**BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY**

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Optimization of Fibrinolytic Protease Production from *Bacillus subtilis* I-2 Using Agro-Residues

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ABSTRACT

The aim of this work was to study the production of fibrinolytic protease by Bacillus subtilis *I-2 on agricultural residues. Molasses substantially enhanced (63%) protease production (652.32 U/mL) than control (398.64 U/mL). Soybean meal supported maximum protease production (797.28 U/mL), followed by malt extract (770.1 U/mL), cotton cake (761.04 U/mL), gelatin (742.92 U/mL) and beef extract (724.8 U/mL). Based on the Plackett-Burman designed experiments, incubation time, soybean meal, mustard cake and molasses were identified as the significant fermentation parameters. Ammonium sulfate precipitation and DEAE sephadex chromatography resulted 4.8-fold purification of protease. Zymography showed the presence of three iso-forms in the partially purified protease preparation, which was confirmed by the SDS-PAGE analysis (42, 48, 60 kDa). Protease exhibited maximum activity at 50^oC and at pH 8.0. Significant stability was observed at 30-50^oC and at pH 7.0-10.0. Mg^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+} and Cu^{2+}, EGTA, EDTA and aprotinin severely decreased the enzyme activity.*

Key words: Fibrinolytic Protease, Production, Agricultural residues, *Bacillus subtilis* I-2

INTRODUCTION

Cardiovascular diseases (CVDs) have emerged as the number one cause of global deaths claiming 17.3 million lives in 2008 and the figure is predicted to rise to 23.6 million by 2030 (Joshi et al. 2008). Thrombosis, manifested in the form of embolism, myocardial infarction, stroke and other cardiac disorders is one of the major causes of CVDs (Mahajan et al. 2012). Under pathophysiological disorders, dynamic balance between the formation and degradation of fibrin is disturbed, leading to accumulation of fibrin causing thrombosis (Simkhada et al. 2010). Therapy for the treatment of acute thrombosis requires intravenous administration of highly specific fibrinolytic agents, which target the preformed fibrin clot hydrolysing the thrombus and restoring the blood flow to the area of ischemia. Although various fibrinolytic agents such as tissue plasminogen activator (t-PA, EC 3.4.21.68), urokinase (u-PA, 3.4.21.73), and bacterial plasminogen activator streptokinase (EC 3.4.24.29) are being extensively investigated (Simkhada et al. 2010) and used widely but most of these thrombolytic agents have got serious limitations such as low fibrin specificity, excessively expensive cost of clinical applications, short half-life, and undesired side effects such as gastrointestinal bleeding, resistance to repercussion and allergic reactions (Mahajan et al. 2012). Therefore, search for novel thrombolytic agents which are safer, cost-effective and more efficient is in progress.

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Enzymes have gained momentum in recent years for therapeutic applications as anticoagulants, oncolytics, thromolytics, anti-inflammatories, fibrinolytics, etc. There are several reports on the isolation and purification of fibrinolytic enzymes with potential thrombolytic applications from various sources such as fermented foods, earthworms and snake venom (Peng et al. 2005). Enormous microbial diversity has been exploited for novel biotechnologically important products, including the potential fibrinolytic proteases with suitability for prospective clinical applications (Mahajan et al. 2012). Furthermore, microbial enzyme production always favours the economy of bulk production (Bajaj et al. 2013). Most of the studies on fibrinolytic proteases involved purification and characterization of enzymes (Mahajan et al. 2012) with relatively a fewer reports on optimization of enzyme production (Mukherjee and Rai 2011). Literature shows that studies on fungal fibrinolytic proteases have been more emphasized than bacterial ones (Simkhada et al. 2010). Biochemical and physiological diversity of bacteria may enhance the scope of targeting better fibrinolytic proteases for potential therapeutic applications. Besides, bacteria grow faster as compared to fungi and can use crude raw material as nutrients, and are easily amenable to genetic manipulations. Substrate being the major cost determining factors for microbial enzyme production, utilization of agroresidues as substrates may help not only in reducing enzyme production cost (Bajaj et al. 2013), but would also lead to valorisation of agro-wastes.

In the current study, production of an efficient fibrinolytic protease from bacterial isolate *Bacillus subtilis* I-2 was optimized by employing cost-effective agricultural residues as substrates and enzyme was partially purified and characterized.

MATERIALS AND METHODS

Isolation and Screening of Fibrinolytic Bacteria
Soil samples from slaughter-houses, dairy, domestic garbage and compost were enriched in nutrient broth and used for the isolation of fibrinolytic protease producing bacteria (Bajaj et al. 2013). Screening of the bacterial isolates was accomplished at primary and secondary levels. In the primary screening, each isolate was examined for proteolytic activity on skimmed milk agar

(SKMA) plates containing skimmed milk powder (0.5%, w/v) and agar (2%, w/v) by spot and well assay (Bajaj and Sharma 2011). Bacterial isolates, which exhibited proteolytic activity, were subjected to secondary screening on fibrin plate to ascertain their fibrinolytic activity. Fibrinolytic activity was determined by the method of Astrup and Mullertz (1952) with slight modifications. The fibrin-agarose gel was prepared, which contained agarose (1%, w/v), fibrinogen (7.0 mg/mL) and thrombin (3.0 U/mL). All the solutions were prepared in normal saline (0.9% NaCl). The clot was allowed to set for 30 min at room temperature. Wells were punctured in the fibrin-agarose gel and crude enzyme (cell-free cultural supernatant obtained after centrifugation of 24 h cultivated bacteria) was poured at 20 µL in each well. Plates were allowed to stand for 2-3 h for fluid to diffuse and then incubated at 37°C overnight. Appearance of the clear zone around the wells on fibrin plate was indicative of fibrinolytic activity of the crude enzyme. The bacterial isolate (I-2), which showed the biggest zone of clearance and also produced maximum fibrinolytic protease under submerged fermentation, was selected for further studies. Bacterial isolate (I-2) was identified by biolog analysis at Centre for Instrumentation Facility, University of Delhi, South Campus, New Delhi, India (http://www.south.du.ac.in/cif/).

Fibrinolytic Protease Production
Submerged fermentation was carried out using the isolate I-2 in protease production medium (PPM), which consisted of (%, w/v) glucose 0.5; peptone 0.5; yeast extract 0.5; K_2HPO_4 0.4; Na_2HPO_4 0.1; $MgCl_2$ 0.01; Na_2CO_3 0.6 at pH 7.0 at 180 rpm and 37°C for 10-12 h (A_{600}, 0.9). This was used as inoculum at 1% (v/v) in the production medium (PPM) and fermentation was carried out at 180 rpm and 37°C. Samples, withdrawn after appropriate time periods, were centrifuged (10,000×g for 10 min at 4°C) and supernatant was used for assaying the protease activity.

Assay of Protease Activity and Protein Content
Protease activity was determined by using casein as the substrate according to the method of Gessesse et al. (2003) with minor modifications. Assay mixture containing 0.5 mL of appropriately diluted enzyme and 0.5 mL of casein (0.65% in phosphate buffer 50 mM, pH 7.0) was incubated at 37°C for 20 min. The reaction was stopped with 2.5 mL of trichloroacetic acid (TCA, 5%, w/v),

and contents were centrifuged (10,000×g for 15 min at 4°C), and supernatant was examined spectrophotometrically (UV-1800 Spectrophotometer, Shimadzu, Japan) for tyrosine content at 280 nm by comparing with tyrosine standard curve. One unit of protease activity was defined as the amount of enzyme required to release 1 µg of tyrosine from casein per min under assay conditions.

The protein content of the enzyme preparation was determined by Bradford method using bovine serum albumin (BSA) as standard.

Effect of Carbon and Nitrogen Sources on Protease Production

The glucose of the PPM was replaced with various crude or refined carbon sources (0.5%, w/v) such as rice husk, wheat bran, maize bran, starch, sucrose, molasses, rice polish, almond hulls, lactose or fructose. The medium was inoculated with 10-12 h grown culture and fermentation was conducted under shaking at 37°C. Enzyme was assayed in periodically withdrawn samples. For determining the effect of various nitrogen sources on protease production, nitrogen source of PPM (peptone and yeast extract) was replaced with different refined or crude nitrogen sources (malt extract, corn steep liquor seasame cake, gelatin, soybean meal, cotton cake, casein, beef extract, mustard cake or chicken feathers, 1.0%, w/v), and fermentation was carried out under shaking at 37°C. Samples withdrawn periodically were assayed for protease activity.

Selection of Significant Variables by Plackett-Burman Design

For the selection of the most significant variables for protease production, a variety of carbon sources, inorganic salts, nitrogen sources and cultivation parameters were tested and identified by the Plackett-Burman (PB) designed experiment. A total of six variables (molasses, starch, mustard cake, soybean meal, incubation time, K_2HPO_4) were selected. Experimental design with the name, symbol code and actual level of variables is shown in Table 1. A set of 12 experiments was generated using the Design Expert 6.0 (Stat Ease, Inc., Minneapolis, USA). The significance level (P value) of each variable was determined using Student's t-test.

Table 1 - Experimental range and levels of the independent variables used in Plackett-Burman Design.

Variables	Units	Symbol code	Experimental values	
			Lower	Higher
Molasses	mL/L	A	5	15
Starch	g/L	B	5	15
Mustard cake	g/L	C	5	15
Soybean meal	g/L	D	5	15
Incubation time	h	E	24	72
K_2HPO_4	g/L	F	1	10

Purification of the Fibrinolytic Enzyme

Fibrinolytic protease was partially purified by ammonium sulfate precipitation and ion exchange chromatography (DEAE sephadex A-50). Crude enzyme (culture supernatant) was subjected to ammonium sulfate fractionation (20-90%). Protein content and enzyme activity of each fraction was determined. Ammonium sulfate precipitated enzyme preparation was collected by centrifugation, re-dissolved in phosphate buffer (0.2 M, pH 8.0), dialysed against the same buffer overnight at 4°C and dialysate was applied to DEAE sephadex A-50 column equilibrated with phosphate buffer (0.2 M, pH 8.0). Elution was effected with 0.25-1.0 M NaCl gradient and fractions collected were examined for protease activity and protein content.

Native-Page, Zymography and Sds-Page Analysis

The polyacrylamide gel was copolymerized with the fibrin substrate and native electrophoresis was performed (Sambrook et al. 1989). After electrophoresis, gel was incubated in phosphate buffer for 1 h at 37°C. The gel was stained with Coomassie brilliant blue (CBB) for 30 min and destained (methanol, glacial acetic acid, distilled water in the ratio of 4.5:1:4.5 (v/v). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by using the stacking gel at 5% (w/v) and resolving the gel at 12% (w/v) in Tris-Glycine buffer, pH 8.3. The gel was stained with CBB for 1 h and destained. The molecular mass of purified enzyme was determined by using a full-range rainbow molecular weight marker (GE Healthcare, USA).

Biochemical Properties of Purified Fibrinolytic Protease

The effect of temperature on enzyme activity was studied by assaying the enzyme at 30°C to 80°C in

0.2 M phosphate buffer, pH 7.4. The thermostability was determined by pre-incubating the enzyme preparation for 60 min at different temperatures (30-80°C), and then determining the remaining activity at pH 7.4 and 37°C. The effect of pH on enzyme activity was studied by using different pH buffers (acetate buffer – pH 5.0 and 6.0; phosphate buffer – pH 7.0 and 8.0, and Tris-HCl buffer – pH 9.0 and 10.0). For the measurement of pH stability, the enzyme was pre-incubated for 60 min. at 4°C at different pH by using appropriate buffers. The residual activity was then determined at pH 7.4 at 37°C.

The effect of different metal ions viz. Fe^{2+}, Mg^{2+}, Zn^{2+}, Co^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+} on the protease activity was determined by including the metal salt in enzyme assay mixture at final concentration of

2 mM. Similarly, ethylene glycol tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), and aprotinin were also evaluated for their influence on enzyme activity by including either of them in enzyme assay mixture at concentration of 2 mM.

RESULTS

Fibrinolytic Bacteria

Among a total of 32 bacterial isolates screened, the isolate I-2 showed maximum fibrinolytic protease activity in plate assay (Fig. 1) as well under submerged fermentation. On the basis of biolog analysis (http://www.south.du.ac.in/cif/), the organism was identified as *Bacillus subtilis* and designated as *B. subtilis* I-2.
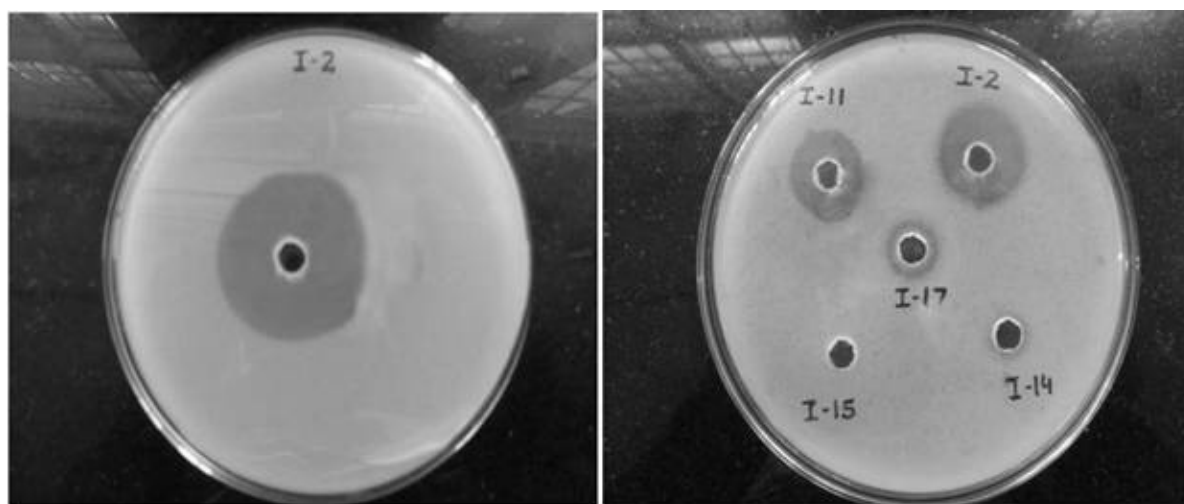

Figure 1 - Screening of bacterial isolates for fibrinolytic activity. Cultural supernatant obtained after centrifugation of 24 h cultivated bacteria was poured at 20 µL in wells punctured in the fibrin-agarose plate (agarose 1%, fibrinogen 7 mg/mL, thrombin 3 U/mL). Plates were incubated at 37°C overnight and observed for clear zone around the wells which was suggestive of fibrinolytic activity.

Effect of Carbon and Nitrogen Sources on Protease Production

Among various carbon sources examined for protease production, molasses supported maximum protease yield (652.32 U/mL), which was substantially higher than control (398.64 U/mL), and was followed by fructose (490.5 U/mL) and starch (413.13 U/mL) as shown in Figure 2. However, other carbon sources did not perform well. All the ten different agriculture based nitrogen sources though supported appreciable protease production but none was able

to induce higher protease production than the control. Soybean meal supported maximum protease production (797.28 U/mL) and was closely followed by malt extract (770.1 U/mL), cotton cake (761.04 U/mL) gelatin (742.92 U/mL) and beef extract (724.8 U/mL) as presented in Figure 3. Other nitrogen sources too gave significant protease yield. Protease production was examined at varying concentration of soybean meal, which showed that its 1.0-1.5% was optimum for protease production.
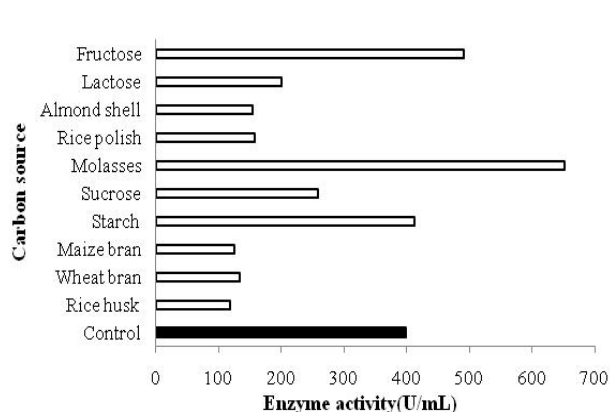
Figure 2 - Fibrinolytic protease production from *Bacillus subtilis* I-2 using various agriculture based carbon sources. Glucose of protease production medium (control) was replaced with either of carbon source (0.5%, w/v), and fermentation was executed under shaking (180 rpm) at 37°C.
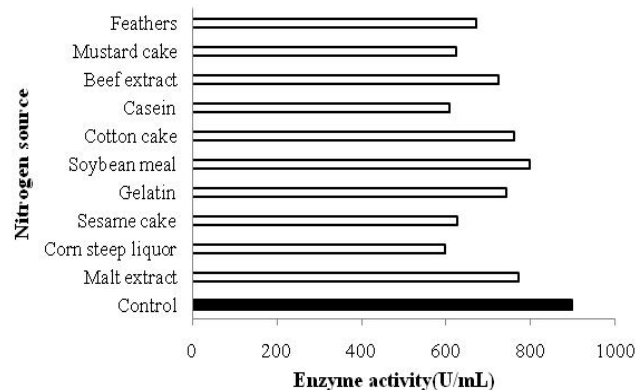

Figure 3 - Fibrinolytic protease production from *Bacillus subtilis* I-2 using various agriculture based nitrogen sources. Peptone and yeast extract of the protease production medium (control) were replaced with either of nitrogen source (1%, w/v), and fermentation was executed under shaking (180 rpm) at 37°C.

Selection of Significant Variables by Plackett–Burman Design

The design matrix selected for the screening of significant variables for protease production and the corresponding responses are shown in Table 2. The adequacy of the model was calculated and the variables showing statistically significant effects were screened by Student's t-test for ANOVA. Incubation time, with a probability value of 0.0012, was the most significant factor, followed by soybean meal (0.0034), mustard cake (0.0076), and molasses (0.0488) as depicted in Table 3. The lower probability values indicated the more significant factors for the production of protease.

Table 2 - Plackett-Burman design for selection of significant fermentation variables for protease production from *Bacillus subtilis* I-2.

Run order	Experimental values						Protease activity (U/mL)	
	A	B	C	D	E	F	Experimental	Predicted
1	15	5	5	5	72	10	559.9	538.01
2	5	15	15	5	72	1	596.14	596.60
3	5	5	5	5	24	1	396.82	390.93
4	15	15	15	5	72	10	625.14	639.48
5	15	5	15	5	24	1	476.55	499.65
6	5	5	5	15	72	10	541.78	579.68
7	15	15	5	15	24	1	534.54	547.68
8	15	15	5	15	72	1	665	651.86
9	5	5	15	15	72	1	663.19	695.52
10	5	15	5	5	24	10	436.69	426.57
11	15	5	15	15	24	10	599.77	584.22
12	5	15	15	15	24	10	581.65	576.97

Table 3 - Results of ANOVA for the Plackett-Burman design.

Variables	Mean square	F-value	P-value
Molasses	4986.99	6.71	0.0488
Starch	3371.78	4.58	0.0863
Mustard cake	13852.29	18.65	0.0076
Soybean meal	20393.18	27.46	0.0034
Incubation time	32565.63	43.84	0.0012
K_2HPO_4	13.42	0.018	0.8983

R- squared- 0.9529, Adj. R- squared- 0.8964, C.V.- 4.90

Purification of Fibrinolytic Enzyme

Ammonium sulfate fraction precipitated at 60% saturation exhibited maximum activity and was subjected to DEAE sephadex A-50 chromatography after dialysis, which resulted in purification by 2.3-fold. Subsequent DEAE sephadex A-50 chromatography led to purification by 4.8-fold, with a recovery of 10.4% as shown in Table 4.

Table 4 - Purification of fibrinolytic protease from *Bacillus subtilis* I-2.

Sample	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude extract	7840	278	28.2	1	100
Ammonium sulfate precipitation (40-60%)	2263	34.8	65	2.3	28.9
Dialysis	1864.3	26.5	70.39	2.5	23.8
DEAE-Sephadex A-50 chromatography)	814.5	6	135.75	4.8	10.4

Effect of Temperature on Activity and Stability of Protease

Fibrinolytic protease showed maximum activity at 50°C; however, considerable activity was observed at 60°C (88.6%) and at 40°C (68.6%). But activity at still higher and lower temperatures decreased severely. Thermostability analysis of the protease showed that enzyme was quite stable at 30-50°C for 60 min. but at 60°C and above activity decreased drastically (Fig. 4).
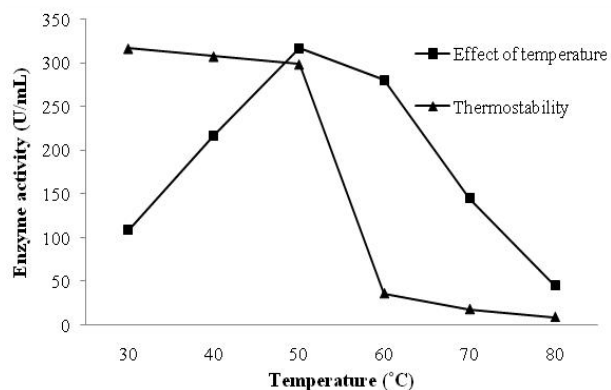
pH 8.0 (407.7 U/mL). Considerable activity was observed at pH 10.0 (84.4%) as presented in Figure 5. However, fibrinolytic protease exhibited poor activity in the acidic pH 5.0-6.0 (53-64%). pH stability analysis of the protease showed that enzyme possessed remarkable stability at pH 7.0-10.0 (98.5-100%). In the acidic pH (5.0-6.0), the enzyme retained 77 and 85% activity, respectively (Fig. 5).
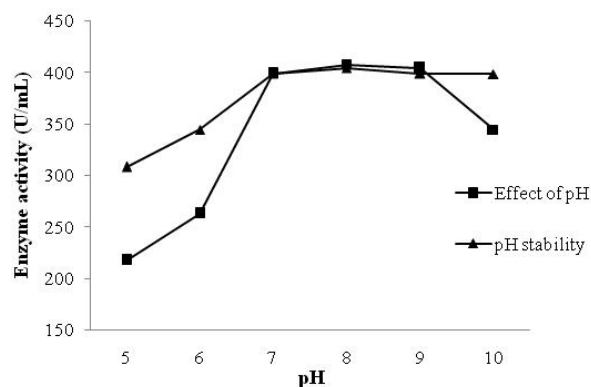

Figure 4 - Activity and stability of protease from *Bacillus subtilis* I-2 at different temperatures. Protease activity assay was conducted at different temperatures (30-80°C), and thermostability was examined by pre-incubating the enzyme at respective temperatures for 1 h, and then assaying the residual activity.

Figure 5 - Activity and stability of protease from *Bacillus subtilis* I-2 at different pH. Protease activity assay was conducted at different pH by using appropriate buffers, and pH-stability was determined by pre-incubating the enzyme at respective pH for 1 h, and then assaying the residual activity.

Effect of pH and pH Stability of Fibrinolytic Protease

The partially purified enzyme exhibited maximum activity in the pH range of 7.0-9.0 with optimum at

Effect of Metal Ions and Additives on Protease Activity

All the metal ions examined in the present study caused severe activity decrease of *B. subtilis* I-2

fibrinolytic protease (52-73.2%), except Fe^{2+}, which reduced the enzyme activity slightly only. EGTA and aprotinin inhibited the activity more strongly as compared to EDTA (Fig. 6).

Fibrin Zymography and SDS-PAGE

Native-PAGE analysis of the purified enzyme preparation followed by zymography showed three
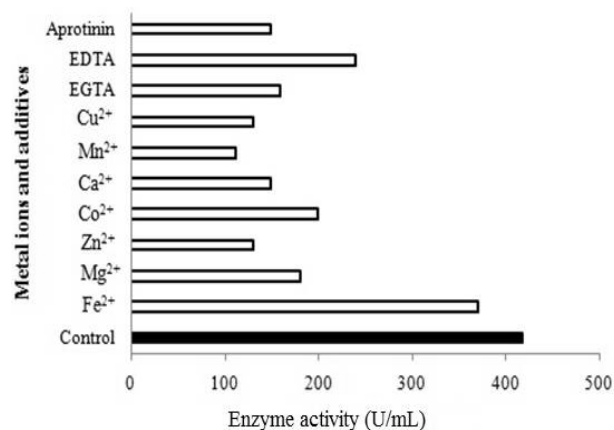
clear bands on the fibrin zymogram, indicating that there were three different proteases with fibrinolytic activity. SDS-PAGE analysis confirmed the fibrin zymography results showing the presence of three protein bands corresponding to molecular weights of approximately 42, 48 and 60 kDa (Fig. 7).
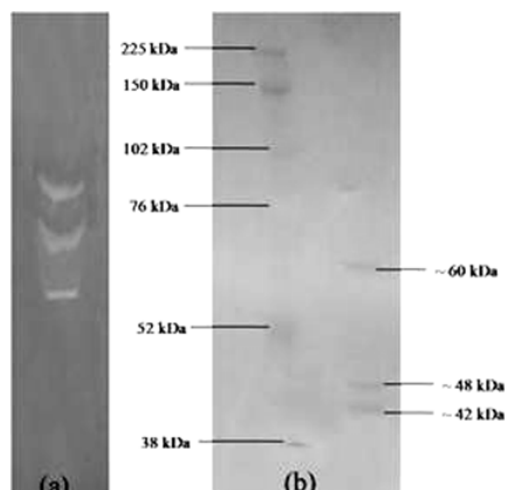

Figure 6 - Effect of metal ions and additives on activity of protease from *Bacillus subtilis* I-2. Either of the metal ion/additive was included in the enzyme assay mixture at final concentration of 2 mM, and protease activity assay was conducted.

Figure 7 - Zymogram analysis (a) and SDS-PAGE (b) of partially purified protease from *Bacillus subtilis* I-2. In-gel zymography was done using 0.1% fibrin. Molecular weight was determined using full-range rainbow molecular weight markers (GE Healthcare, USA).

DISCUSSION

Enormous natural microbial diversity has been exploited for the isolation of potential microorganisms for the production of numerous biotechnologically important products, including fibrinolytic proteases (Agrebi et al. 2009; Kim et al. 2011; Bajaj et al. 2013). Uesugi et al. (2011) reported a highly potent fibrinolytic serine protease from *Streptomyces omiyaensis* isolate, which had about 18-fold higher activity than that of plasmin.

Economic viability of enzyme production process is necessary considering the huge quantities of enzymes required for the industrial applications. Enzyme overproduction by genetic manipulation and/or culture medium engineering can significantly contribute towards process economy (Bajaj et al. 2013). Substrate cost substantially influences the economy of bulk production of

industrial enzymes. Agricultural residues, available in abundance in India, may serve as low-cost substrates for economical production of enzymes including proteases (Bajaj and Sharma 2011; Bajaj et al. 2012; Bajaj et al. 2013). Extracellular protease production by the microorganisms is strongly influenced by the cultural and environmental variables (Bajaj and Sharma 2011). Generally, the moderately complex substrates serve as conditioned carbon sources for microbial enzyme production realizing the slow release of carbon and obviating the catabolite repression (Bajaj and Singh 2010). In the current study, molasses as carbon source substantially enhanced the protease production (63%) by *B. subtilis* I-2, which was in accordance with the results of Qureshi et al. (2011) who showed that *B. subtilis* EFRL 01 produced maximum protease when molasses was used at 1% as a sole carbon source. Similar results have been obtained by

others (Calik et al. 2003; Shikha et al. 2007). Molasses, a by-product from sugar industries is rich in nutrients and minerals, and at the same time is cheap and available in plenty, and could be exploited for the production of other industrial products (Bajaj and Sharma 2010). Various crude substrates have been reported to serve as excellent substrates for fibrinolytic protease production from *Bacillus* spp. (Wang et al. 2009; Agrebi et al. 2010; Bajaj et al. 2013).

The type and availability of nitrogenous precursors in the medium influence the production of extracellular enzymes (Bajaj and Sharma 2011). Besides, nitrogen source can significantly affect the medium pH during the course of fermentation, which in turn may influence enzyme activity and stability. *B. subtilis* I-2 successfully utilized various complex low-cost nitrogen sources, *viz.* soybean meal, cotton seed cake, gelatin, beef and malt extract and produced appreciable titres of fibrinolytic protease. Cotton seed cake enhanced the protease production substantially from *B. cereus* NS-2 (Bajaj et al. 2013). Various complex materials such as yeast extract, soy/casein peptone, soybean powder, shrimp shell powder, etc. have been reported as reasonably good nitrogen sources for protease production from *Bacillus* spp. (Wang et al. 2008; Wang et al. 2009; Agrebi et al. 2010; Mahajan et al. 2012).

The most effectual fermentation variables, which influence the protease production maximally can be identified by using the traditional 'one-variable-at-a-time' approach or by PB designed experiments (Bajaj and Wani 2011; Mukherjee et al. 2012). Since the PB design study is fast, less laborious, efficient and reliable, is widely preferred choice (Stanbury et al. 1995). Among a total of six fermentation variables examined, four (incubation time, soybean meal, mustard cake and molasses) influenced the protease production maximally by *B. subtilis* I-2. Mukherjee and Rai (2011) explored six fermentation variables by PB designed experiments and reported that three factors, *viz.* casein, ammonium sulphate and medium pH stimulated the fibrinolytic protease production by *Bacillus* sp. strain AS-S20-I. Similarly, four variables (hulled grain of wheat, NaCl, K_2HPO_4 and KH_2PO_4) were found as the most significant ones among a total of 11 investigated for protease production from *B. mojavensis* A21 by PB designed experiments (Haddar et al. 2010).

Salt precipitation and chromatography have commonly been employed for the purification of enzymes (Bajaj and Singh 2010; Mahajan et al. 2012). *B. subtilis* I-2 protease was purified by 4.8-fold with a recovery of 10.4%. Wang et al. (2008) purified *B. subtilis* LD-8547 fibrinolytic enzyme by 32.42-fold employing ammonium sulfate precipitation, DEAE-sephadex A50 and gel filtration chromatography with 12.4% recovery. *B. subtilis* ICTF-1 fibrinolytic enzyme was purified by ammonium sulphate precipitation, sepharose anion exchange and butyl sepharose FF hydrophobic interaction chromatography with a 34.42-fold increase in specific activity and 7.5% recovery (Mahajan et al. 2012). *B. subtilis* I-2 produced three proteases corresponding to molecular weight of 42, 48 and 60 kDa as determined by SDS-PAGE. Similar to the present results, *B. subtilis* P13 has been reported to secrete multiple proteases (Pillai et al. 2011). In contrast however, *Streptomyces* sp. CS624 (Mander et al. 2011) and *Bacillus* sp. AS-S20-I (Mukherjee et al. 2012) produced single protease.

B. subtilis I-2 protease showed maximum activity and stability at 50°C. Several *Bacillus* spp. fibrinolytic proteases have shown maximum activity at 35-40°C (Bajaj et al. 2013). However, temperature optima of fibrinolytic protease from *B. subtilis* A26 (Agrebi et al. 2009) and *Streptomyces* sp. CS624 (Mander et al. 2011) was 60°C. Alkaline protease with fibrinolytic activity from *S. gulbargensis* showed maximum activity at 45°C (Vishalakshi et al. 2009). Fibrinolytic proteases from various *Bacillus* spp. showed thermostability in the range of 40-50°C (Agrebi et al. 2010; Bajaj et al. 2013). *B. subtilis* I-2 protease showed maximum activity at pH 8.0 and stability at pH 7.0-10.0, which suggested potential application of this enzyme in various biotechnological industries. The fibrinolytic enzymes from different *Bacillus* spp. exhibited maximum activity at pH 8.0-9.0 (Vishalakshi et al. 2009; Agrebi et al. 2010; Mahajan et al. 2012; Bajaj et al. 2013). The activity and stability of *Streptomyces* sp. CS684 fibrinolytic enzyme showed shear activity loss in acidic and basic environment (Simkhada et al. 2010). In contrast, fibrinolytic proteases from *B. subtilis* A26 (Agrebi et al. 2009) and *B. amyloliquefaciens* An6 (Agrebi et al. 2010) showed stability over a broad range of acidic and alkaline pH.

Metal ions can influence the activity of enzymes in several ways (Palmer 2001). *B. subtilis* I-2 protease showed inhibition in the presence of various metal ions. However, *B. cereus* NS-2 fibrinolytic protease was activated by Fe^{2+} but inhibited by Pb^{2+} and Hg^{2+} (Bajaj et al. 2013). *B. subtilis* A26 fibrinolytic protease remained unaffected in the presence of Ca^{2+}, got slightly influenced by Na^+, K^+, Ba^{2+}, Cu^{2+}, Mg^{2+} and Mn^{2+}, but significantly inhibited by Hg^{2+} and Zn^{2+} (Agrebi et al. 2009). Fibrinolytic activity of *B. subtilis* ICTF-1 protease was activated by Ca^{2+} and inhibited by Zn^{2+}, Fe^{3+} and Hg^{2+} (Mahajan et al. 2012). EDTA caused inhibition to the activity of *B. subtilis* I-2 protease. Similarly, fibrinolytic enzymes from *B. amyloliquefaciens* An6 (Agrebi et al. 2010) and *Streptomyces* sp. CS684 (Simkhada et al. 2010) have been reported to be inhibited by EDTA.

CONCLUSIONS

It was concluded that *B. subtilis* I-2 due to its capability to successfully utilize various agricultural residues as substrates could be of potential use for cost-effective production of fibrinolytic protease. The stability of protease under alkaline pH suggested its possible applications for detergent industry. Further enhancement of protease production from *B. subtilis* I-2 could be attempted by using statistical optimization approaches and genetic engineering tools.

ACKNOWLEDGEMENTS

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