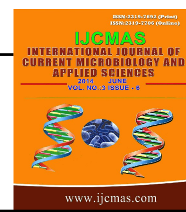

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Original Research Article

Optimization and Production of Alkaline Protease enzyme from *Bacillus subtilis* 168 isolated from food industry waste

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Keywords

Bacillus subtilis, 16S rRNA analysis, alkaline protease, optimization, submerged fermentation.

A B S T R A C T

The demand for alkaline proteases in industries is increasing worldwide. The present study intended to isolate a suitable higher alkaline protease producing bacteria and its identification, optimize conditions for alkaline protease production such as carbon sources, nitrogen sources, pH, temperature, inoculums size and mass production of alkaline protease by submerged fermentation. In order to isolate the protease producing organisms, solid wastes from industries were collected and primary screening was achieved by skim milk casein hydrolysis method. The organism showing maximum (17 mm) hydrolysis was selected and identified by microscopic, biochemical and 16S rRNA phylogenetic analysis as *Bacillus subtilis* 168. Purification of crude enzyme was carried out by ammonium sulphate precipitation and dialysis. The apparent molecular weight of purified enzyme was determined as 55 kDa. The maximum alkaline protease production was achieved with 2% inoculums size at pH 13 and 35ºC temperature, maltose as best carbon source, yeast extract as good nitrogen source, and using wheat bran as important substrate. According to our knowledge, this study demonstrated the first report on alkaline protease producing *B. subtilis* 168 isolated from food industry waste. Purified enzyme can be used in textile, leather and food industries.

Introduction

Proteases, one among the three largest groups of industrial enzymes, accounts for about 60% of the total worldwide sale of enzymes from biological sources since they possess almost all characteristics desired for their biotechnological applications (Adinarayana et al., 2003). Among the various proteases, bacterial protease was the most significant compared with animal, fungi and plant protease. Bacterial alkaline proteases are characterized by their high activity at alkaline pH. Optimal temperature required for alkaline protease production is around 60°C. Due to these properties of bacterial alkaline proteases make them suitable for industrial applications. *Bacillus* species were specific producers of extracellular

protease under extremes of conditions using simple carbon sources (Gupta et al., 2002).

Alkaline proteases of microbial origin posses considerable industrial potential due to their biochemical diversity and wide applications in detergents, leather processing, silver recovery, medical purposes, food processing, feeds, and chemical industries, as well as tannery waste treatment (Vijay et al., 2010).

Production of alkaline protease was carried out generally using submerged fermentation. It is advantageous than other methods due to its consistent enzyme production with defined medium, better process conditions and improved downstream processing (Prakasham et al., 2006). Microbial proteases producing industries are always in search of new and cheaper methods to enhance the protease production as well as to decrease the market price of this enzyme (Mukherjee et al., 2008). The use of cost effective growth medium for the production of alkaline proteases from an alkalophilic microorganisms is especially important, because these enzyme account for approximately 25% of the world wide enzyme consumption (Gessesse, 1997).

The growth and enzyme production of the organism are strongly influenced by medium components like carbon and nitrogen sources. Besides the nutritional factors the cultural parameters is the primary task in a biological process. So, the media components and cultural conditions are need be optimized. It is essential that these organisms be provided with optimal growth conditions to increase enzyme production. The culture conditions that promote protease production were found to be significantly different from the culture conditions promoting cell growth. In the industrial production of alkaline proteases, technical media were usually employed that contained very high concentrations (100–150 g dry weight/liter) of complex carbohydrates, proteins, and other media components (Aunstrup, 1980). With a view to develop an economically feasible technology, research efforts are mainly focused on to improve the yields of alkaline proteases and to optimize the fermentation medium and production conditions. The present study was aimed to evaluate the usage potency of *Bacillus* sp., and to optimize various parameters for alkaline protease production under submerged fermentation.

Materials and Methods

Collection of samples

Three different solid samples (leather, food industrial waste and slaughter house waste) were collected in sterile container according to microbiological procedures and shifted to the laboratory for further analysis.

Screening of protease producers

The collected samples were serially diluted and streaked on skin milk agar plates. The plates were incubated for 48h at 37°C and protease producers were selected by observation of zone of hydrolysis around the colonies (Genkal et al., 2006).

Identification of isolates

All the screened organisms were identified based on morphology, cultural and biochemical characteristics (Koneman et al., 1994). The higher yielding strain was identified by making use of 16S rRNA

sequencing using forward (5' –AGAGTTTGATCCTGGCTCAG-3') and reverse (5' –TACCTTGTTACGACTT 3') primers. The sequence similarity search was done for the 16S rRNA sequence using online search tool called BLAST (http://www.ncbi.nlm.nih.gov/blast). The unknown organism was identified using the maximum aligned sequence through BLAST search (Krishnan et al., 2012).

Characterization of protease enzyme

The total protein contents of the samples were determined according to the method described by Lowry's method using Bovine Serum Albumin (BSA) as standard. Enzyme activity was determined using culture supernatant collected by centrifuging culture broth at 10, 000 rpm for 15min. Protease activity was measured by standard assay procedure proposed by Akcan and Uyar, 2011. About 0.5ml of 0.5% casein and 1.25ml of tris buffer (pH-8.0 to 14.0) was added into 0.2ml of each of the culture supernatant separately. Mixture was incubated for 30 min at 37^0C. About 3ml of trichloroacetic acid was added and incubated at 40^0C for 10 min to form precipitate. The mixture was centrifuged at 10,000rpm for 15min and 0.5ml of supernatant was collected.

Reagent containing sodium carbonate, copper sulphate, sodium potassium tartarate was mixed with 1ml of Folin-phenol reagent. The mixture was incubated at dark for 30 minutes to form blue colour. The absorbance was read at 660 nm to determine the optical density of each sample. The obtained OD was extrapolated in the standard graph. The standard curve was obtained for series of known concentrations of bovine serum albumin. From the graph, the amount of protein liberated due to the action of enzyme protease in the supernatant was determined. One unit of protease activity was defined as the amount of enzyme required to liberate 1 µg/ml tyrosine under the experimental conditions.

Enzyme activity = OD value X amount of protein released (µg)/ concentration of substrate X time of incubation X weight of the sample

Optimization of conditions for enhanced enzyme production (Das and Prasad, 2010)

Standard methods were adopted to optimize the parameters like cultural conditions, carbon, nitrogen, temperature, pH, inoculum size and substrates.

Mass production of alkaline protease

The fermentation was carried out in a sterile Stirred Bed Reactor (SBR). The vessel was maintained at optimized temperature, pH and other & incubated for 48h in a shaking incubator. At the end of fermentation period, the whole culture both was centrifuged at 10,000 rpm for 15 minutes, to remove the cellular debris and the clear supernatant was used for enzyme analysis.

Characterization of partially purified alkaline protease

The culture filtrate (crude protease) was collected aseptically after upstream production in a SBR under controlled conditions. The required volume of the spent media was centrifuged at 10,000 rpm for 15 min at 4ºC in order to obtain a cell free filtrate. About 200 ml of the cell free filtrate containing protease were collected and their proteolytic activity was determined. Protease enzyme was purified by ammonium sulfate fractionation the concentration of ammonium sulphate

required for precipitation varies from protein to protein and should be determined empirically. The two milliliter of the crude protease enzyme was first brought to 20% (w/v) saturation with solid ammonium sulfate (enzyme grade) and 100% saturated dialysis against distilled water in a dialysis bag (cut off 30) for 3 h, followed by dialysis against phosphate buffer at pH 7.0. The obtained protease enzyme preparation was concentrated against crystals of sucrose and kept in the refrigerator at 4ºC. The enzyme activity and protein content was determined for salted out dialyzed enzyme fractions. The enzyme activity of the purified fractions of the alkaline protease after harvesting, ammonium sulfate precipitation and dialysis was determined by the method of Gomori (1955). Separation and size determination of enzyme was performed by SDS-PAGE (Joo et al., 2002).

Results and Discussion

Alkaline proteases has considerable industrial potential in detergents, leather processing, silver recovery, medical purposes, food processing, feeds and chemical industries, as well as tannery waste treatment . At present, the largest part of the hydrolytic enzyme market is occupied by the alkali proteases. Extreme environments are important sources for isolation of microorganisms for novel industries and enzymes production.

Hence, in this present study the protease producing bacteria were isolated from tannery, food industry; and slaughter house industrial effluent discharge site. Five different protease producers were selected based on zone of hydrolysis and named as PD – 1, PD – 2, PD – 3, PD – 4 and PD – 5. Among the five isolates, the isolate PD - 4 isolated from food industry showed maximum of 17 mm diameter of zone of hydrolysis.

The zone of hydrolysis was due to protease enzyme produced by the isolates on Skim milk agar media. Narendra et al., (2012) reported that about 25 organisms were recovered from different fields near to Ravulapalem village, East Godavari district, Andhra Pradesh, India. Five isolates were considered as protease positive strain. These researchers explained that, Indian soil is best for alkaline protease producing bacteria. The isolated proteolytic strain (PD4) was spore-forming, gram-positive rod, which was identified as *Bacillus* sp. Siddalingeshwara et al., (2010) similarly isolated fifty-three bacterial isolates from various fields of cosmopolitan city of Karachi, out of which 25 were alkaline protease producers.

Alkaline protease activity of the protease producing isolates

After isolating five different bacterial strains, each isolates was screened for their enzymatic activity at different alkaline pH conditions (pH 8.0 to 14.0). During this screening step, PD4 exhibited more enzymatic activity in all pH conditions when compared to all other isolates. The maximum alkaline proteolytic activity was exhibited at pH 13.0 (170.32 ± 1.5IU/ml). The difference in enzymatic activity at different pH among the five different isolates was presented in Figure 1.

Optimization of conditions for enhanced enzyme production

The enzyme production was optimized under different parameters like growth in different carbon sources, nitrogen sources, pH, temperature, and substrates. All the

parameters were analyzed using PD- 4 strain to determine the enzymatic activity of protease (Table. 1).

Of various carbon sources used, maltose exhibited maximum enzyme activity of about 493.5 ± 5.0IU/ml. Previous works substantiates that depending on the species and source of the organism carbon source requirement varies. Isolated organism prefers to utilize the maltose as the carbon source. Next to the carbon, nitrogen was served as important nutrient source for the protease production. Of the various nitrogen sources used; yeast extract exhibited the highest enzyme activity of about 696.3 ± 4.4IU / ml for PD-4. Atalo and Gashe (1993) showed that yeast extract and peptone can induce the alkaline protease production in glucose medium. PD4 strain exhibited best production of protease was at 35◦C with the yield of 696.3±4.41 IU/ml. two percentage inoculum yielded good enzyme productivity.

Irfan et al., (2009) found that the optimum inoculum size of 2% showed the maximum activity. However a further increase in inoculum size decreased enzyme production by *B. megaterium*. During the recent years, efforts have been directed to explore the means to reduce the protease production cost through improving the yield, and the use of either cost free or low cost feed stocks or agricultural by products as substrate like green gram husk which are highly involved in the protease enzyme production (Prakasham et al., 2006). The effect of agro based by products as alternative substrate on bacterial protease production under submerged fermentation was studied using five different substrates (Rice bran, wheat bran, sugarcane molasses, sago waste and Black gram bral) wheat Bram exhibited maximum enzyme activity of 585.5 ± 3.3 IU /ml. In the present study, wheat bran was found to be the best inducer of protease enzyme production.

Since the organism has industrial potentiality it was identified by 16S rRNA method, which is found to be the novel and accurate method for identifying unknown species. The DNA from the isolate PD4 was isolated and the 16s rRNA was amplified and sequenced. The BLAST analysis of PD4 using its 16S rRNA sequence data showed that strain had highest homology (100%) with *Bacillus subtilis* 168. The sequence has been submitted to the Genbank (KJ668820).

Bulk production of *B. subtilis* (PD4) was carried out under controlled cultural conditions in a stirred bed reactor. At the end of each fermentation period, the culture broth was harvested to remove the cellular debris and the clear supernatant was used for characterization of enzyme. Harvested media containing the crude protease was partially purified by ammonium sulfate precipitation and dialysis. The supernatant collected after centrifugation of harvest media was precipitated with 100% saturated ammonium sulfate. Very sharp needle-shaped precipitates were obtained during the incubation of crude enzyme and ammonium salt mixtures (Plate- 2). In the present study, the enzyme alkaline protease from *Bacillus subtilis* was purified using ammonium sulphate precipitation method. The needle shaped protein precipitate exhibited 55kDa in size. The molecular weight of the protease reported by Sousa et al., 2007 that the purified enzyme migrated as a single band with an apparent molecular weight of 46 kDa in SDS-PAGE.

Figure.1 Alkaline protease activity of the isolates at various pH

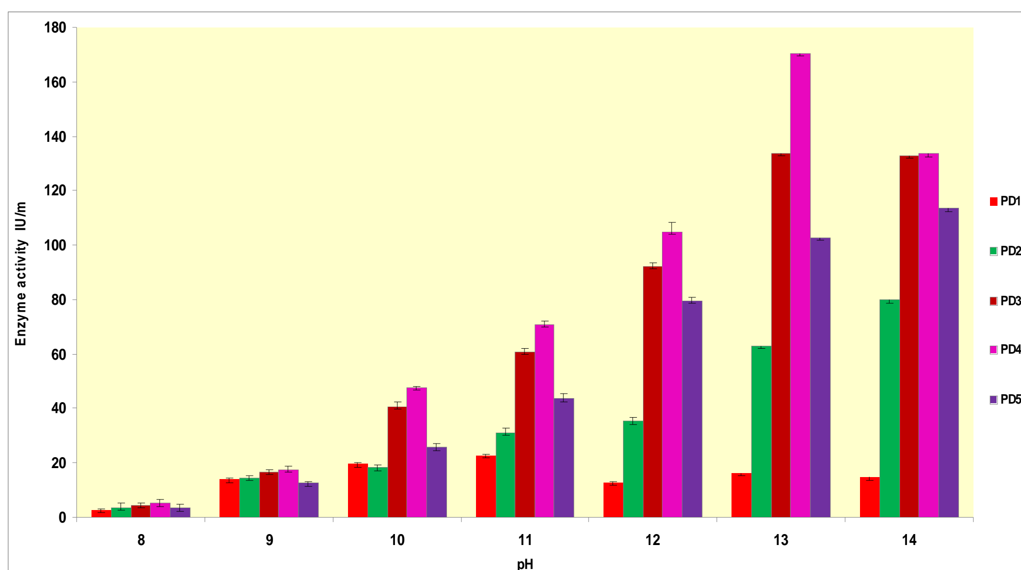

Table.1 Optimization of conditions for alkaline protease production using *B. subtilis* 168

Parameters		Enzyme activity (IU/ml))
Carbon sources	Glucose	344.7± 1.5
	Sucrose	182.2± 6.0
	Maltose	**493.5± 5.0**
	Lactose	211.7± 2.1
	Fructose	126.0±4.0
Nitrogen sources	Peptone	495.6 ± 4.7
	Yeast extract	**696.3 ± 4.4**
	Ammonium sulphate	195.2 ± 2.5
	Ammonium chloride	354.2 ± 3.5
	Beef extract	208.3 ± 5.0
Temperature	30^0	275.5 ± 3.9
	35^0	**442.6 ± 7.4**
	40^0	174.3 ± 6.0
	45^0	75.4 ± 4.7
	50^0	137.0 ± 8.4
Inoculum size (%)	2	**464.9 ± 1.5**
	4	238.6 ± 1.7
	6	163.8 ± 4.5
	8	50.9 ± 1.5
	10	20.8 ± 1.0
Substrates	Rice bran	11.9 ± 1.8
	Wheat bran	**585.5 ± 3.3**
	Sugarcane molasses	208.2 ± 1.3
	Sago waste	190.8 ± 2.9
	Black gram bran	460.7 ± 1.6

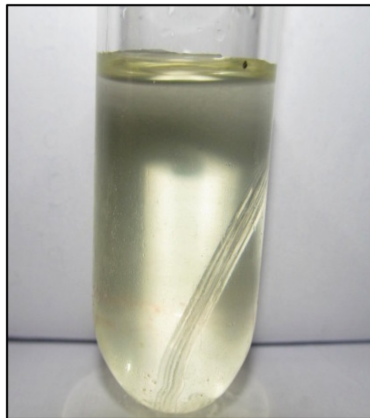

Figure.3 Needle shaped enzyme precipitate

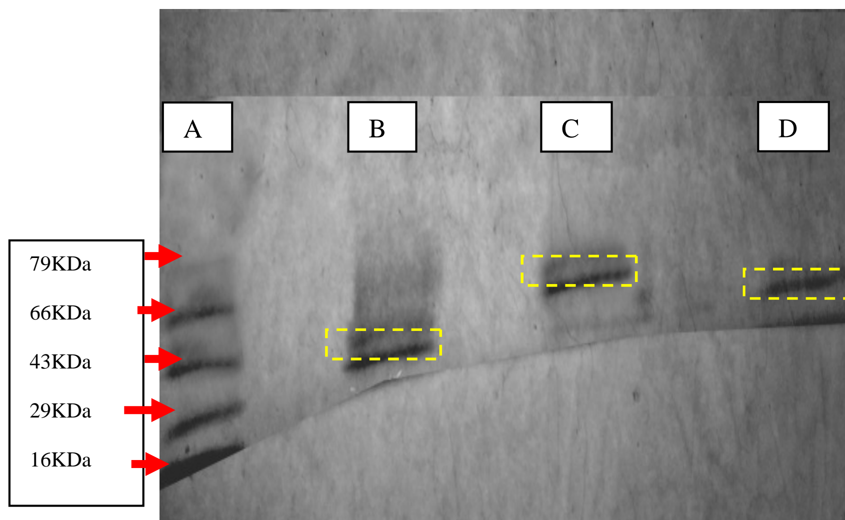

Figure.4 SDS – PAGE pattern of Protease enzyme

These results were in accordance with literature reports, where most of the molecular mass of protease from *Bacillus genusis* was less than 50 kDa. The alkaline proteases of some bacteria such as *Bacillus subtilis* RM 615, *Bacillus* sp. Y, *Bacillus* sp. KSM-K16, *Vibrio metschnikovii* RH530 and *Fiennoac6nomyces* sp. HS 682 have been reported to be stable towards detergents to some extent. Of these the M-protease of *Bacillus* sp. KSM-K16 has been reported to be retaining 70% of its original activity after incubation for three weeks in a commercial heavy-duty liquid detergent with pH 9.6 at 40°C (Kwon et al., 1994). The study therefore concludes that because of the higher enzyme stability under various conditions and production at lower cost can be commercialized for industrial applications

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