogen concentration. Table 4 shows 37°C is the suitable temperature for protease production throughout the study. Based on the result Abebe et al. (2014) reported that the temperature, 37-45°C is suitable for protease production. Fermentation period was contacted on every 12 h of incubation up to 96 h. The highest enzyme activity was recorded in period of 72 h, this variation is shown in Table 5. Similar researcher (Ponnuswamy et al., 2014) proved 72 h is suitable for protease production under solid state fermentation condition using cow dung as a substrate. Therefore, the results of this study showed that protease production when increased with incubation time up to 72 h, after 72 h of incubation, the enzyme activity was considerabily decreased due to nutrient depletion. Agitation rang was investigated for protease production upto (80-200 rpm), the result is shown in Table 6. The highest enzyme activity was recorded after 140 rpm. Therefore the agitation rate gradually increase the protease production.

Quantitative analysis and identification/support protease producer

The selected seven primary bacterial isolates were separately checked for quantitative analysis of protease production in selected media (Genckal and Tari, 2006) at 72 h incubation, 37°C and 140 rpm under shack flask fermentation condition. After incubation and assay, it was found that the isolate IAS01 produced highest yield of protease activity (332.08±18.82 U/ml), protein (3.30±0.07) and cell biomass (2.99±0.01) followed by standard culture of *B. subtilis* (MTCC-1789) with their enzyme activity of 326.06±18.85U/ml, protein 2.52±0.09 and cell biomass 2.05±0.01, this variance is shown in Table 7. Similar researcher (Vanitha et al., 2014) reported the food waste isolate for *B. subtilis* "168" exhibited enzyme activity of 170.32 ± 1.5l U/ml. Another researcher (Hanan, 2012) reported that the marine isolate *Bacillus* sp. 2 EHN produced maximum yield of protease with the enzyme activity of 243 U/ml. Another

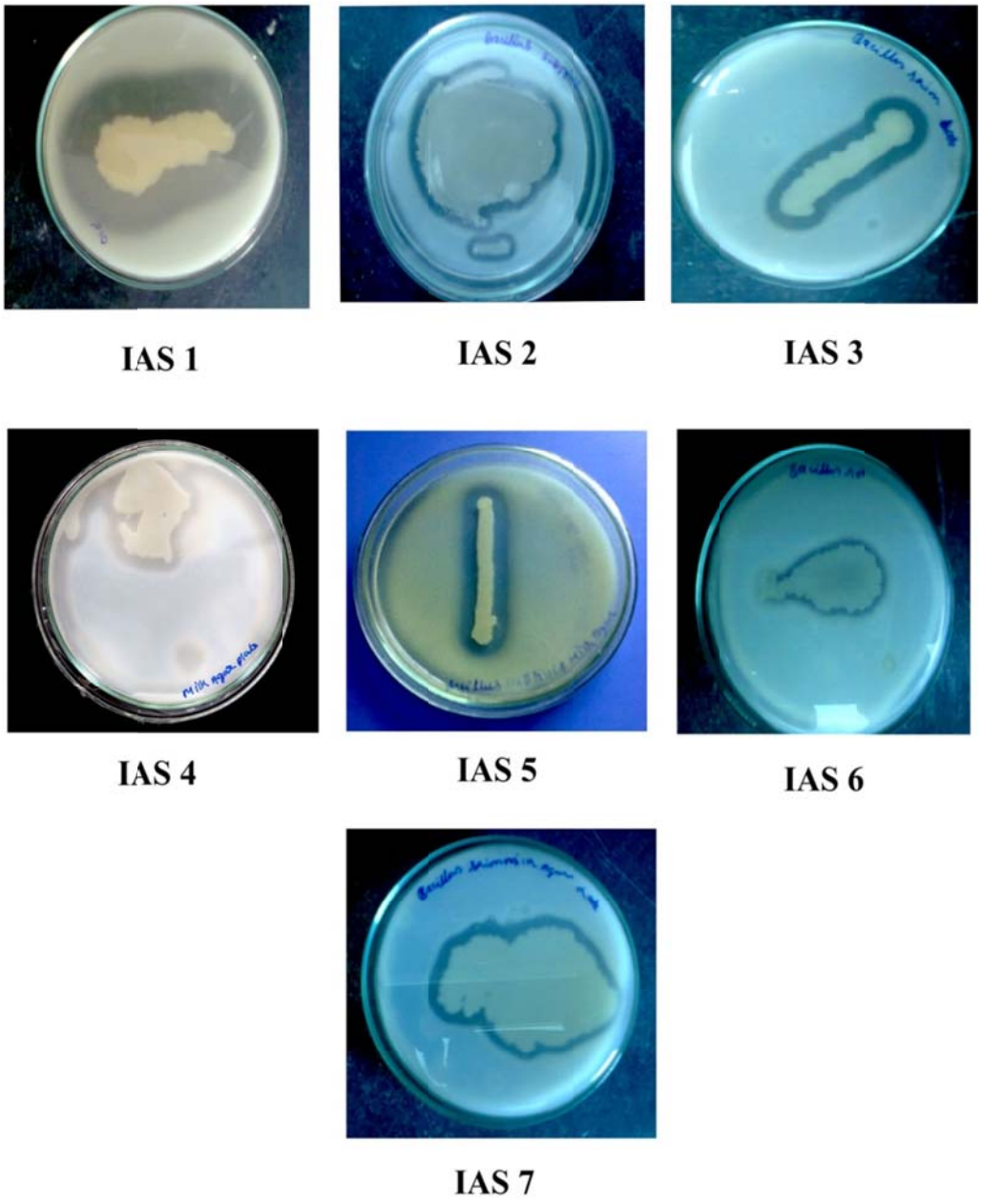

Figure 1. Effect of protease activity on skim milk agar plate.

researcher (Yun-Zhu et al., 2015) reported statistically produced alkaline protease optimized from *Penicillium citrinum* YL-1 under solid state fermentation exhibited activity of 94.30 U/ml. Earlier, the alkaline protease producing bacteria was also isolated, which shows that both *Bacillus anthraces* S-44 and *Bacillus cereus* S-98 exhibited their maximum ability to biosynthesize proteases within 60 h incubation period since the productivity reached up to 126.09 U/ml for *B. anthracis* S-44 corresponding to 240.45 U/ml for *B. cereus*, *Bacillus* sp. JB-99 respectively (Johnvesly et al., 2012). Therefore, similarly an isolate '1AS01' produced highest yield of protease activity (Table 5), chosen for further study. The selected isolate was confirmed as *B. subtilis* based on morphological SEM (Figure 2), biochemical tests performed according to Bergey's manual (Grimont and Grimont, 1984; Holt et al., 1994) indicating that this isolate (Tables1 and 2) belong to the genus *Bacillus* and 16S rRNA characterization study revealed that the species was confirmed as *B. subtilis* with approximately 1440 bp nucleotide sequence of 16s rRNA gene. The gene sequence was deposited in Gen bank (Accession number KF 761633) also phylogenetic tree was constructed (Figure 3).

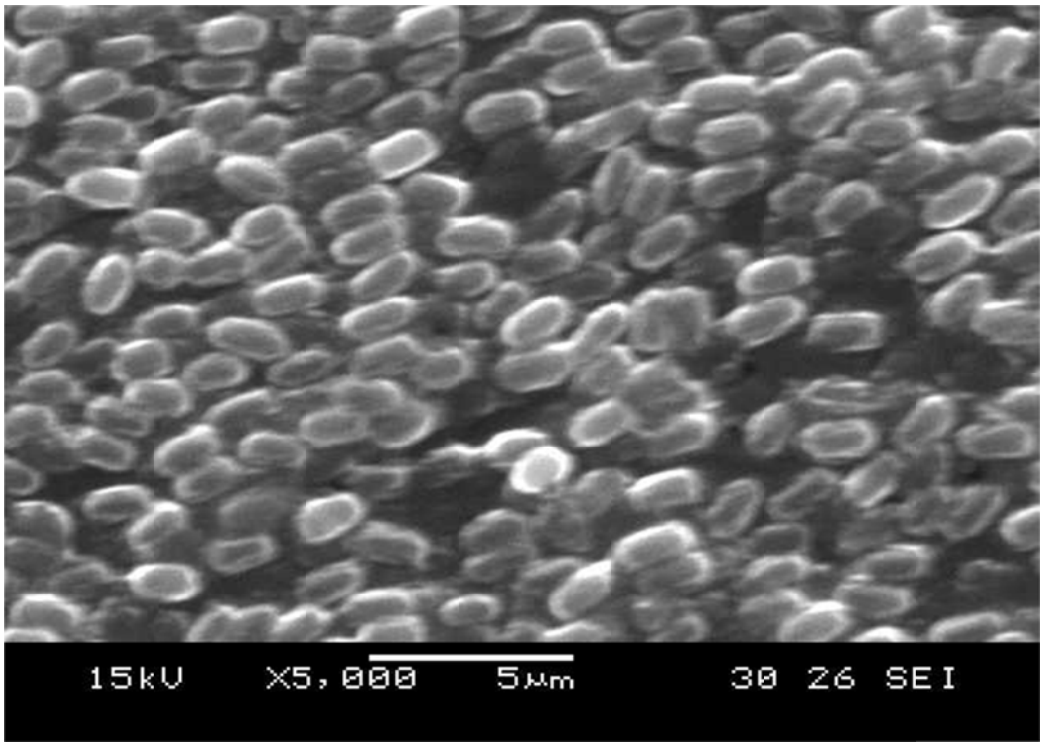

Figure 2. SEM picture revealing rod shape of the Gram positive bacteria.

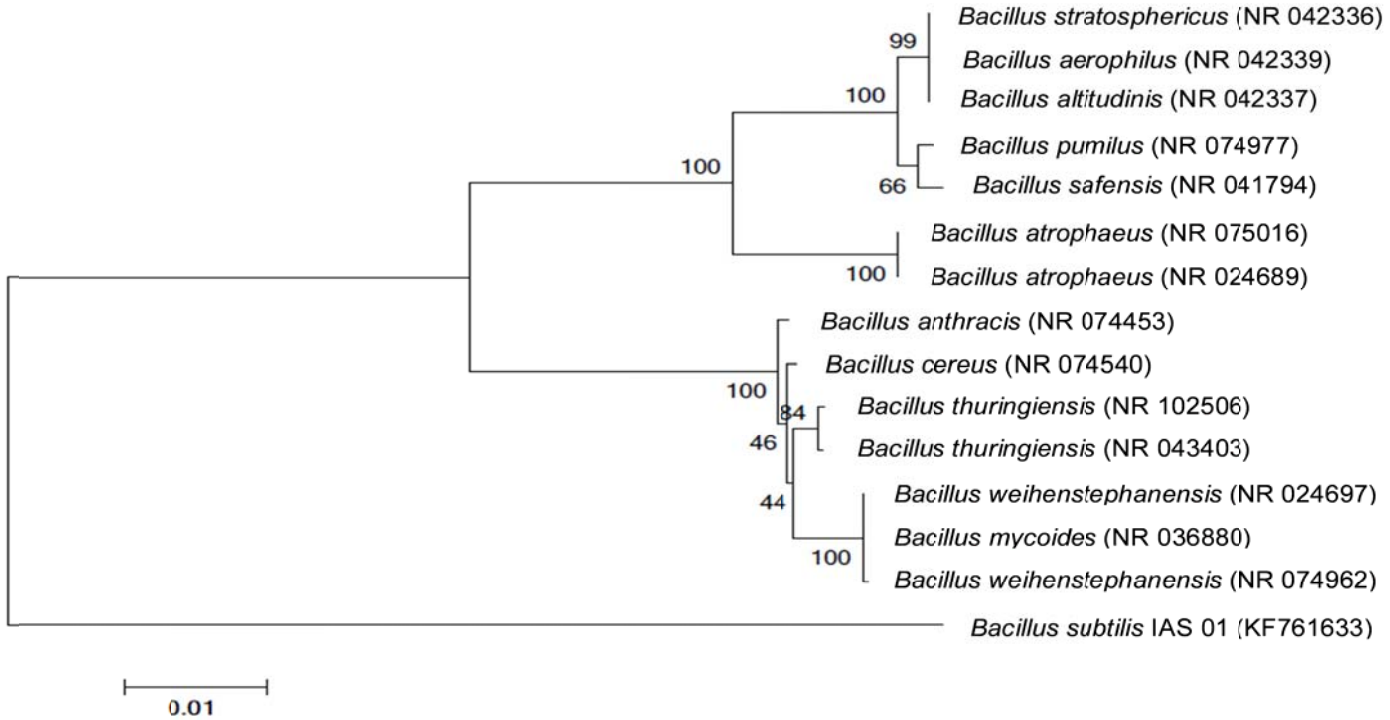

Figure 3. Phylogenetic tree predicted by the neighbour joining method using 16s rRNA gene sequences. The bootstrap considered 1000 replicates. The isolate IAS01 belong to the *B. subtilis*. Taxa are represented by type strain with Gen bank accession number. The scale bar represents the expected number of substitution average to over all the analyzed sites. Number in bracket indicates accession number.

Table 1. Biochemical result for selected strain, IAS01.

Biochemical characteristic	Result
Nitrate reduced to nitrite	+
Voges-Proskauer test	+
Urea test	-
Utilization of citrate test	+
Utilization of propionate	-
Formation of indole test	-
Hydrolysis of casein, starch and gelatin test	+

+, 90% or more strain positive; -, 90% or more strain negative.

Table 2. Classical biochemical test for selected strain IAS01.

Sugar fermentation test		Fluorogenic substrate test		Other specific test	
Parameters	**Result**	**Parameters**	**Result**	**Parameters**	**Result**
Glucose	+			Methionine	+
Glycerol	+			Aesulin	+
Maltose	+	β – D galactopyenoside	-	Ornithin and D alanine	-
Mannitol	+			Arginine	+
Rhamnose	-			Leucine	+
Sucrose	+	Valanine AMC	-	Serine and Urea	-

The selected IAS1 was tested for their biochemical nature by using diagnostic test kit TREK. The Sensititre GPID plate is an in vitro diagnostic product for the automated identification of Gram positive bacteria

Table 3. Effect of pH on growth and alkaline protease production by isolated strain "IAS01" after 48 h of incubation at 30°C temperature and 100 rpm agitation speed in shake flask culture.

pH	Protease activity (U/ml)	Protein (mg/ml)	Cell biomass (g/l)
5	101.88±3.28^{g}	0.88±0.07	1.26±0.02
6	119.33±15.36^{f}	1.27±0.07	1.49±0.03
7	154.83±2.48^{e}	1.57±0.10	1.63±0.03
8	193.74±2.84^{c}	1.67±0.09	1.71±0.02
9	235.05±2.91^{a}	2.45±0.08^{a}	2.04±0.02
10	209.38±2.84^{b}	1.99±0.07	1.93±0.02
11	180.50±3.13^{d}	1.41±0.16	1.47±0.05

Each value is an average of six replicates, ± indicates standard deviation (SD) among the replicates. Values of a given column are statistically compared by employing single factor analysis of variance and Duncan's multiple range test using CoStat software. Values followed by different letters in respective column differ significantly from each other at p≤0.05.

Table 4. Effect of temperature on growth and alkaline protease production by isolated strain "IAS01" at pH 9.0 and agitation speed of 100 rpm after 48 h incubation in shake flask culture.

Temperature (°C)	Protease activity (U/ml)	Protein (mg/ml)	Cell biomass (g/l)
30	210.19±3.10^{d}	2.01±0.09	1.26±0.02
35	246.89±2.21^{c}	2.21±0.11	1.93±0.03
37	272.76±1.64^{a}	2.55±0.10	2.14±0.02

Table 4. Contd

40	257.72±2.45[b]	1.74±0.11	1.97±0.01
45	227.43±2.63[e]	2.12±0.56	1.24±0.02
50	196.55±2.48[f]	1.99±0.07	0.94±0.02
55	163.05±1.26[g]	1.04±0.18	0.56±0.01

Each value is an average of six replicates, ± indicates standard deviation (SD) among the replicates. Values of a given column are statistically compared by employing single factor analysis of variance and Duncan's multiple range test using CoStat software. Values followed by different letters in respective column differ significantly from each other at p≤0.05.

Table 5. Effect of incubation period on growth and production of alkaline protease by isolated strain "IAS01" at pH 9.0, temperature 37°C and agitation speed of 100 rpm in shake flask culture.

Incubation (h)	Protease activity (U/ml)	Protein (mg/ml)	Cell biomass (g/l)
24	167.26±2.94[f]	1.08±0.10	0.57±0.02
36	200.96±2.52[e]	2.06±0.09	0.93±0.02
48	237.26±1.77[c]	2.52±0.09	2.05±0.01
60	261.93±1.45[b1]	2.63±0.12	2.18±0.02
72	289.81±2.33[a]	3.23±0.07	2.30±0.12
84	262.93±4.54[b]	2.02±0.18	2.51±0.02
55	221.82±1.96[d]	1.46±0.09	1.84±0.02

Each value is an average of six replicates, ± indicates standard deviation among the replicates. Values of a given column are statistically compared by employing single factor analysis of variance and Duncan's multiple range test using CoStat software. Values followed by different letters in respective column differ significantly from each other at p≤0.05.

Table 6. Effect of agitation speed on growth and production of alkaline protease by isolated strain "IAS01" at corresponding pH 9.0 and temperature37°C after 72 h of incubation in shake flask culture.

Agitation (rpm)	Protease activity (U/ml)	Protein (mg/ml)	Cell biomass (g/l)
80	230.04±1.77[f]	2.50±0.06	2.48±0.01
100	244.08±1.92[e]	2.62±0.12	2.66±0.02
120	285.80±1.65[c]	3.20±0.05	2.92±0.02
140	332.13±1.31[a]	3.30±0.07	2.99±0.01
160	305.25±2.36[b]	1.78±0.09	3.06±0.01
180	272.36±2.10[d]	2.56±0.12	2.30±0.05
200	200.16±2.10[g]	2.08±0.09	1.57±0.31

Each value is an average of six replicates, ± indicates standard deviation (SD) among the replicates. Values of a given column are statistically compared by employing single factor analysis of variance and Duncan's multiple range test using CoStat software. Values followed by different letters in respective column differ significantly from each other at p≤0.05.

Table 7. Quantitative estimation of protease production by test cultures as compared to standard culture, *B. subtilis* (MTCC-1789) under optimizing condition: pH 9.0, temperature 37°C, incubation period 72 h and 140 agitation in shake flask cultures.

Name of the isolates	Protease activity (U/ml)	Protein (mg/ml)	Cell biomass (g/l)
MTCC-1789	230.04±1.77[e]	2.52±0.09	2.05±0.01
IAS-01	332.13±1.31[a]	3.30±0.07	2.99±0.01
IAS-02	285.80±1.65[c]	3.19±0.05	2.95±0.02

Table 7. Contd

IAS-03	222.59±11.14^{f}	1.99±0.07	1.93±0.02
IAS-04	305.25±2.36^{b}	3.25±0.07	2.30±0.12
IAS-05	272.36±2.10^{d}	2.55±0.10	2.14±0.02
IAS-06	200.16±2.10^{g}	1.67±0.09	1.71±0.02

Each value is an average of six replicates, ± indicates standard deviation (SD) among the replicates. Values of a given column are statistically compared by employing single factor analysis of variance and Duncan's multiple range test using CoStat software. Values followed by different letters in respective column differ significantly from each other at p≤0.05.

Conclusion

The present study achieved the isolation of an effective protease producing *Bacillus* sp. such as *B. subtilis* IAS01 from coastal regions of rhizosphere soil and investigated the ability of the strain based on qualitative and quantitative examination with standard culture of *B. subtilis* (MTCC-1789) under alkaline condition. As a result, the isolate *B. subtilis* IAS01 produced highest yield of alkaline protease (332.13±1.31 U/ml), protein concentration (3.30±0.07 mg/ml) out of twenty seven isolates. Therefore, IAS01 was found to be an efficient isolate and it can be studied further for its biochemical characteristics and applicability in industrial applications.

Conflict of interest

The authors did not declare any conflict of interest.

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