Defatted seed meal of soybean —an inexpensive substrate for alkaline protease production from selected isolate

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Abstract

Industrially important efficient alkaline protease producer was isolated from soil samples collected from mango garden of S.R.T.M. University campus in Nanded district of Maharashtra and identified based on its morphological, microscopic and physiological characters, biochemical pattern and enzyme profile as *Cyclobacterium* sp. APM2. Production of alkaline protease was carried out in nutrient salt solution supplemented with 10 g/L defatted seed meal of soybean and 350 U/mL production of alkaline protease was recorded after 48 h. APM2 alkaline protease has shown remarkable catalytic efficiency at pH range 7-9 and temperature range 30-60 ^{o}C as well. Therefore alkaline protease from *Cyclobacterium* sp. APM2 can be used in different biotechnological industries where hydrolysis of protein is carried out at alkaline pH and elevated range of temperature.

Keywords: alkaline protease, *Cyclobacterium* sp., defatted seed meal of soybean, protease activity

1. Introduction

From the beginning of 19th century, researchers have isolated many alkaline protease producers that mainly belonged to firmicutes and proteobacteria phyla from the diverse habitats like soda deserts, soda lakes, alkaline springs, glaciers, brine samples, tannery effluents, garden soil and animal manure etc. (Satyanarayana *et al.*, 2005; Joshi *et al*., 2008; Dhakar and Pandey, 2016). Isolation of alkaline protease producers from new sources is necessary to study their phylogenetic affiliation.

Proteases account for 65 % of total worldwide enzyme sales. Proteases having their pH optima in the range of pH 8-11 are regarded as alkaline proteases (E.C.: 3.4.11-19) and these enzymes hydrolyze proteins into polypeptides, oligopeptides, monopeptides and free amino acids. At present alkaline proteases are considered as the most versatile industrial enzyme that execute a wide variety of functions and have tremendous important biotechnological applications in detergent, tannery, food, silk, pharmaceutical, and agricultural industries (Rathod and Pathak, 2014a,b). Alkaline proteases are used in formulation of diagnostic reagents, peptide synthesis, silver recovery from used photographic films, preparation of organic fertilizers, contact lens cleaning, pest control, degumming of silk and waste treatment as well. Alkaline protease enzyme based cleaners are used to wash surgical instruments adhered by blood proteins. Moreover, alkaline proteases are used to remove stains of grass, blood, egg and human sweat. Alkaline proteases solubilize alkaline proteinaceous waste and thus help to lower the biological oxygen demand (BOD) of aquatic systems. Some industrially important alkaline proteases are Alcalase™, Savinase™, Esperase™, Biofeed™ pro, Durazym™, Novozyme™ 471MP, Novozyme™ 243 and Nue™ (Kumar and Takagi, 1999).

Cost effective production of enzymes at large scale is a challenging task in industries. Therefore researchers are on the lookout for different inexpensive agro-industrial residues due to the ease of availability and remarkable presence of nutrient contents like proteins, carbohydrates, amino acids and fatty acids (Sharma *et al*., 2015). The defatted seed meal of mustard and sesame contain more than 30 % crude protein (Sharma, 2013; Sharma et al., 2013; Yasothai, 2014). The highest presence of crude protein (> 44 %) was reported in soybean deoiled cake (Arif *et al.,*2012). Therefore, in present investigation we have selected defatted seed meal of soybean for production of alkaline protease from a newly isolated efficient alkaline protease producer.

2. Materials and Methods

2.1. Isolation and screening

Twenty soil samples were collected from mango garden of S.R.T.M. University campus. Collected soil samples were dried, crushed and sieved and mixed in equal proportion to form a composite soil sample. pH of composite soil sample was recorded. Aliquots of diluted soil samples were spread on casein agar (pH 9.0) plates and these plates were incubated at 30 ^{o}C for 24 h in an incubator (Kumar make, Mumbai). Morphologically distinct colonies were isolated and further spot inoculated on casein agar (pH 9.0) plates. These plates were incubated at the same temperature and incubation period. After incubation, the plates were observed either for presence or absence of zone of clearances around the grown cultures. The efficient alkaline protease producer was selected based on the size of zone formed and further purified by repeated subculturing on casein agar plates by streak plate method (Pathak and Rathod, 2013; Rathod and Pathak, 2014a,b).

2.2. Identification of alkaline protease producer

Typical morphological characters viz. shape, size, margin, surface, elevation, consistency, colour and opacity and microscopic characters viz. cell shape, Gram nature and motility of the selected isolate were recorded. Gram staining was performed by using Gram staining kit (HiMedia, Mumbai) and motility test was performed by using hanging drop technique (Polkade *et al.*, 2015). Sugar utilization pattern of the selected isolate was recorded by performing carbohydrate fermentation tests in basal medium. The selected sugars were sucrose, maltose, lactose and glucose. IMViC test was performed as per the standard procedure. To perform catalase test, 3 % H_2O_2 was poured on grown culture of selected isolate and culture was observed for evaluation of bubbles. To perform oxidase test, selected isolate was placed on strips of 1 % tetramethyl-p-phenylenediamine dihydrochloride and observed for the change in colour of selected isolate. Amylase production test was performed by using starch agar (pH 9) plates (Khairnar *et al*., 2012; Pathak *et al*., 2016). Optimum temperature and pH required for the growth of selected isolate was determined. Selected isolate was identified by comparing its morphological, microscopic and physiological characters, biochemical pattern and enzyme profile with standard reference strain given in Bergey's manual of systematic bacteriology (Kriege *et al.,* 1984; Sharma *et al*., 2009; Pathak and Sardar, 2012, 2014; Pathak *et al*., 2012; Kolekar et al., 2013; Hingole and Pathak, 2013; Pathak and Rathod, 2013, 2014, 2015; Pathak *et al*., 2014a,b; Sardar and Pathak, 2014; Rathod and Pathak, 2014a,b; Pathak et al. 2015a,b,c,d,e; Dastager et al., 2015; Sonalkar et al., 2015; Gavali and Pathak , 2015; Pathak and Rathod, 2016).

2.3. Production of alkaline protease

Volume of 4 mL fresh culture of selected isolate was inoculated in a 1 L conical flask containing 500 mL of nutrient salt solution (KH_2PO_4 3 g/L, ammonium nitrate 5 g/L, NaCl 1 g/L and $MgSO_4$ 0.5 g/L) supplemented with 10 g/L defatted seed meal of soybean. pH of this

medium was adjusted to 9.0. The flask was incubated in orbital shaking incubator (CIS-BL 24, Remi Make, Mumbai) at 30 °C and 120 rpm agitation speed for 48 h (Bhunia *et al*., 2013b; Rathod and Pathak, 2014 a,b).

2.4. Extraction of alkaline protease and quantitative assay

After completion of production period, whole fermented broth was centrifuged at 10,000 rpm for 15 min at 4 ^{o}C in a cooling centrifuge (BioEra, India). The cell-free supernatant was collected and used for quantitative alkaline protease activity assay. Crude alkaline protease (1 mL) from selected isolate was added to the test tube containing 1 mL of 1 % casein solution prepared in 0.2 M Glycine-NaOH buffer pH 9.0. This mixture was shaken and incubated at 30 °C for 10 min. The reaction was terminated by adding 3 mL of trichloroacetic acid (5 %). The supernatant was collected by centrifuging the content at 10,000 rpm for 10 min. Absorbance of supernatant was measured at 280 nm using a spectrophotometer (Shimadzu corporation). The control was maintained by terminating reaction at zero hour. One unit activity of alkaline protease was defined as the amount of enzyme required to liberate 1 µmol min^{-1} mL^{-1} of product expressed as tyrosine equivalent, under the assay conditions (Bhunia *et al*., 2011; Pathak and Rathod, 2013, Rathod and Pathak, 2014a,b).

2.5. Characterization of partially purified alkaline protease

2.5.1. Effect of pH on catalytic activity of alkaline protease

Catalytic activities of crude alkaline protease from the selected isolate were recorded at pH 5, 7, 8 and 9 using 0.1 M citrate buffer, phosphate buffer and Glycine-NaOH buffer under standard assay conditions as described previously (Pathak and Rathod, 2013; Rathod and Pathak, 2014 a,b).

2.5.2. Effect of temperature on catalytic activity of alkaline protease

Catalytic activities of crude alkaline protease from the selected isolate were recorded at temperatures 10, 30, 40, 50 and 60 ^{o}C under standard assay conditions as described previously (Bhunia *et al*., 2013a; Pathak and Rathod, 2013; Rathod and Pathak, 2014 a,b).

3. Results and Discussion

3.1. Isolation of alkaline protease producer

pH of composite soil sample was recorded as 8.1. Total 97 colonies were appeared on casein agar plates. Of these, 15 morphologically distinct colonies were designated as APM1 to APM15. After screening the isolate APM2 has shown a largest zone of clearance on casein agar plate. Therefore, APM2 was selected for its identification and production of alkaline protease.

3.2. Identification of alkaline protease producer

Morphological, microscopic and biochemical characters and enzyme profile of APM2 are given in Table 1. The isolate APM2 grew optimally at 30 ^{o}C and at pH 9. Based on these characters selected alkaline protease producer was identified as *Cyclobacterium* sp. APM2.

Table 1: Morphological, microscopic and biochemical characters and enzyme profile of *Cyclobacterium* sp. APM2

Morphological characters	*Cyclobacterium* sp. APM2.	**Microscopic characters**	*Cyclobacterium* sp. APM2.	**Biochemical characters**	*Cyclobacterium* sp. APM2.
Shape	Circular	Cell shape	Ring	Glucose	+
Size	2.5 mm	Cell motility	Non motile	Maltose	+
Margin	Entire	Gram stain reaction	Negative	Lactose	-
Surface	Shiny	**Enzyme profile**	**APM2**	Sucrose	+
Elevation	Convex	Catalase	+	Indole test	-

Consistency	Non sticky	Oxidase	+	MR test	-
Colour	Pale yellow	Amylase	+	VP test	-
Opacity	Opaque	Protease	+	Citrate test	-

3.3. Production of alkaline protease

Production of crude alkaline protease from *Cyclobacterium* sp. APM2 was recorded 350 U/mL after 48 h of incubation period.

3.4. Characterization of crude alkaline protease

3.4.1. Effect of pH on alkaline protease activity

Crude alkaline protease from *Cyclobacterium* sp. APM2 exhibited maximum catalytic efficiency at pH 8 (1050 U/mL) followed by at pH 9 (350 U/mL), pH 7 (320 U/mL) and pH 5 (60 U/mL) (Fig. 1).

3.4.2. Effect of temperature on alkaline protease activity

Crude alkaline protease from *Cyclobacterium* sp. APM2 exhibited maximum catalytic efficiency at 30 ^{o}C (275.01 U/mL) temperature followed by at 50 ^{o}C (231 U/mL), 40 ^{o}C (110 U/mL), 60 ^{o}C (49.50 U/mL) and 10 ^{o}C (24.75 U/mL) (Fig. 2).

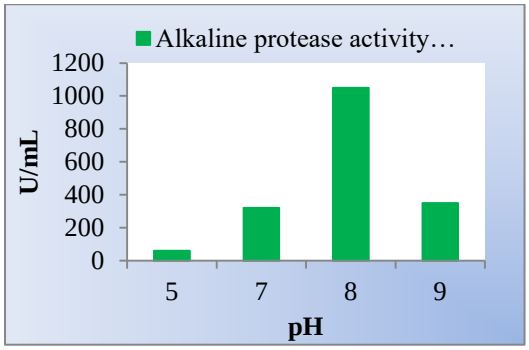

Fig. 1: Effect of pH on catalytic activity of alkaline protease from *Cyclobacterium* sp. APM2

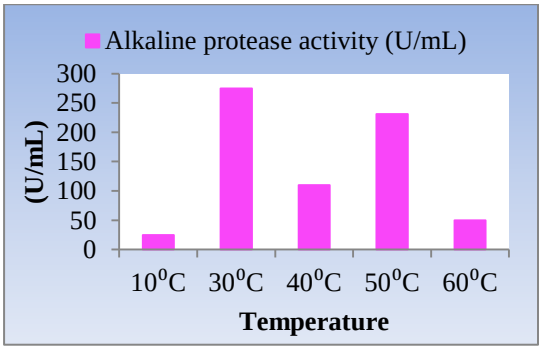

Fig. 2: Effect of temperature on catalytic activity of alkaline protease from *Cyclobacterium* sp. APM2

4. Conclusions

Efficient alkaline protease producer was isolated and identified as *Cyclobacterium* sp. APM2. Remarkable production of alkaline protease (350 U/mL) was recorded by using defatted seed meal of soybean from *Cyclobacterium* sp. APM2. Beside pH 8, crude APM2 alkaline protease has also shown remarkable catalytic efficiency at alkaline pH 9. APM2 alkaline protease was catalytic active at elevated temperatures (50 and 60 ^{o}C) as well. Therefore alkaline protease from *Cyclobacterium* sp. APM2 can be used in different biotechnological industries where hydrolysis of protein is carried out at alkaline pH and elevated range of temperature.

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