International Scholars Journals

African Journal of Parasitology Research ISSN 2756-3391 Vol. 12 (9), pp. 001-006, September, 2024. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research paper

Extraction, Purification, and Scalable Production of Protease Enzyme from *bacillus subtilis*

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Accepted 26 April, 2024

Screening and isolation of protease producing strains of bacteria were carried out from four different soil samples collected from various places in Bangalore. The isolates were positive on skim milk agar (1%) and thus are selected as protease producing strain. The organisms were tested for various biochemical tests, which lead to their identification as *Bacillus subtilis* producing protease enzyme. These *Bacillus subtilis* could group upto 40ºC and pH range 6-9 with optimal growth temperature and pH at 37ºC and 8.0 respectively. It was also optimized for carbon test and nitrogen test with optimal growth in dextrose and peptone respectively. Enzyme production was carried in 1 litre of optimized media in the fermenter at 37ºC for 48 hours at pH 8.0. Harvested protease product was purified by salt precipitation method. Finally the enzyme protease was purified by column chromatography. The protein was characterized using SDS-PAGE. This result showed that *Bacillus subtilis* under study is a good producer of extra cellular protease, which can be beneficial for industries.

Keywords: *Bacillus subtilis*, protease, fermenter, chromatography

INTRODUCTION

Enzymes are delicate protein molecules necessary for life. Protease are the single class of enzymes which occupy a pivotal position due to their wide applications in detergent, pharmaceutical, photography, leather, food and agricultural industries. An important biotechnological application of protease is in bioremediation processes (Gupta et al, 2002). Among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases. And among bacteria, Bacillus species are specific producers of extracellular proteases. These proteases have wide applications in pharmaceutical, leather, laundry, food and waste processing industries (Pastor et al; 2001), (Ward; 1985). Global requirements of thermostable biocatalysts are far greater than those of the mesophiles of which proteases contribute two thirds (Beg et al; 2003). Thermostable proteases are advantageous in some applications because higher processing temperatures can be employed, resulting in faster reaction rates, increase in solubility of nongaseous reactants and products and Reduced incidenceof microbial contamination by mesophilic organisms. Thermophilic bacteria from hot springs produced unique thermostable enzymes (Rao et al; 1998). *Bacilus subtilis* is one of the most widely used bacteria for the production of specific chemicals and industrial enzymes and also a major source of amylase and protease enzymes.Protease has wide application in detergent, pharmaceutical, photography, leather, food and agricultural industries. It is also used in baking, brewing, meat tenderization, peptide synthesis, medical diagnosis, cheese making, certain medical treatments of inflammation and virulent wounds and in unhairing of sheepskins. It has also wide application in Bioremediation process (Anwar and Saleemudin, 1998; Gupta et al., 2002). In this study an attempt was made for, the isolation and selection of a thermophilic bacterial strain that is potent producer of extra cellular alkaline protease, and the optimization of culture conditions required for enzyme production.

Table 1: Precipitation of the enzyme using ammonium sulphate method.

Initial % of ammonium sulphate	Final concentration of ammonium sulphate (% saturation)																	
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Gram solid ammonium sulphate to add to 100ml of solution																
0	10.7	13.6	16.6	19.7	22.9	26.2	29.5	33.1	36.6	40.4	44.2	48.3	52.3	56.7	61.1	65.9	70.7
5	8	10.9	13.9	16.8	20	23.2	26.6	30	33.6	37.3	41.1	45	49.1	53.3	57.8	62.4	67.1
10	5.4	8.2	11.1	14.1	17.1	20.3	23.6	27	30.5	34.2	37.9	41.8	45.8	50	54.5	58.9	63.6
15	2.6	5.5	8.3	11.3	14.3	17.4	20.7	24	27.5	31	34.8	38.6	42.6	46.6	51	55.5	60
20	0	2.7	5.6	8.4	11.5	14.5	17.7	21	24.4	28	31.6	35.4	39.2	43.3	47.6	51.9	56.5
30			0	2.8	5.7	8.7	11.9	15	18.4	21.7	25.3	28.9	32.8	36.7	40.8	45.1	49.5
35				0	2.8	5.8	8.8	12	15.3	18.7	22.1	25.8	29.5	33.4	37.4	41.6	45.9
45						0	2.9	6	9.1	12.5	15.8	19.3	22.9	26.7	30.6	34.7	38.8
50							0	3	6.1	9.3	12.7	16.1	19.7	23.3	27.2	31.2	35.3
55								0	3	6.2	9.4	12.9	16.3	20	23.8	27.7	31.7
65										0	3.1	6.4	9.8	13.4	17	20.8	24.7
70											0	3.2	6.6	10	13.6	17.3	21.2
75												0	3.2	6.7	10.2	13.9	17.6
80													0	3.3	6.8	10.4	14.1
85														0	3.4	6.9	10.6
95																0	3.5

MATERIALS AND METHOD

Collection and isolation of sample

Soil samples were collected from lake at Basava Nagar, community garbage dump at Vignan Nagar, Domlur and compost pit in Indira Nagar Park in Bangalore. The samples were labeled after collection. These were spreaded onto isolation media and incubated at 35˚C for 48 hours after serial dilution of 10^{-4} to 10^{-9} times.

Identification of bacteria

The isolated bacteria were identified based on cellular morphology, growth condition, gram staining, endospore staining, capsule staining and biochemical tests (Sneath and Halt; 1986).

Optimization of media for extraction of protease

The media used for optimized production of protease enzyme consisted of glucose 1% (w/v), casein 0.5%, yeast extract 0.55, KH_2PO_4 0.2%, Na_2CO_3 1%, $MgSO_4$. $7H_2O$ 0.2%, and pH 8.0.

Effect of pH on protease production

The effect of pH on alkaline protease production from *Bacillus subtilis* under study was carried out using different pH like 6-9. The optimization media with the above P^H were inoculated with the test sample and the protease assay was done after 24hrs. The best P^H was concluded by reading the absorbance at 660nm.

Effect of temperature on protease production

The effect of temperature on protease production was studied by taking various temperatures like 0˚C, 25˚C 37˚C and 40˚C. The optimization media was inoculated with the test sample at different temperatures and the protease assay was done after 24hrs.

Mass production of alkaline protease through fermentation

The culture media used for mass production of protease contains Dextrose 1%(w/v), peptone 0.5%, KH_2PO_4 0.2%, $MgSO_4$. $7H_2O$ 0. 2%, Casein 1% and pH 8.0. It was maintained at 37˚C for 48hrs. in a shaking incubator. After inoculation, fermentation was carried out at 37˚C at 200 rpm for 48hrs. At the end of each fermentation period, the whole culture broth was centrifuged at 10,000 rpm for 15 minute, to remove the cellul;ar debrises and the clear supernatent was used as enzyme preparation (Olajuyigbe and Ajela, 2005). The purification of the enzyme was done by ammonium sulphate precipitation method.

Purification

About 1 ml of the enzyme precipitate in 50% saturated from ammonium sulphate salted out was taken out in a dialysis bag for dialysis. The bag was then dipped in 100 ml of 0.025M phosphate buffer and was kept for 24 hrs. for stirring on a magnetic stirrer. Further purification of dialyzed protein was done by column chromatography using Diethyl amino ethyl (DEAE) column.

RESULTS AND DISCUSSION

Five bacterial isolates were obtained from soil samples of which P5 strain was identified as *Bacillus subtilis* , morphologically and biochemically. The colonies were subjected to grams staining; capsule staining by negative staining method and endospore staining. The colonies, which were positive for Grams staining, capsule and endospore staining were considered for further studies (Table 1 2, 3 & 4) . The selected colonies were streaked on skim milk agar plates. The plates were subjected to incubation for a period of 24 hours at 37°C.The plates which showed clear zone around the streaked area of

Table 2: Tabulation for results of Colony Characteristics.

Strain No.	Colony Surface	Colony Colour	Visual Characteristics	Shape of the Colony	Height of the Colony
P1	Smooth	Brown	Opaque	Irregular	Raised
P2	Smooth	Off white	Translucent	Irregular	Flat
P3	Smooth	Off white	Opaque	Circular	Raised
P4	Smooth	Off white	Opaque	Circular	Raised
P5	Smooth	Off white	Opaque	Irregular	Flat

P 1-P5 designates the different kinds of bacterial colonies isolated after serial dilution.

Table 3: Tabulation for results of staining techniques.

Strain No.	Gram Staining	Morphology (*Bacillus*/ Cocci)	Endospore Staining	Capsule Staining
P1	Negative	Rod	Positive	Positive
P2	Positive	Rod	Positive	Positive
P3	Positive	Rod	Positive	Positive
P4	Positive	Both Rod & Cocci	Positive	Positive
P5	Positive	Cocci	Positive	Positive

P5 designates the different kinds of bacterial colonies isolated after serial dilution.

Table 4: Tabulation for results of Various Biochemical Tests.

Sl. No.	Samples	Indole	MR	VP	Amylase	Nitrate	Oxidase	Catalase	Urease	Gelatin	Caesin	TSI
1	P1	-ve	-ve	+ve	+ve	-ve	+ve	-ve	-ve	-ve	+ve	+ve
2	P2	-ve	-ve	+ve	+ve	+ve	+ve	-ve	+ve	-ve	+ve	-ve
3	P3	-ve	+ve	-ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve	+ve
4	P4	-ve	+ve	-ve	+ve	+ve	-ve	-ve	-ve	-ve	+ve	+ve
5	**P5**	-ve	-ve	+ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	+ve

P 1-P5 designates the different kinds of bacterial colonies isolated after serial dilution.

test organism was selected as protease producing strain. The organism named P5 showed the inhibition zone and was subjected to various biochemical tests (Table 3). P5 showed the following results for the biochemical test. It was positive for Methyl red test, starch hydrolysis, citrate utilization test, oxidase test, TSI, gelatin hydrolysis, urease test and nitrate reduction test, and was negative for Voges Praskauer test, indole test and catalase test. After biochemical tests the test organism was confirmed to belong to the *Bacillus* species producing Protease. The *Bacillus* species was then tested for its growth under various parameters like carbon, nitrogen source, pH and temperature on production media (Figure. 1, 2, 3 & 4). The media selected for optimization-contained glucose, casein, yeast extract, potassium dihydrogen phosphate, sodium carbonate and magnesium sulphate. For optimization of carbon source, glucose was substituted with different carbon source such as sucrose, lactose, dextrose and maltose. Dextrose was considered the suitable carbon source as compared to glucose according to the O.D readings at 660nm. Similarly yeast extract was substituted by different nitrogen sources like

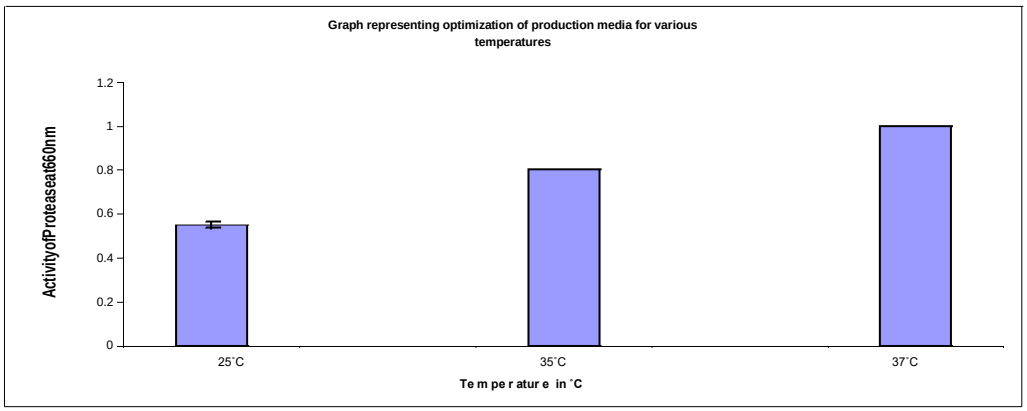

Fig 1: Optimization of production media for various temperatures

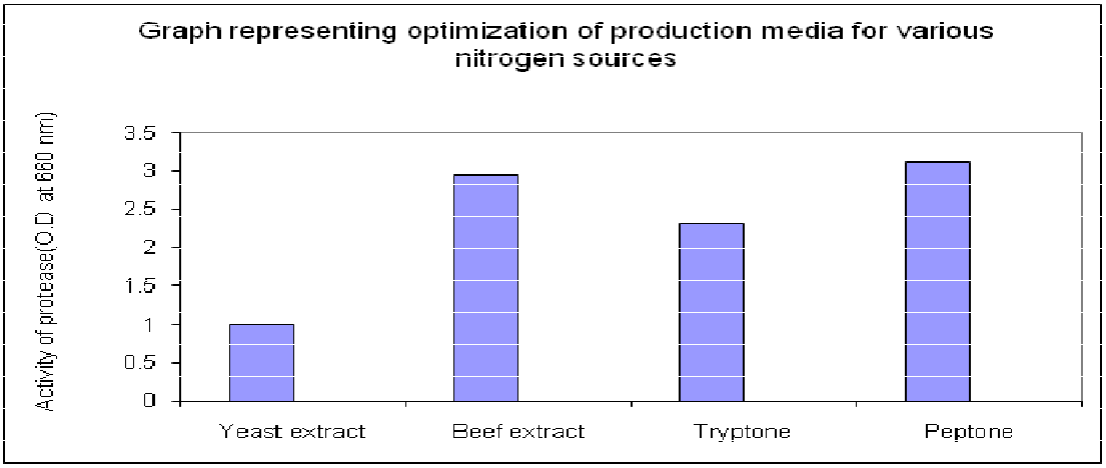

Figure 2: Optimization of production media for various nitrogen sources

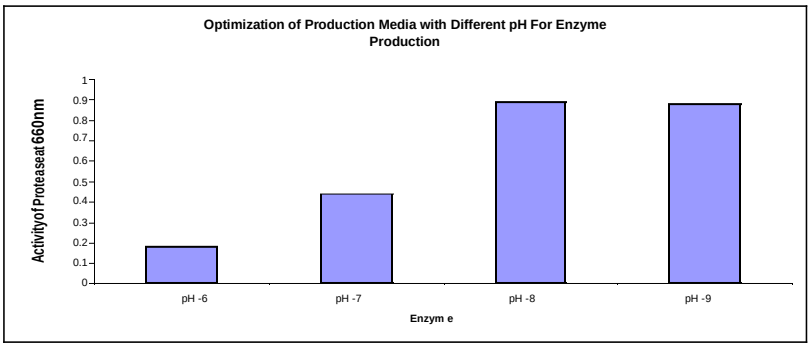

Figure 3. Optimization of production media for various pH

beef extract, tryptone and peptone. Peptone was considered as the best nitrogen source according to the absorbance read at 660 nm. Among physical parameters, pH and temperature was considered. Among various pH ranging from 6-9, the pH of 8.0 was considered to be the best. Among various temperature like 25°C, 37°C and 40°C, the temperature of 37°C supported the growth of test organism. After optimization, the mass production

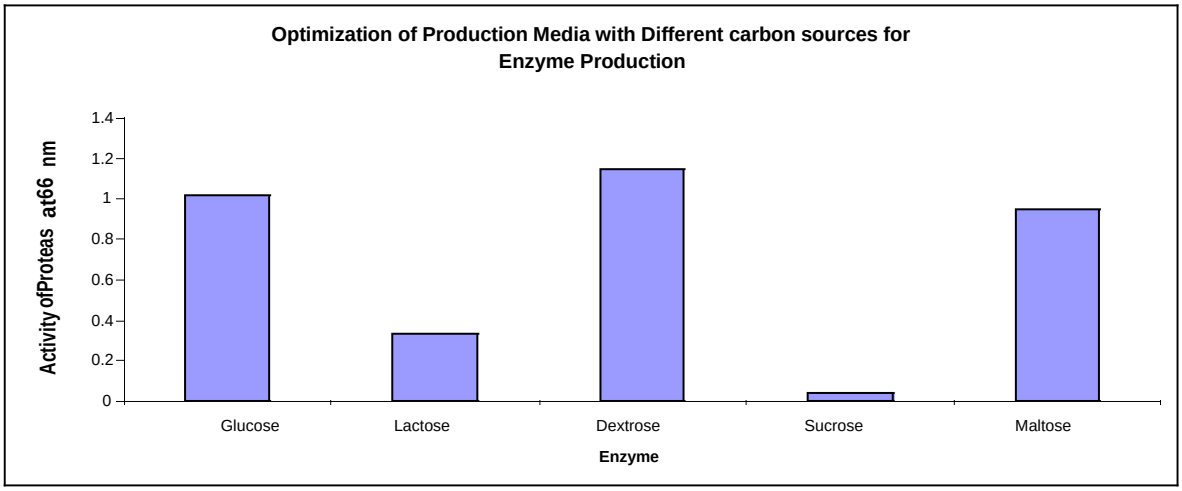

Figure 4. Optimization of production media for various Carbon sources

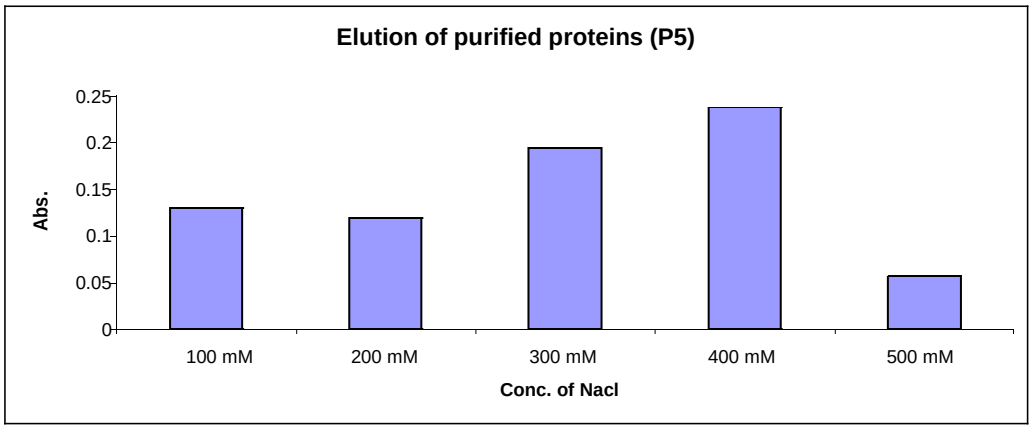

Figure 5.Column chromatography elutents.

was carried in 1 litre of optimized media, in the fermenter (Sartorius) at 37°C for 48 hours at a pH of 8.0 with the stirrer maintained at a speed of 200 rpm. After 48 hours of incubation the culture was centrifuged and crude extract of the enzyme was obtained. The harvested protease product was treated with 50 % ammonium sulphate for purification by salt precipitation method. Then the culture was purified by dialysis.

Finally the enzyme protease was purified by column chromatography (Fig-5). The purified enzyme was loaded onto holes punched on skim milk agar. After 24-48 hours of incubation it was observed that clear zones were formed successfully. The bacterial isolate named P5 is almost similar with respect to the control (*Bacillus subtilis*), morphologically and bio-chemically. Also the O.D values of column chromatography elutant values differ only by 0.01 nm, which is very negligible amount so we may conclude that the organism named P5 may be the *Bacillus subtilis.*

Thus at last it can be said that this enzyme has wide application in various industrial and medical fields and it can be produced in large scale from the microorganism *Bacillus subtilis* isolated from soil sample by applying the fermentation technology.

ACKNOWLEDGEMENT

We are grateful to the authorities of Sangenomics Research Lab, Bangalore, India, for laboratory facilities.

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