Fermentation Technology
ISSN: 2167-7972

Qureshi et al., Ferment Technol 2018, 7:1
DOI: 10.4172/2167-7972.1000151

Research Article Open Access

Production, Purification and Partial Characterization of Organo-Solvent Tolerant Protease from Newly Isolated *Bacillus* sp. BBXS-2

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Received date: January 12, 2018, **Accepted date:** February 01, 2018, **Published date:** February 08, 2018

Abstract

Proteolytic enzymes are applied in various industries such as detergent, leather, food, textile, cosmetics, pharmaceutical, and synthetic biotechnology. To meet commercial needs, microbial strains of high value in terms of cost-effective production have been focused. In this study, thermophilic strain, *Bacillus* sp. BBXS-2 was activated on simple growth medium and then transferred to Luria Bertani (LB) medium. Maximum protease concentration of 6723 U/mL under optimized fermentation conditions (molasses, corn steep liquor, pH 9.0, and 45°C). The results showed, there was no difference observed in bacterial growth and protease titer while replacing yeast extract with corn steep liquor so, in this way, about 90% cost of nitrogen source can be saved. The extracellular enzyme was purified to homogeneity from cell-free supernatant by ammonium sulphate precipitation followed by dialysis and ion exchange chromatography, recovery yield reduced from 100 to 22% and purification fold increased from 1 to 9.82. The enzyme was active in broad pH and temperature range 8-12 pH and 30-60°C, with maximum activity at pH 10.0 and 60°C, respectively. Protease retained more than 90% activity after incubation at 40°C for 2 weeks in the presence of (40% v/v) organic solvents including ethanol, methanol, and isopropanol. Overall, research suggests that this strain is a more promising candidate and possess practical ability to use in industries.

Keywords: *Bacillus* sp. BBXS-2; Corn steep liquor; Organic solvent; Tolerant protease; Molasses; Purified protease

Introduction

Proteases are applied in several industries, including detergent, leather, food, textile, cosmetics, pharmaceutical, and synthetic biotechnology [1,2]. Proteases are major industrial enzymes that accounts for 60% of the total enzyme sale, and a major proportion of proteases is produced from microorganisms [3]. Among available proteases, many are produced from *Bacillus* species perhaps due to simple medium and growth conditions requirements, extracellular secretion of enzymes, rapid cell growth, and availability of fully sequenced genome [4]. Several *Bacillus* species are exploited for extracellular protease including, *Bacillus subtilis* EFRL 01 [5], *Bacillus licheniformis* [6-8], *B. pseudofirmus* [9] and other *Bacillus* sp. [10]. However, the search of new bacterial strains for protease production with unique properties such as organic solvent tolerant microbes and enzymes, alkaline pH stability, thermostability, stability in the presence of a surfactant, bleaching and oxidizing agents is continued.

Enzymatic reactions in the presence of organic solvents provide various industrially important benefits, for example, enhanced nonpolar substrates solubility, inhibition or decrease of water-dependent side reactions and controlling the microbial contamination [11-13]. Synthetic biology applications need protease stable active in the organic solvents for a longer duration. Organic solvent tolerant proteases are produced by several bacterial species, including *Pseudomonas* [14-21], *Enterobacter* [22], *Rhodococcus* [23], and *Geomicrobium* [24]. However, only a few studies are available on organo-solvent proteases from *Bacillus* species [12,13]. Thus, there is a huge gap to be filled with new organic solvent stable proteases from unique microbial strains to meet increasing demand. To obtain highest protease titer, cultural conditions must be optimized in terms of carbon and nitrogen sources, pH, temperature, inoculum size, and time of incubation [5,25,26]. A major difficulty in the commercialization of fermentation processes is the cost of nutrients (carbon and nitrogen sources) and purification. Thus, the use of agro-industrial residue as nutrient source could be a promising solution to dispose of the waste and cost-effective protease production.

Therefore, this research work describes protease production from thermophilic *Bacillus* sp. BBXS-2 using molasses and corn steep liquor as cost-effective carbon and nitrogen sources. Protease fermentation was also confirmed in a 2 L conical flask with a titer of 6723 U/mL under optimized fermentation conditions (molasses, corn steep liquor, pH 9.0, and 45°C). In addition, results show that bacterial growth and protease titer have certainly no difference when yeast extract or corn steep liquor was used as nitrogen source. About 90% of nitrogen source cost was saved by replacing yeast extract with corn steep liquor. The purified protease of *Bacillus* sp. BBXS-2 has characterized that displayed excellent characteristics, for instance, stability in alkaline pH,

thermostability, stability in presence of surfactant, oxidizing and bleaching agents, and tolerance against organic solvents. These results suggest that strain possess practical potential to be used in industrial production units and an enzyme having excellent properties make it suitable for biotechnological applications.

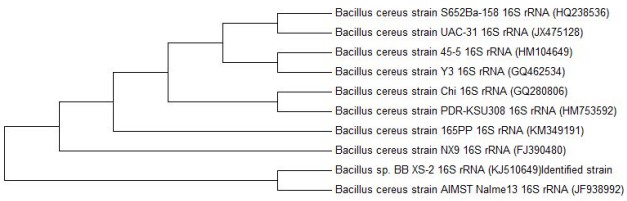

Figure 1: Phylogenetic tree showing the relationship of isolate *Bacillus* sp. BBXS-2 (KJ510649) to other *Bacillus cereus* strains values shown in the parenthesis are accession number.

Materials and Methods

Strain

Bacillus sp. BBXS-2 was isolated from the soil sample collected from Xuhui district of Shanghai, China. The strain was grown on simple growth medium containing glucose 20 g/L, peptone 10 g/L, and sodium chloride 5 g/L. pH was adjusted to 7.0, the culture was incubated for 18 h at 37°C in shaking incubator. This culture was used as inoculum in protease fermentation medium. *16S rRNA* gene sequence of the strain was deposited in GenBank database under accession number KJ510649.

Enzyme production

Bacillus sp. BBXS-2 seed culture was inoculated enzyme production medium composed of glucose 10 g/L, peptone 5 g/L, $MgSO_4.7H_2O$ 2 g/L, KH_2PO_4 3 g/L and NaCl 2 g/L, as described elsewhere [5]. The culture was incubated in shaking incubator for 120 h at 37°C and samples were harvested after every 12 h for growth and enzyme production. The cell-free clear sample was collected after centrifugation at 11,300 × g for 10 min and used for biochemical analysis by spectrophotometric methods.

Effect of carbon and nitrogen sources

Effect of various carbon sources, for instance, glucose, molasses, starch, maltose, galactose, fructose, mannose, lactose, sucrose, and xylose (10 g/L) for bacterial growth and protease yield was analyzed. To investigate the effect of nitrogen sources, production medium was supplemented (5 g/L) with either of peptone, yeast extract, urea, casein, meat extract, tryptone, ammonium chloride, ammonium nitrate, and ammonium sulphate. The culture was incubated in an orbital shaking incubator at 37°C, 150 rpm, and pH 7.0 for 48 h.

Influence of pH and temperature on protease production

Bacillus sp. BBXS-2 was grown in a fermentation medium with different initial pH ranging from 5.0-12.0 adjusted with 2.0 M H_2SO_4 or 5.0 M NaOH. For evaluating the effect of temperature on bacterial growth and protease titer, *Bacillus* sp. BBXS-2 was cultivated in shaking incubator at a different temperature range of 30-55°C, pH 10.0 for 48 h.

Evaluation of corn steep liquor as cost-effective nutrient

The different initial concentrations of corn steep liquor (0, 5, 10, 20, 30, 40 g/L) were added to optimize fermentation medium instead of yeast extract.

Protease assay

Protease production was monitored according to the following method adapted from [27]. 0.5 mL of the appropriately diluted enzyme was added in 0.5 mL of a casein solution (10 g/L, adjusted to pH 10.0) and 1.5 mL of glycine-NaOH buffer (pH 10.0). The reaction mixture was kept at 60°C for 30 min in a shaking water bath. 2 mL of sample was removed at the end of the reaction and quenched at once by mixing with 2 mL of trichloroacetic acid (15 g/L). The mixture was centrifuged at 4000 × g for 10 min to remove the protein precipitate, whereas 1 mL of the (supernatant) sample was mixed with 4 mL of 0.5 M NaOH, 1 mL of Folin phenol reagent and 4 mL of double distilled water. Absorbance was detected at 625 nm against an appropriate substrate and enzyme blank. The difference in absorbance between the initial reaction mixture and the sample was taken at 30 min, indicated the proteolytic activity in terms of released tyrosine. The standard graph for calculating protease concentration was prepared with 10-50 µg/mL of tyrosine solutions. One unit of protease activity was defined as the amount of protease that liberated 1 µmol of tyrosine under the assay conditions.

Confirmation experiment in 2 L conical flask

Protease fermentation was performed in 2 L conical flask to scale up the protease production from the new thermophilic strain, experiments were conducted in parallel. 18 h old seed culture of *Bacillus* sp. BBXS-2 at the rate of 10 (% v/v) was transferred as inoculum to enzyme production medium and culture was incubated at 45°C for 48 h in constant shaking conditions, pH was adjusted to 9.0. Samples were collected at regular interval for monitoring the bacterial growth and protease activity.

Protease purification and characterization

Cell-free clear liquid (culture broth) was precipitated by addition of ammonium sulphate (30-100%), with optimum precipitation at 75%. The fraction was obtained by centrifugation at 10,000 g for 10 min. Precipitates were dissolved in 20 mM Tris-HCl buffer (pH 8.0). Dialysis was applied to the collected extract then, undissolved proteins were removed by centrifugation at 11,300 × g for 5 min. Clear supernatant 10 mL was applied to ion exchange column and equilibrated with 10 mM Tris-HCl buffer (pH 8.0). Protein samples were measured at 280 nm and protease activity was checked according to the method described in materials and methods section, and then enzyme bands were analyzed on SDS-PAGE.

To monitor pH optimum for protease activity with a maximal substrate, the conversion was investigated by incubating reaction mixture at different pH range 6.0-12.0. Following buffer solutions were used for changing pH values: phosphate buffer pH 6.0-7.5; Tris-HCl buffer pH 8.0-8.5; glycine-NaOH buffer pH 9.0-12.0. Protease stability at different pH values (6.0-12.0 pH) was monitored by placing purified enzyme different buffer solutions at 35°C for 1 h, and the protease

activity was measured under assay conditions. The optimum temperature for the maximal catalytic reaction was checked by determining protease activity at different temperature ranges (30-85°C) at fixed pH and substrate concentration for 30 min. Thermostability was monitored prior to incubate enzyme solution for 1 h at different temperature ranges 30-85°C. Metal ions influence on protease activity was monitored by pre-incubating enzyme with 12 mM of these metal ions (Ca^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, Ni^{2+}, Mg^{2+}) at the concentration of 12 mM.

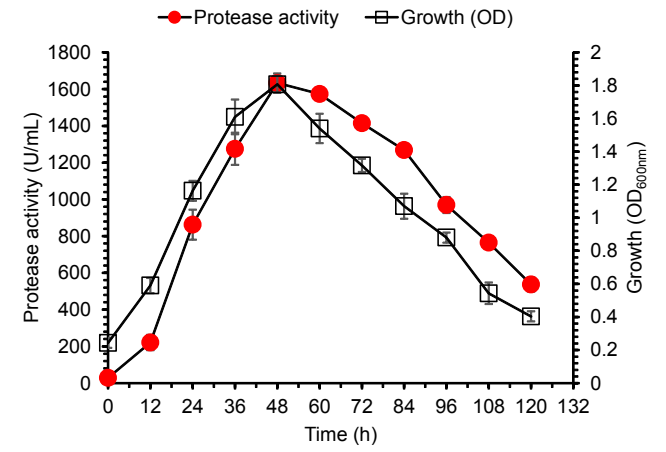

Figure 2: Batch profile of protease titer and bacterial growth in basal medium containing (g/L) glucose 10, peptone 5, $MgSO_4.7H_2O$ 2, KH_2PO_4 3 and NaCl 2 and incubated in an orbital shaking incubator at 37°C with initial pH 7.0. Results are the average of a triplicate experiment.

Enzyme stability in surfactant, oxidizing and bleaching agents

For applicability of protease in biotechnology industries (synthetic biotechnology, detergent industry, and leather industry) its stability in the presence of organic solvents, surfactants, oxidizing, and bleaching agents was determined. Protease stability was measured by incubating enzyme in presence of different organic solvents (40% v/v), including ethanol, methanol, isopropanol, acetone, toluene, n-hexane, xylene, and DMSO for 2 weeks at 40°C in shaking incubator. Stability of protease was assayed in presence of Triton X-100, Tween 80, SDS (surfactants), H_2O_2 (oxidizing agent), and sodium hypochlorite (bleaching agent). Residual activity was determined under optimized assay conditions (pH 10.0 and 40°C). Protease activity was measured as% of relative activity as compared to control (without additives) that was taken as 100%.

Accession number

The genome sequence data have been deposited in GenBank under accession number KJ510649.

Statistical analysis

Three replicates of each sample were used for statistical analysis. Data were reported as means ± S.D. Analysis of variance and least significant difference tests were conducted to identify differences among means. t-test was performed to determine significant differences at $p<0.05$.

Results

Screening, isolation, and identification of protease producing strain

In the present study, 33 bacterial strains were isolated from soil samples of Xuhui, Shanghai, China and were screened for proteolytic activity using casein agar medium and clear zone of proteolysis was detected on the casein agar plates. Among these strains with larger zone were selected for further confirmation of extracellular protease activity in fermentation medium (data not shown). Finally, a strain that performed well in plates and liquid fermentation was identified as a member of the genus *Bacillus* behalf of *16S rRNA* gene sequence similarity. The nucleotide sequence (KJ510649) reported here has been assigned from NCBI GenBank database. The phylogenetic tree was plotted by comparing the gene sequences of earlier submitted data using molecular evolutionary genetics analysis (MEGA-6) [28], and a phylogenetic tree is shown in Figure 1.

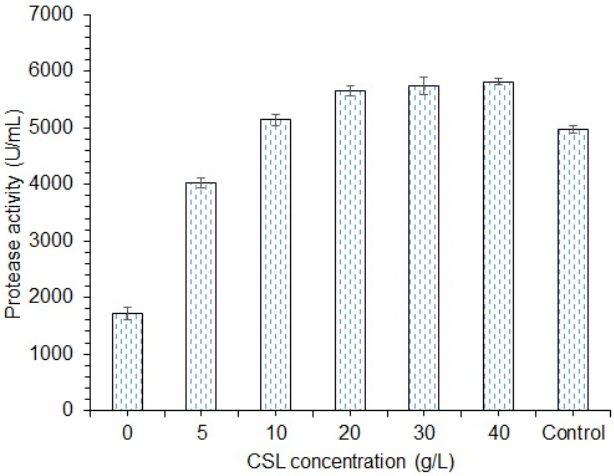

Figure 3: Effect of corn steep liquor (CSL) concentration (0, 5, 10, 20, 30, 40 g/L) on protease production under optimized conditions (48 h, molasses, 9.0 pH, and 45°C). The control experiment was designed to compare the efficiency of CSL with yeast extract (5 g/L). In the control all other conditions were same. Results are the average of triplicate experiments.

Optimization of fermentation conditions for protease production

A time course of protease concentration and bacterial growth was developed for newly isolated *Bacillus* sp. BBXS-2 in basal medium containing: 10 g/L of glucose, 5 g/L of peptone, 2 g/L of $MgSO_4$, 3 g/L of KH_2PO_4, and 2 g/L of NaCl (Figure 2). Maximum protease yield of 2060 U/mL obtained when molasses was supplemented as sole carbon source compared to other pure sugars, results are shown in Table 1.

Carbon source	Glucose	Molasses	Maltose	Galactose	Fructose	Mannose	Starch	Lactose	Xylose	Sucrose
Protease activity	1450	2060	1622	1488	1245	1109	1886	1423	1125	1624
Absorbance 600 nm	1.82	2.23	1.65	1.21	1.38	1.91	1.22	1.17	1.09	1.54
Nitrogen source	Peptone	Yeast extract	Urea	Casein	Meat extract	Tryptone	Ammonium chloride	Ammonium nitrate	Ammonium sulphate	
Protease activity	2060	2579	979	1664	1739	1452	928	814	657	
Absorbance 600 nm	2.25	2.52	0.98	1.31	1.32	1.13	0.98	0.72	0.82	
pH	5	6	7	8	9	10	11	12		
Protease activity	1094	1588	2579	3070	3260	2938	2650	2346		
Absorbance 600 nm	1.14	1.53	2.57	2.72	3.19	2.67	1.96	1.83		
Temperature	30	37	40	42	45	48	50	55		
Protease activity	2386	3260	3746	4321	4974	4841	4421	3623		
Absorbance 600 nm	2.71	3.12	3.20	3.47	3.59	3.37	3.14	2.75		

Conditions for carbon sources effect (10 g/L) on protease titer and bacterial growth at 48 h of fermentation (37°C, initial pH of 7.0). Conditions for nitrogen sources effect (5 g/L) on protease production and cell growth at 48 h of fermentation (37°C, initial pH of 7.0) in a molasses (10 g/L initial concentration) mineral medium. Conditions for initial pH effect on protease fermentation and microbial growth for 48 h at (37°C) in a mineral medium containing molasses and yeast extract as carbon and nitrogen sources, respectively. Conditions for the influence of temperature on protease production and *bacillus* growth at 48 h. The medium initially contained 10 g/L molasses and 5.0 g/L yeast extract and initial pH was adjusted to 9. Results are the average of a triplicate experiment.

Table 1: Optimization of cultural conditions for protease production (U/mL) from *Bacillus* sp. BBXS-2.

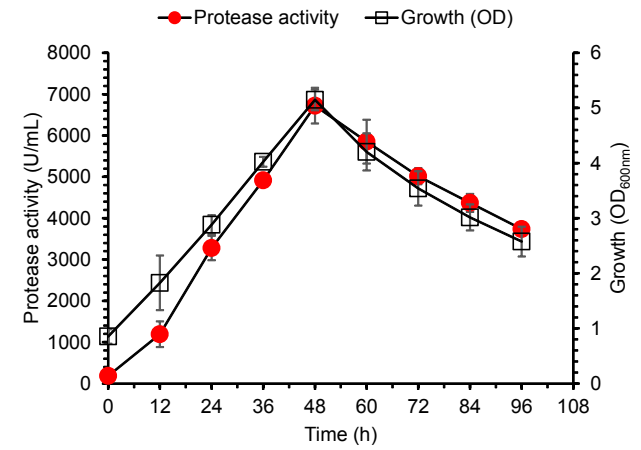

Figure 4: Confirmation experiment for protease production using optimized medium and cultural conditions in a 2 L conical flask. Molasses and corn steep liquor were used as sole carbon and nitrogen source. The culture was incubated at 45°C for 96 h, pH was adjusted to 9.0. Results are average of a triplicate experiment. Error bars are shown for standard deviation.

Effect of organic and inorganic nitrogen sources was evaluated on cell growth and protease production. Highest enzyme yield obtained when yeast extract (2579 U/mL) used followed by peptone (2060 U/mL) and meat extract (1736 U/mL) was used. Inorganic nitrogen source showed lowest protease titer, perhaps due to reduced cell growth, results are shown in Table 1. Effect of initial pH on the protease titer and growth of *Bacillus* sp. BBXS-2 is shown in Table 1. Initial pH of the medium was adjusted in the range of 5.0-12.0 before sterilization of medium. The culture was inoculated in each flask then, incubated at 37°C for 48 h in shaking incubator. Maximum protease activity (3260 U/mL) was obtained at initial pH of 9.0, it indicates the alkaliphilic nature of strain. Generally, the microbial strain could grow in alkaline pH range are very important due to increasing demands of alkaline stable enzymes. Literature reports that alkaline stable proteases are applied in detergent and leather industries.

Further, an effect of temperature (30 to 55°C) on enzyme production and growth of *Bacillus* sp. BBXS-2 was studied. Protease titer increased with increasing temperature and reached maximum to 4974 U/mL at 45°C. Further increase in temperature reduced protease concentration perhaps due to the decrease in cell growth, results are shown in Table 1.

Evaluation of corn steep liquor (CSL) as cost-effective nitrogen source

In routine fermentation experiments, at a laboratory and industrial scale, yeast extract (YE) and or peptone are used as nitrogen source but due to increasing nutrients cost, inexpensive nitrogen sources are certainly required to increase the economic viability of commercial enzyme production. Therefore, in this study, the efficiency of corn steep liquor (CSL) as nitrogen source was investigated. Corn steep liquor at various concentrations (0, 5, 10, 20 30, 40 g/L) were supplemented in the fermentation medium and results were compared with control (yeast extract 5 g/L). Maximum protease titer was observed when 20 g/L of CSL used, there was no significant increase in the protease concentration when 30 and 40 g/L of CSL used. Protease concentrations were (5654 U/mL) and (4974 U/mL) in case of CSL and

yeast extract, respectively, results are shown in Figure 3. The price of yeast extract is 9.2 $/kg equivalent to 9,200 $/ton and that of CSL is 0.18 $/kg equivalent to 180 $/ton as reported elsewhere [29]. Results clearly showed the significance of our study to reduce enzyme production cost using CSL and molasses as nitrogen and carbon sources.

Purification step	Total activity (U)	Total Protein (mg)	Specific activity (U/mg protein)	Purification fold	Yield (%)
Crude	5654	63	89.74	1	100
Ammonium sulphate precipitation	2945	18	163.61	1.82	52.08
Dialysis	2004	8	250.5	2.79	35.44
ion exchange chromatography	1234	1.4	881.42	9.82	21.82

Table 2: Purification of protease produced from *Bacillus* sp. BBXS-2.

In the next step, confirmation experiment for protease production in 2 L conical flask was performed and 6723 U/mL of protease was obtained as shown in Figure 4, and 5654 U/mL protease activity was noted in 250 mL conical flask containing 50 mL of fermentation medium at the end of 48 h with 1.18-fold increase in protease titer at large scale fermentation. Previous studies have also reported similar pattern for scaling up studies. Thus, the strain shows great potential to be applied for large-scale protease production.

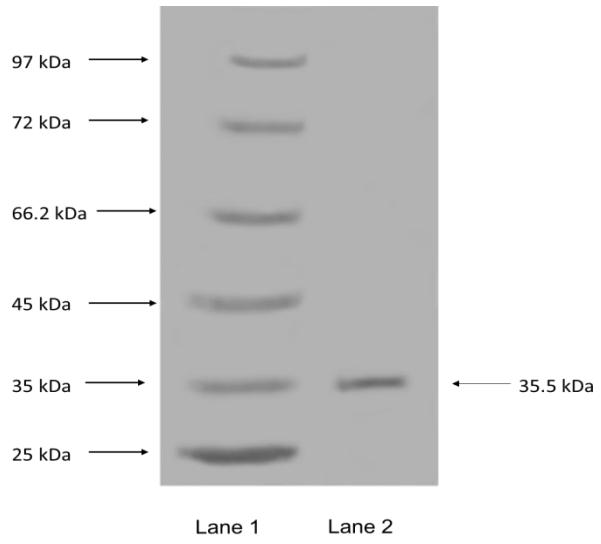

Figure 5a: SDS-PAGE, (Lane 1) Standard protein marker (97.0 kDa-Phosphorylase; 66.2 kDa-Bovine serum albumin (BSA); 45.0 kDa-Ovalbumin; 35.0 kDa-Lactate dehydrogenase; 25.0 kDa-REase Bsp981, (Lane 2) Purified protease from *Bacillus* sp. BBXS-2.

Enzyme purification and characterization of the protease of *Bacillus* sp. BBXS-2

Cell-free clear solution (fermentation broth) was used for ammonium sulphate precipitation (30-100%) showed protease activity at 75% saturation with 1.82-fold purification, then precipitates were dissolved in 20 mM Tris-HCl buffer (pH 8.0). Then enzyme buffer mixture was dialyzed and finally partially purified sample with 2.79-fold purification and 35.44% yield was subjected to ion exchange chromatography. Enzyme purification fold increased to 9.82 with 21.82% yield. Finally, purified enzyme was loaded on the SDS-PAGE gel to checking homogeneity and a single band of 35.5 kDa was obtained (Figure 5a). Purification steps from culture both to final products are detailed in Table 2.

Surfactant	Protease activity (%)
Triton X-100 (0.5% v/v)	115.2 ± 1.9
Tween-80 (1.0% v/v)	110.6 ± 1.4
SDS (0.5% v/v)	142.2 ± 1.1
SDS (1.0% v/v)	133.1 ± 2.4
H2O2 (1.0% v/v)	105.1 ± 2.2
Sodium hypochlorite (1.0% v/v)	111.1 ± 1.7
Control	100
Organic Solvent	
Ethanol	96.6 ± 1.4
Methanol	93.27 ± 2.2
Isopropanol	90.33 ± 1.4
Toluene	86.6 ± 1.9
Xylene	81.8 ± 1.7
n-Hexane	65.3 ± 1.9
DMSO	54.1 ± 2.5
Acetone	81.1 ± 2.4
Metal Ions	
Mg^{2+}	124.2 ± 1.9
Ca^{2+}	146.4 ± 1.5
Fe^{2+}	112.1 ± 1.2
Zn^{2+}	24.5 ± 2.7
Co^{2+}	41.3 ± 1.4
Ni^{2+}	55.3 ± 2.5

Conditions: Preincubation (1 h, 40°C, pH of 10) of the purified enzyme with various metals (12 mM), organic solvents (40% v/v), and surfactant on protease activity (1.0% v/v). The reaction was incubated at 40°C for 1 h in glycine-NaOH buffer (pH 10). Results are shown as a percentage of relative activity comparing to that of control (no additive). Results are average of triplicate experiments.

Table 3: Effect of different metal ions (12 mM), detergents and oxidizing agents (1%), and organic solvents (40% v/v) on the activity of protease from *Bacillus* sp. after 1 h at 40°C.

Characterization of an enzyme is significantly important for applications and enzyme stability in terms of pH, temperature, surfactants, oxidizing and bleaching agents, and organic solvents is required for the local detergents formulations, leather industry, and synthetic biotechnology. Optimum pH for enzyme activity was checked at different pH values (6.0-12.0), casein used as substrate and reaction was incubated at 35°C, results are shown in Figure 5b. Protease activity increased till pH (10.0) and then showed a gradual decrease as pH increased. Moreover, protease remained stable in broad pH range of (8.0-12.0) with maximum activity at pH (10.0). However, maximum enzyme activity (75.91%) was achieved at pH (12.0) as shown in Figure 5b.

On the other hand, maximum protease activity appeared at 60°C, as shown in Figure 5c. The relative activities at 30 and 70°C were about 39.45 and 69.66%, respectively. The enzyme was thermo-stable in the range of 25-60°C and retained 95.22, 75.46 and 26.67% initial activity after 1 h incubation at 60, 70 and 80°C, respectively as shown in Figure 5c.

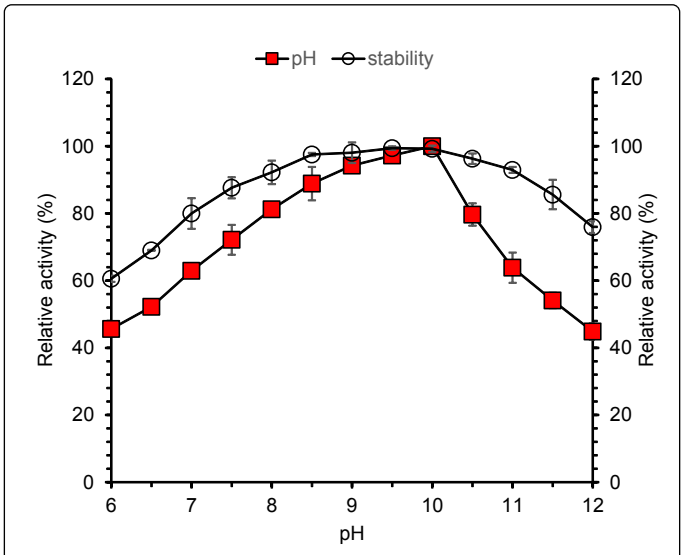

Figure 5b: Effect of pH on protease activity and stability. For evaluating pH activity, a percentage of relative activity was measured by incubating enzyme with the substrate at different pH values (6.0-12.0). Buffers used are: pH stability of purified protease was determined by measuring protease activity. The purified enzyme was incubated for 1 h, at specified pH values (above-mentioned buffers) at 35°C, prior addition of substrate, and% of relative activity was determined. Results are the average of a triplicate experiment.

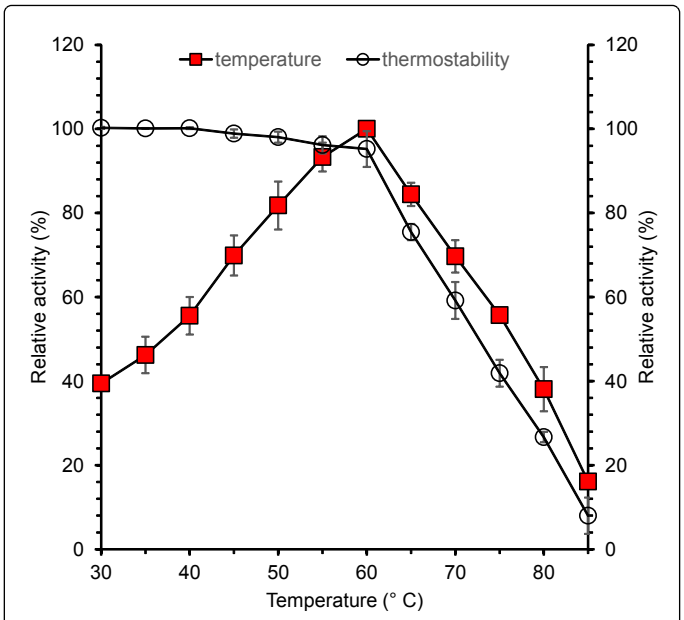

Figure 5c: Effect of temperature on protease activity. The enzyme was mixed with the substrate and incubated at different temperature for 30 min. % of relative activity was measured under assay conditions. Thermostability of protease was determined by incubating enzyme at different temperature without the addition of substrate for 1 h. Percentage of relative activity was checked under assay conditions. Results are the average of a triplicate experiment.

The protease was stable against all surfactants especially, SDS (0.5% v/v) that increased to 142.2% when treated at 40°C. However, with SDS (1.0% v/v) it reduced to 133.1% (Table 3). Protease relative activity was enhanced by the addition of all tested surfactant, oxidizing, and bleaching agent that obviously indicate the suitability of protease for detergent applications. Table 3 shows that alkaline protease is stable in the presence of various solvents (40% v/v). The protease was found stable in presence of several organic solvents, with maximal activity (98%) in the presence of 40 (% v/v) ethanol after 2 weeks of incubation at 40°C compared to control. In addition, our protease was stable at high temperature and pH.

In addition to solvent tolerability, protease activity was evaluated with different metal ions (Table 3). Protease activity increased with addition of Ca^{2+}, Mg^{2+} and Fe^{2+} to 46.4%, 24.2% and 11.2%, respectively. Whereas, protease activity was significantly reduced by addition of Zn^{2+}, Co^{2+}, and Ni^{2+} compared to control (Table 3). Unique characteristics of protease: stability in surfactants, bleaching agent, alkaline pH, and high temperature make it suitable candidature in biotechnological applications.

Discussion

In our study, more than thirty bacterial strains were isolated and screened for proteolytic activity using casein agar plates. Some of these strains showed protease activity with clear zone formation, then strain with larger zone was identified as a member of the genus *Bacillus* on behalf of *16S rRNA* gene sequence similarity, nucleotide sequence was submitted to NCBI GenBank database with the accession number of KJ510649. Zone formation was due to proteolytic activity of microbial

strains, size of zone was directly related to enzyme secretion. Protease titer increased with the passage of time reached to maximum level after 48 h of incubation then gradually decreased. These results were in line with Ibrahim et al. [30], as they attained maximum protease titer after 36 h from *Bacillus* sp. NPST-AK-15. In addition to protease activity and microbial growth, total sugar, total protein, and final pH were also determined (data not shown). It was noted that final pH reduced with the passage of time perhaps due to formation and accumulation of organic acid. This could also be a reason for reduced growth with the time of incubation. Glucose is commonly used as carbon source in growth and fermentation media but increasing demands of fermented products and cost of pure sugars has compelled researchers to search for new cost-effective and abundant energy sources. However, several researchers have exploited waste residues: olive pomace for lipase production, agricultural waste for protease production, feather waste for protease production, and corn stover for ethanol production [31-34].

Strain	Carbon source	Nitrogen source	Reference
Bacillus licheniformis YP1A	Starch 5 g/L	Yeast extract 1.0 g/L+Degossy cotton seed meal 5.0 g/L	[13]
Kluyveromyces marxianus IFO 0288	Glucose 10 g/L	YNB 5 g/L+ Albumin 0.4 g/L	[40]
Bacillus sp. JER02	Sucrose 5 g/L+Citric acid 5 g/L	Yeast extract 10 g/L	[12]
Bacillus sp. SM2014	Glucose 0.5 % w/v	Casamino acid 0.5 % w/v+Yeast extract 0.5 % w/v	[41]
Pseudomonas putida SKG-1	Glucose 10 g/L	Peptone 10 g/L Yeast extract 5 g/L	[37]
Bacillus sp. BBXS-2	Molasses 10 g/L	Corn steep liquor 20 g/L	This study

Table 4: Comparison of carbon and nitrogen sources used for protease production.

Previous studies have proved the potential of agro-industrial residues as a cost-effective energy source. Therefore, in this study, an efficacy of molasses as carbon source was evaluated. Molasses, industrial residues obtained from sugar industry that contains carbohydrates, proteins, minerals, and growth regulators. Thus, molasses provides sufficient nutrients for better bacterial growth and enzyme production. Microbial growth and protease activity varied with nitrogen sources. No doubt, maximum protease yield attained when the microorganism is grown in mineral medium containing yeast and beef extract as evident from various studies [35,36] but researchers are focusing to utilize inexpensive nitrogen sources like corn step liquor and seed cake. Therefore, we used corn steep liquor as a cost-effective nitrogen source for bacterial growth and protease fermentation. Temperature is one of the important parameters, most of the reported protease work used mesophilic strains, for example, Li et al. [13] obtained protease from mesophilic *Bacillus licheniformis* YP1A at 30°C. Singh et al. [37] produced protease from *Pseudomonas* putida SKG-1 at 25°C. However, thermophilic microorganisms could produce thermostable enzyme at high temperature and this might eliminate the chances of contamination during fermentation. However, isolation of thermophilic microorganisms is certainly required to meet increasing demands for thermostable proteases. Our study provides a suitable source of thermostable protease production from newly isolated thermophilic bacterial strains, this enzyme could be a good candidate for various industrial processes.

From an application point of view protease with better properties in terms of stability in an organic solvent, alkaline pH, and high temperature could be the best choice. Previous studies reported proteases stability at moderate organic solvent concentration 20-25% v/v [37-41]. However, our results are showing more advantages of stability at a high organic solvent concentration (40% v/v), alkaline pH (10 pH), and thermostability (60°C). These properties make enzyme suitable for detergent and synthetic biology application. Our results are also compared with some published research articles in various journals, data is shown in Table 4. The comparison is based on only carbon and nitrogen sources used. The full comparison is not possible due to the difference in media composition, protease activity unit, strains, and other fermentation conditions. In available literature, most of the researchers have performed fermentation in the basal medium using pure sugars (glucose, sucrose, and starch) and expensive nitrogen sources (peptone, yeast extract, albumin, and casamino acids), as shown in Table 4. Our results show that bacterial growth and protease titer have certainly had no difference when yeast extract or corn steep liquor was used as nitrogen source (Figure 3). About 90% of nitrogen source cost was saved when yeast extract was replaced with corn steep liquor. In addition, scale-up experiments proved the practical potential of new thermophilic and organo-solvent tolerant strain for industrial scale protease production using cost-effective carbon and nitrogen sources.

Conclusion

Organo-solvent tolerant and thermophilic strain was isolated and identified as *Bacillus* sp. BBXS-2, the strain was hyper protease producing that was stable in a high concentration of organic solvents. Protease fermentation was scaled up in a 2 L conical flask with a titer of 6723 U/mL under optimized fermentation conditions (molasses, corn steep liquor, pH 9.0, and 45°C). In addition, results showed that bacterial growth and protease titer have certainly no difference when yeast extract or corn steep liquor was used as nitrogen source. About 90% of nitrogen source cost was saved when yeast extract was replaced with corn steep liquor. The extracellular enzyme was purified and partially characterized. Purified protease displayed excellent tolerance towards alkaline pH (8.0-12.0), temperature (60°C), organic solvents (40% v/v), surfactants and oxidizing & bleaching agents. Results suggest that strain possess practical potential to be used in industrial production unit and an enzyme having excellent properties which make it suitable for biotechnological applications.

Acknowledgment

Funding was provided by the Natural Science Foundation of China (No.31300603), Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning (No. 2012-28), Fundamental Research Funds for the Central Universities (No. 15D110527, No.15D110508, No.13D110522, No. 15D110568, No. 15D310523, No. 15D110568), the National College Student Innovation Experiment Program (No. 14T10501), General Financial Grant from the China Postdoctoral Science Foundation (2015M571455).

Conflict of Interest

The authors declare no competing interests.

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