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Production Partial Purification and Industrial Applications of Alkaline Protease Produced by Bacillus Lichenifomis

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Abstract: ***Proteases are very important industrial enzymes, which contribute maximum of the total world enzyme market. Besides their use in normal physiology, proteases are used in various industries including pharmaceuticals, detergents, food and waste processing. The most common and widely used bacteria belongs to the genus Bacillus. The aim of the work is based upon an extracellular alkaline protease produced by Bacillus licheniformis, under optimized conditions. The alkaline protease production was optimized. The optimum day 2 days, pH 8.5; temp 40°c, were determined. The enzyme was further partially purified using acetone, followed by dialysis (75 kDa) and sephadox G100 column chromatography. The molecular weight of the enzyme was found to be 320KDa using SDS PAGE. The extracted enzyme was used for hair removal on "goat skin". The extracted enzyme was found to be better agent to remove the hair efficient as an eco – friendly method. This property of the enzyme is very useful for application in leather industry.***

Keywords: Alkaline Protease, *Bacillus Licheniformis*, Dehairing

1. Introduction

Proteases are the most important classes of enzymes, plays an major role of about 60% of the world enzyme market[8]. It is one of the 3 largest group of industrial enzymes. Proteases produced from microorganism's play important role in several industries such as detergents, tanning, photographic industries, Pharmaceutical and waste treatment, etc.[2].Among this family of enzymes, thermophilic proteases play an important role in bioengineering and biotechnological application, due to its better resistance of chemical denaturant and organic solvents.

Alkaline Protease: Among all protease alkaline proteases are primarily used as detergents additives, which also play a specific role in the hydrolysis of protein stain such as blood, milk, human, sweet, etc [2]. In view of this microbial strains have been exploited for the production of alkaline proteases, several microbial strains including fungi (Aspergillus flavus, Fusarium, Graminarum, Penicillum, griseafulvin etc.,) and Bacteria (Bacillus licheniformis, Bacillus, firmus, Bacillus subtilis, etc.,) are defined as those proteases, which are active in neutral to alkaline pH range. The most commercialized alkaline proteases are produced by bacteria especially bacillus.[3].The first alkaline proteases (Carlberg) from Bacillus licheniformis was commercialized as an additive in detergents in 1960's [7]. A number of Bacillus derived alkaline protease have been purified and characterized because of their significant proteolytic activity, stability, broad substrate specifically short period of fermentation. Simple down stream purification and low cost [6].

Microbial source and Application: Protease are widely spread in nature. Microbial cells are the usual sources of proteases for industrial use, because of their rapid growth, the limited space required for their cultivation and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various application [4].The genus bacillus is an important source at industrial alkaline proteases and are probably the genera being commercialized for alkaline protease production [1].Thus, the goal of the work is to purify and produce the protease enzyme from the organism, Bacillus licheniformis. This enzyme is gone under the application of deharing in the lab conditions.

2. Materials and Methods

Source of inoculum

Bacillus licheniformis culture obtained from sewage water and was subcultured on nutrient agar medium and stored at 4°C for further use. A slant culture of strain was inoculated into 45 ml of sterilized media of skim milk agar is prepared in the composition of casein enzymic hydrozylate. 5.00g / lit; yeast extract 2.50g / lit; Dextrose 1.00 gl lit, skim milk powder 28.00g/lit, thus 0.7725 g of skim milk agar is taken and make upto 25 ml with distilled water agar 15.00 g / lit and a pinch of agar is added for the quicker solidification. 25ml of nutrient broth is also prepared which is inoculated by the same strain for sub culture media.

Here 0.325g of nutrient broth is made upto 25ml with distilled water. The agar plate is inoculated by "simple strik" method. Both the media is incubated for 24 hours @ 37°C.

Production of Enzyme

Production of alkaline protease from Bacillus licheniformis was carried in lab scale biosectors. 25ml of casein media is used for the culture of organism, which constitutes of yeast extract 0.06 g/l; casein enzyme hydrosylate 0.125 g/l; dextrose 0.025 g/l at optimum condition. Cell free supernatant was used for subsequent studies. Here plug

method is held for the inoculation of organism from agar media. Enzyme array; protease activity was determined by the pH of substrate and incubation temperature. The reaction mixture containing 1 ml of 1% casein solution is dissolved in 0.1 M Tris Hll buffer (pH 8.0) and 1 ml of enzyme solution was incubated at 37°C for 30 mins. The buffer increases the reactivity of the casein. The reaction was stopped by adding 1 ml of 5% TCA. The precipitate is obtained. After that entire mixture was centrifuged at 3000 rpm for 10 mins. The supernatant was collected and added 2ml of 0.4M sodium carbonate. Mix well and added the colouring reagent Folin's Thenol of 1 ml. The absorbance / OD value of the liberated protein was measured at 660 nm against blank in spectrophotometer.

Extraction and Estimation

The extraction of contents of fermented containing protease was filtered and then preserved in the refrigerator at 4°C as a crude protease. The growth of organism was obtained by the measure of BIOMASS of the optimum day, which is used to calculate the enzyme activity.The concentration of protein activity in the protease was determined by the Lowry's method.

Purification Acetone precipitation:

Cell free supernatant was precipitated by adding 20 ml of pre-chilled acetone. The enzyme solution was stirred for 1 hr. The protein precipitated was collected by centrifugation at 3000rpm for 2 – 3 minutes, and resuspended in minimum volume of 0.05 M Tris HCl buffer. pH 8.0 to get the concentrated enzyme suspension. After that enzyme suspension was dialyzed against the same buffer.

Sephadex G-100 gel filtration chromatography

The concentrated enzyme sample was purified on sephadex G-100 column. The sephadex column was equilibrated with 0.05M Tris HCl buffer of pH 8.0. The dialyzed enzyme sample was loaded onto a sephadex G-100 column and then eluted with the same buffer. Each fractions of 1ml were collected at flow rate of 11ml/hr by fraction collector. The fractions were analyzed for protease activity and the active fractions were pooled and concentrated and stored at refrigerator for further studies of protein estimation. The molecular weight of purified protease were determined through 15% SDS – PAGE on vertical gel apparatus

SDS – PAGE

SDS – PAGE was performed using a mini slab gel apparatus. The molecular weight was determined using standard molecular weight markers. The sample for SPS – PAGE was prepared by mixing the dialyzed fraction of alkaline protease with the dye in a ratio 30µl: 70µl. The mixture was heated at 72°C for 5 mins by suspending the sample tubes in the boiling water. Using micropipette, 10µl of each sample was loaded onto the well formed in the stacking gel and electrophoresis was carried out. After complete electrophoresis, the gel assembly was removed, and stacking gel was cut off and discarded. All the steps were carried out at room temperature. The gel was transferred into a fixative or staining solution of coomassie Brilliant blue R.250 for about 30 min with gentle agitation at room temperature. After staining, the gel was destained using a destaining solution until the stained background had been satisfactorily removed and protein bands became clear.

3. Results and Discussion

Production of Enzyme

The level of growth by the bacterial strain is achieved by determining the BIOMASS for the optimum Day. The strain were isolated on skim Milk Agar Medium and for BIOMASS test is done by (Casein) liquid Medium.

Table 1: Biomass Calculation

Day Weight	*Tube With Pellet Weight*	*Pellet Weight*
	DAY 1	
1.20	1.22	0.02
1.20	1.23	0.03
	DAY 2	
1.20	1.23	0.03
1.20	1.23	0.03

Thus, these values are used to find the activity of the enzyme after the assay.

Enzyme Assay:

Table 2: Measurement of Enzyme Activity

Day	Bio – Mass	Optical Density	Enzyme Activity U/Ml
0	0.00	0.00	Nil
1	0.02	0.18	1.7501
2	0.03	0.010	145.8A

Protease activity from factorial strain is obtained at varied lends. It was found that maximum production occurred at end of exponential phase.

Purification

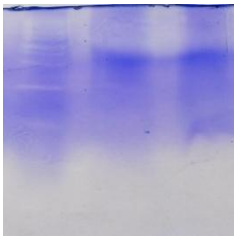

Figure 1

The enzyme purity was confirmed by SDS-PAGE which demonstrated a single band.

Application:

Dehairing :

Figure 2

The result was observed that by comparing the buffer and the tap water, the protease enzyme removed the hair from the skin of goat at 100%. Thus, such property of the enzyme is very useful for the application in lather industries

4. Conclusion

The alkaline protease enzyme of *Bacillus licheniformis* was purified up to homogeneity level by employing acetone precipitation (60-70%) and gel filtration through Sephadex G-100 chromatography. After final purification step, the enzyme was purified with a specific activity. The molecular weight of the enzyme was estimated to be 320 kDa by SDS-PAGE. The enzyme was also found compatible and stable with most of surfactants and oxidizing agents tested and retained its more than 100% residual activity. These properties indicate the possibilities of commercial exploitation of the alkaline protease in detergent formulations and also in dehairing formulation.

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