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PRODUCTION, PURIFICATION AND OPTIMIZATION OF MEDIA FOR ALKALINE PROTEASE FROM *BACILLUS SUBTILIS* MTCC1028.

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ABSTRACT

The main aim of this study was production, partial purification and media optimization of alkaline protease enzyme by *Bacillus subtilis* MTCC 1028. *Bacillus subtilis* was allowed to grow in broth culture for purpose of producing protease enzyme. Alkaline Protease production by *Bacillus* subtilis was performed with optimum substrate concentrations, optimum incubation temperature, optimum pH, optimum carbon sources and optimum nitrogen source for protease production. The protease enzyme was purified by Ammonium Sulfate precipitation. A trial for the purification of protease resulted in an enzyme with specific activity of 25 (IU/mg). The protease activity increased as the increase in enzyme concentration with optimum substrate concentration. Wheat bran optimum incubation temperature was 37 ºC. Purified protease enzyme had a maximum activity at pH 8.0 of phosphate buffer, with the optimum incubation time of 72 h.

KEYWORDS: Proteases, enzyme, purification, ammonium sulphate.

INTRODUCTION

Proteases are one of the most important classes of enzymes and are expressed throughout the animal kingdom, plant and as well as microbes .The proteases obtained from plant and animal sources were unable to meet current world demands that led to an increased interest in microbial proteases.[1,2] In addition, proteases from microbial sources are preferred than other sources, since they possess almost all characteristics desired for their biotechnological applications.[3] There are acidic, alkaline and neutral proteases, but of all these, alkaline proteases are used primarily as cleansing additives. One of the most physiologically and commercially important groups of enzymes is alkaline protease. Alkaline proteases are defined as those proteases which are active in a neutral to alkaline pH range. They either have a serine center (serine protease) or are of metallo- type (metalloprotease); and the alkaline serine proteases are the most important group of enzymes so far exploited.[4-7] Protease constitute 60-65% of the global industrial market most of which are alkaline proteases.[8] Among the various proteases, Bacterial proteases are more significant compared with animal and fungal proteases.[9] And among Bacteria *Bacillus subtilis* are the specific producers of extracellular proteases. Most of these find applications in the Pharmaceutical, Food industry, Leather industry, Peptide synthesis, for infant formula preparations.[10] 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.

MATERIALS

Bacillus subtilis strains were taken from IMTECH, Chandigarh. Wheat bran, Rice bran, Maize bran and Soya bean have been procured from Market. All the chemicals were of analytical grade and purchased from Loba chemicals & Hi-media.

METHODS

Protease Production from *Bacillus subtilis* MTCC1028

The culture was spread on casein agar medium containing casein 3.0%; peptone, 0.5%; NaCl 0.5% and agar 1.5% in 100 ml flask and incubated at pH 8, 37°C for 24 h.[11]

Extraction and purification of protease

Enzyme extraction

The enzyme from the bacterial bran was washed twice with tap water. The slurry was squeezed through damp cheese cloth. Extracts were pooled and centrifuged at 4°C for 15 min at 10,000 rpm to separate small particles of different substrates, cells and spores. The brown, clear

supernatant was used in enzyme assays. The culture fluid from the production media was collected and centrifuged. The culture supernatant was collected as crude enzyme extract for purification. To the culture supernatant, three volumes of 95% cold ethanol was added and the mixture was maintained in ice for 1 h with agitation. The precipitated crude extract was harvested by centrifugation and dissolved in 0.1 M Tris HCl buffer (pH 7.0).

Enzyme Purification by Ammonium Sulphate precipitation

Enzyme obtained from the culture filtrate was precipitated with solid Ammonium Sulphate (60-80%) on magnetic stirrer. The precipitate was collected by centrifugation (8000 rpm, 20 min), dissolved in citrate buffer (0.05 M, pH 5.0) and dialyzed against the same buffer for overnight.[12]

Protease Assay

Alkaline protease activity was determined by the standard assay. The reaction mixture contained 5 ml of casein (prepared in 50 mM of Tris buffer, pH 8.0) and an aliquot of 1.0 ml of the enzyme solution and incubated for 30 min. The reaction was stopped by adding 5 ml of trichloroacetic acid solution (TCA) (0.11M). After 30 minutes the mixture was filtered and 2 ml of the filtrate was added to 5.0 ml of 0.5 M sodium carbonate and 1.0 ml of Folin - Ciocalteu's phenol reagent and kept for 30 minutes at 37°C. The optical densities of the solutions were read against the sample blank at 660 nm using UV - Visible Spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine per ml per min under assay conditions.[13,14]

Determination of Total Protein Content

The total protein contents of the samples were determined according to the method described by Lowry's method, the protein standard used was Bovine Serum Albumin (BSA) (1 mg/ml).

Procedure

Different dilutions of BSA solutions were prepared by mixing stock BSA solution (1 mg/ ml) and water in the test tube. The final volume in each of the test tubes was 5 ml. From these different dilutions, pipetted out 0.2 ml protein solution to different test tubes and added 2 ml of alkaline copper sulphate reagent (analytical reagent). Mixed the solutions well.

This solution was incubated at room temperature for 10 minutes. Then added 0.2 ml of reagent Folin Ciocalteau solution (reagent solutions) to each tube and incubated for 30 min. Zero the colorimeter with blank and observed the optical density (measured the absorbance) at 660 nm.[15]

Gel Filtration Chromatography

Preparation of Column: 2g of Sephadex G-200 was dissolved in 50mM Buffer Tris- HCl pH 7.2. Size of the column was noted as 10cm X 3cm. The slurry was added to the column by mixing with glass rod, keeping the funnel on the top of column, matrix was uniformly added to the column, and the equilibration of column was done with the Equilibration buffer.

Procedure: 2ml of crude enzyme was added to the column. Mobile phase was 50mM of Buffer Tris- HCl. Fractions of 3ml were collected with the help of gravity and Enzyme activity assay done by Protease Assay. Then the protein content was determined and absorbance was noted down at 505 nm.[16]

SDS PAGE

SDS-PAGE (Sodium dodecyl sulphate polyacrylamide gel electrophoresis) [17] was performed to determine the molecular weight and the purity of the sample. SDS was performed in 10% to check the molecular weight and purity of enzyme. The protein staining was done by using Commassive brilliant blue. For sample buffer and electrode buffer, composition is mentioned in table 1 and 2 respectively, other solutions include:

Separating (4x) gel buffer: Tris-HCl (18.3g) was dissolved in 100 ml of distilled water and pH was adjusted 8.8 with 1M HCI (table 3).

Stacking (4x) gel buffer: Tris-HCl (6.055g) was dissolved in 100 ml of distilled water and pH was adjusted 6.8 with 1M HCl (table 4).

Bisacrylamide (30%): 29.2 g acrylamide was mixed with 0.8g of bis-acrylamide and mixture was dissolved in total 100 ml of distilled water.

Sample buffer

Table 1: Composition of Sample buffer.

Component	Quantity
Tris-HCI (pH 6.8) buffer	0.4 ml
SDS (10%)	2.5%
2-mercaptoethanol	0.4ml
Glycerol	2.0 ml
Bromophenol blue	0.002 gm
Distilled water	4.7ml

Electrode buffer

Table 2: Composition of Electrode buffer.

Component	Quantity
Tris-HCI	6.05 g
SDS	2 gm
Glycine	28.8 gm
Distilled water	2.0 L

Separating gel

Table 3: Composition of separating gel.

Component	Quantity
Distilled water	19.5 ml
Bisacrylamide (30%)	10 ml
4x separating gel buffer	10 ml
SDS (10%)	0.8 ml
Glycerol (10%)	0.35 ml
TEMED	20 µl
APS (2%)	0.6 ml

Immediately the whole mixture was poured in a vertical mould and saturated butanol was added and the gel was allowed to polymerize. After ½ hr butanol was removed and upper portion of gel was washed with deionized water.

Stacking gel

Table 4: Composition of stacking gel.

Component	Quantity
Distilled water	6.3 ml
Bisacrylamide (30%)	2 ml
4x separating gel buffer	2.5 ml
SDS (10%)	0.2 ml
Glycerol (10%)	0.15 ml
TEMED	10 µl
APS (2%)	0.13 ml

This mixture was poured in vertical moulds of plates on the separating gel. Comb was placed in it and gel was allowed to settle for 30 minutes. After the stacking gel was polymerized the comb was removed. Sample was prepared by heating in boiling water bath for 2-3 minutes and the sample was loaded in sample wells with the help of auto pipette. Electrophoresis was carried out at 50V uptill dye front reached into the separating gel and the voltage was increased to 100V. After the run is complete the gel was taken out and washed with water. Then comassive blue staining was carried out.

The staining solution consisted of 90ml water, 90ml methanol, 10ml acetic acid and 0.25g Commassive blue dye. While the destaining solution consisted of 90ml water, 90ml methanol, and 10ml acetic acid.

Procedure

Placed the gel in 100 ml of staining solution for 30 minutes for staining the protein in the gel.

Then placed the gel in the destaining solution for destaining the gel for overnight.

Protease Production in SSF (Solid State Fermentation)

To choose a potential substrate for SSF which supports Alkaline Protease enzyme production in 100 ml of MTCC specified media containing various agro residues was carried out by taking 3 g of dry substrate Wheat bran, Rice bran and Maize bran -3 g (g/l) Glucose 5g, Peptone 7.5 g, NaCl 5g, $MgSO_4$ $7H_2O$ 5g, $FeSO_4$ $7H_2O$. 0.1g distilled water was added to adjust the required moisture level with the pH of 8.0. The contents of the flasks were mixed and autoclaved at 121°C for 15 min. The medium was incubated for 48 h in shaker incubator (150 rpm) at 37^OC. The fermented broth was filtered and the filtrate was centrifuged at 5000 rpm for 5 minutes to extract the crude extracellular Protease. The culture filtrate was used for further assay procedures.[18]

Determination of parameters controlling protease production[19-22]

Effect of Fermentation

The test organism was grown in nutrient broth containing 1% casein and 3% NaCl. It was incubated at 37°C for 24, 48, 72 and 96 hr in an orbital shaker at 150 rpm. The contents were then centrifuged at 10,000 rpm at 4°C for 10 min and protease activity was checked in the cell free extract.

Effect of using different substrates

Enzyme was produced by using three different substrates Wheat bran (WB), Rice bran (RB), Maize bran (MB) with 1% casein and 3% NaCl. It was incubated at 37°C for 48 hr in an orbital shaker at 150 rpm. The contents were then centrifuged at 10,000 rpm at 4°C for 10 min and protease activity was checked in the cell free extract.

Effect of pH

The test organism was grown in nutrient broth containing 1% casein and 3% NaCl. Effect of pH on enzyme production was studied by adjusting the culture media (pH 6, 7, 8 and 9) prior to sterilization. It was incubated at 37°C for 48 hr in an orbital shaker at 150 rpm. The contents were centrifuged at 10,000 rpm at 4°C for 10 min and protease activity was checked in the cell free extract.

Effect of Temperature

The optimum temperature of the alkaline protease was determined by incubation at four different temperatures (25, 30, 35 and 40ºC) were added to nutrient broth. The organism was inoculated and incubated for 48h. Enzyme activity was assayed in the culture supernatant.

Effect of Carbon Sources

To find the optimum carbon source for alkaline protease, four carbon sources (1%) (Glucose, sucrose, lactose and fructose) were selected and added to nutrient broth. The organism was inoculated and incubated for 48 hr at 70°C and the enzyme activity was assayed in the culture supernatant.

Effect of Nitrogen Sources

To optimize the nitrogen source for alkaline protease, four different nitrogen sources (1%) (Ammonium nitrate, casein, urea and ammonium sulphate) were added to nutrient broth. Organism was inoculated and incubated

for 48h. The enzyme activity was assayed in the culture supernatant.

Effect of different metal ions

The role of metal ions on alkaline protease production was analysed by the addition of metal ions like $CaCl_2$, $MnSO_4$, $MgSO_4$, $COCl_2$, $MnCl_2$ of 0.5% concentration and was assayed.

RESULTS

Casein hydrolysis by *Bacillus subtilis*

The culture was spread on casein agar medium and the clear zone of casein hydrolysis was observed (Fig 1) alongwith the growth of *Bacillus subtilis* which is a clear indication of protease production.

Figure 1: a) Casein hydrolysis at 37°C b) *Bacillus Subtilis* growth after 24 hr.

Protease assay

OD was determined at 660nm (Table 5) and a standard curve was plotted between casein concentration and OD as shown in Fig 2.

Table 5: Standard of Alkaline Protease.

1%Casein (µl) (w/v)	Tri-HCl buffer pH 8.0 (µl)		10% TCA (µl)	0.5 M Na_2CO_3 (ml)	1N Folin - Ciocalteu's phenol reagent	O.D at 660 nm
0	2000	Incubation at 37°C for 20 min.	200	2.5	0.5	0
100	1900		200	2.5	0.5	0.109
200	1800		200	2.5	0.5	0.195
300	1700		200	2.5	0.5	0.274
400	1600		200	2.5	0.5	0.356
500	1500		200	2.5	0.5	0.418
600	1400		200	2.5	0.5	0.514
700	1300		200	2.5	0.5	0.576
800	1200		200	2.5	0.5	0.639
900	1100		200	2.5	0.5	0.694
1000	1000		200	2.5	0.5	0.753

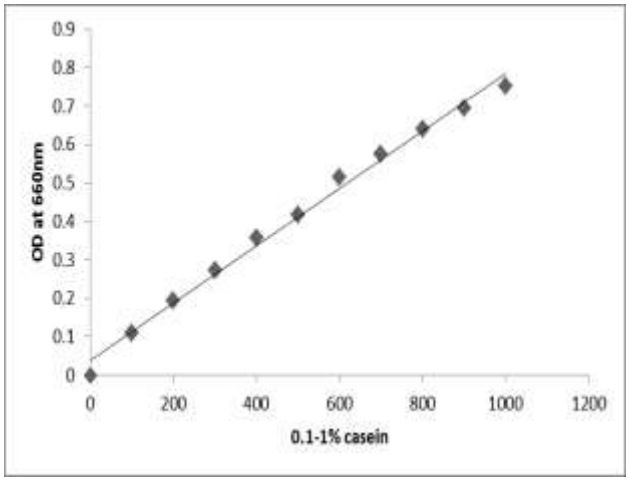

Figure 2: Standard curve of 1% casein

Protease activity was calculated for three different substrates (wheat bran, Rice bran and Maize bran) as shown in table 6. High protease activity in case of Wheat bran corresponds to their high Protein contents.

Table 6: Enzyme activity of Protesase in different substrates samples.

Substrates	Protease activity (U/ml)
Wheat bran	15.4
Rice bran	13.9
Maize bran	14.6

Determination of Total Protein Content

OD was measured at 660nm and a standard graph was plotted between OD and BSA concentration (Fig 3). The protein activity of alkaline protease was estimated to be around 0.67 mg/ml.

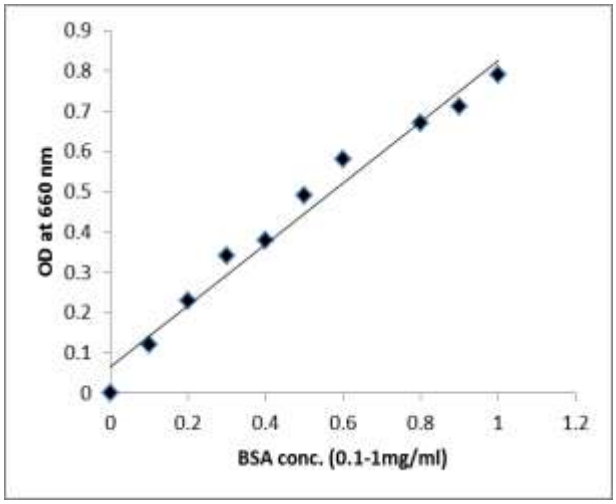

Figure 3: Standard Graph for estimation of Protein

Enzyme Purification

The crude protease when precipitated with ammonium sulphate showed 1378.54 U/ml of protease activity with protein concentration of 2.546 mg/ml.

Gel Filtration Chromatography

Only three of the fractions showed enzyme activity and protein content. Highest enzyme activity was obtained with fraction no. 5 with 75 IU/ml while for the same fraction protein content was also found to be higher with 1.8 mg/ml (table 7). Percent recovery was found to be 77%.

Table 7: Enzyme activity and Protein Content during Gel filteration chromatography.

Fraction no	Enzyme Activity(IU/ml)	Protein Content(mg/ml)
1	N.D	N.D
2	N.D	N.D
3	N.D	N.D
4	68	0.5
5	75	1.8
6	30	0.2
7	N.D	N.D

SDS-PAGE

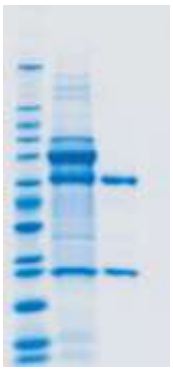

Figure 4: Lane markers of purified alkaline protease (lane 1: Crude alkaline protease; lane 2: Partially purified Alkaline protease; lane 3: Purified Alkaline protease)

SDS-PAGE results showed the presence of multiple bands, since along with protease some other proteins can be produced by the organisms. But the presence of protein bands near the molecular weight 27 kDa confirms the presence of enzyme (Fig 4). It has been previously reported that the molecular weight of most of the alkaline proteases from *Bacillus subtilis* lies between 16 and 32kDa.

Determination of parameters controlling protease production

Effect of Fermentation Period

There was a gradual increase in production which occurred from beginning to 48 h (Fig 5) and production was higher at 72 h with the enzyme activity of 42 U/ml, while the protein content was higher at 96 hr pertaining to about 70% (Fig 6).

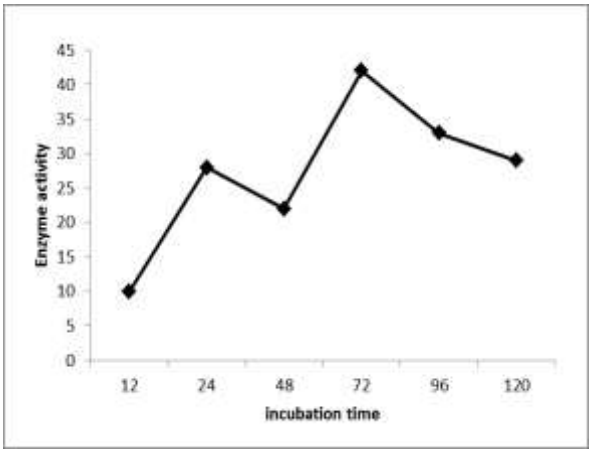

Figure 5: Effect of incubation time on the production of *B. subtilis* MTCC1028

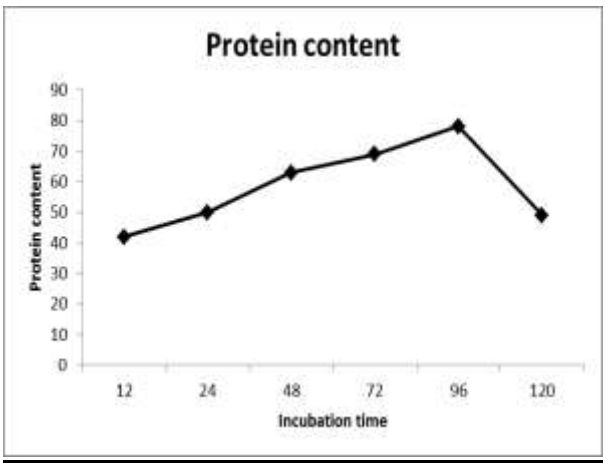

Figure 6: Effect of incubation time on the total protein content of *B. subtilis* MTCC1028

Effect of using different substrates

The production of Protease was done by replacing the Nitrogen sources and common substrates with Maize bran, Soybean, Wheat bran and Rice bran. The maximum production occurred when wheat bran was used as natural substrate (75.96 U/ml) (Fig 7). The result indicates that the wheat bran can also be used as the cheap substrate for Protease production (Fig 8).

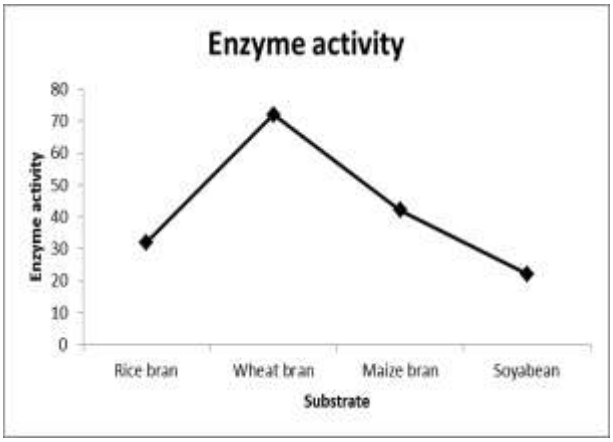

Figure 7: Effect of substrates in the production of *B. subtilis* MTCC1028

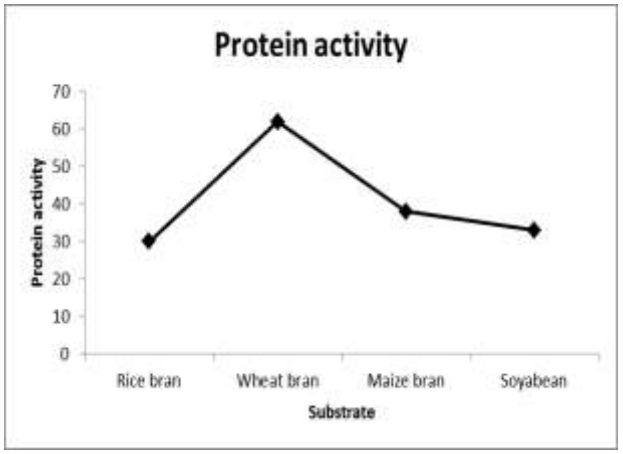

Figure 8: Effect of different substrates in the total protein content of *B. subtilis* MTCC1028

Effect of Temperature

The higher protease activity was found to be 72 U/ml at 37 °C for the protease production (Fig 9). The temperature requirement of the organism is based on the nature of organisms. Bacterial and fungal alkaline protease production was lower at 25°C and moderate at 50 °C (Fig 10).

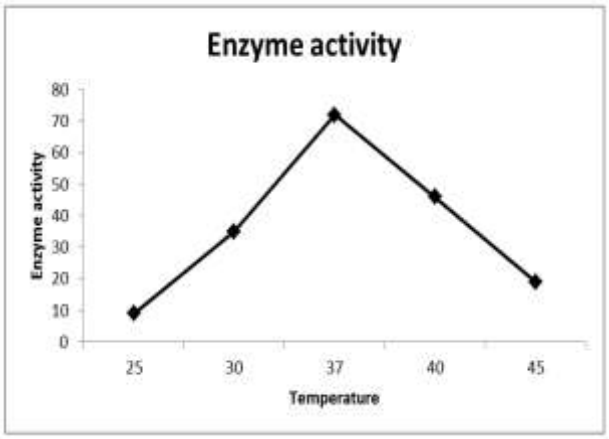

Figure 9: Effect of temperature on the production of *B. subtilis* MTCC1028

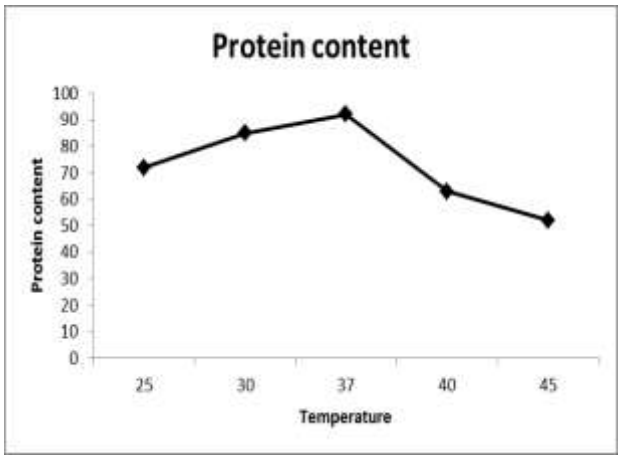

Figure 10: Effect of Temperature on the total protein content of *B. subtilis* MTCC1028

Effect of pH

Medium composition, pH, and aeration are the important variables that affect the production of enzyme in Solid State Fermentation (Fig 11). Initial pH of the production medium is the most important factor that significantly influences the production of proteases (Fig 12). Proteases having optimum pH between 8 and 12 have potential applications in the fields of detergent application, dehairing of hides, and silver recovery from waste X-ray. Enzyme activity was found to be maximum at 50 U/ml with pH 8.0.

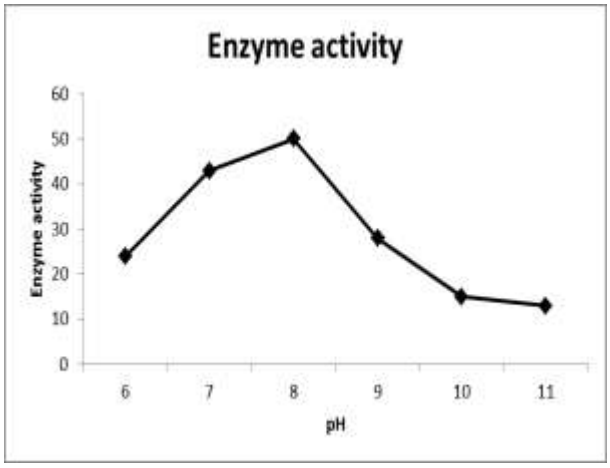

Figure 11: Effect of pH on the production of *B. subtilis* MTCC1028

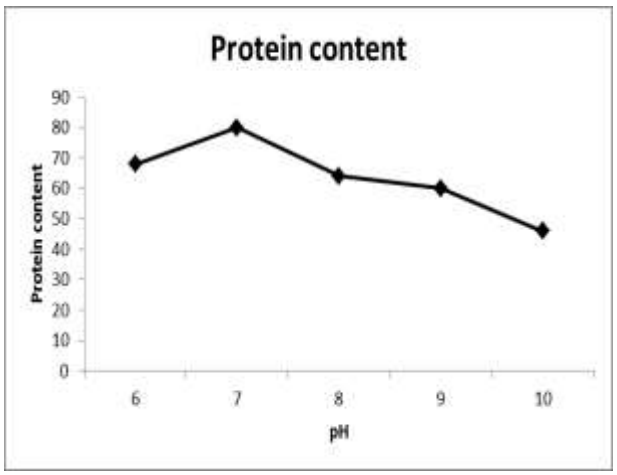

Figure 12: Effect of pH on the total protein content of *B. subtilis* MTCC1028

Effect of Carbon Sources

Nutrient sources were found to be the next important factor for the Protease production. Since carbon is considered as the primary nutrient for the bacteria, different carbon sources like Sucrose, Glucose, Lactose, Starch and Fructose were analyzed for the protease production. Maximum production of Protease of 82 U/ml was observed when Glucose was served as the carbon source (Fig 13). Hence, glucose was served as the better carbon source for the Protease production (Fig 14).

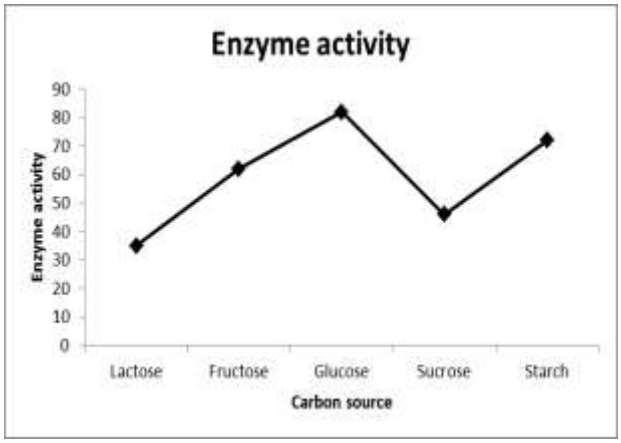

Figure 13: Effect of carbon source in the production of *B. subtilis* MTCC1028

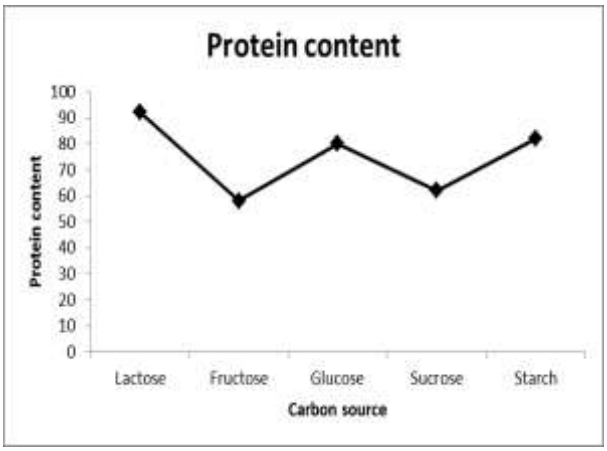

Figure 14: Effect of carbon source on the total protein content of *B. subtilis* MTCC1028

Effect of Nitrogen Sources

Nitrogen was served as important nutrient source for the Protease production. Hence, different nitrogen sources like Peptone, Yeast extract, Beef extract, Casein, Ammonium nitrate, Sodium nitrate, Urea were applied as nitrogen sources for the Protease production. Casein (Fig 15,16) is found to be the better nitrogen source as it increases the production of protease up to 42 U/ml.

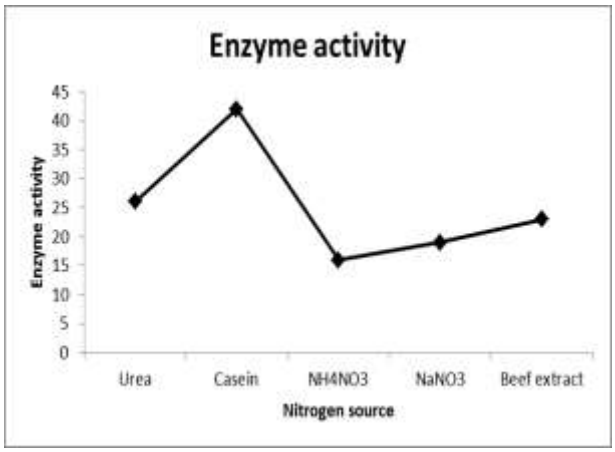

Figure 15: Effect of nitrogen source in the production of *B. subtilis* MTCC1028

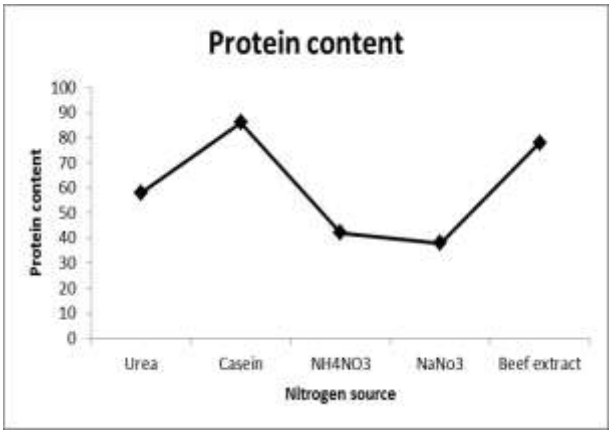

Figure 16: Effect of nitrogen source in the total protein content of *B. subtilis* MTCC1028

Effect of different metal ions

The metal ions were considered to be important cofactors for an enzyme to function and hence they were analysed. From the result, it was clear that $MgSO_4$ with 84 U/ml played a better role in the alkaline protease activity (Fig 17,18). The result supported the statement that supplementation of Mg2+, Ca2+ and K+ salts to the culture medium exhibited slightly better production of protease.

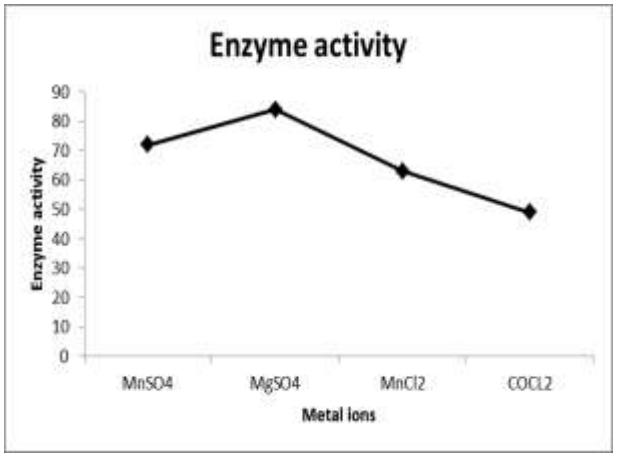

Figure 17: Effect of metal ions in the production of *B. subtilis* MTCC1028

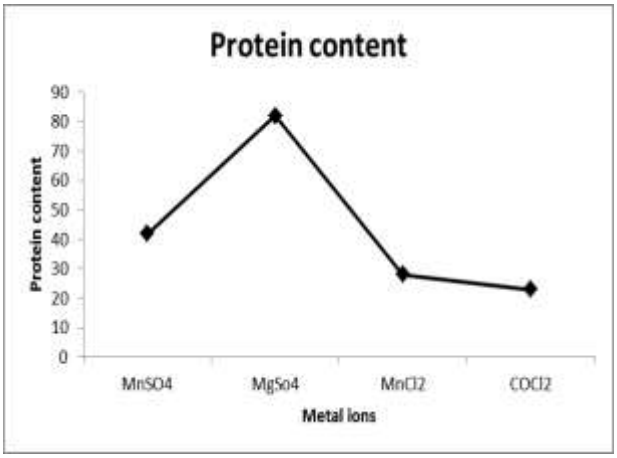

Figure 18: Effect of metal ions in the total protein content of *B. subtilis* MTCC1028

CONCLUSION

The use of waste raw materials is cheaper and more advantageous than conventional substrates for alkaline protease production. Alkaline protease production by *B. subtilis* strain under solid-state fermentation was influenced by physiological and chemical nature of the Wheat bran and was associated with growth of the microbial strain. Furthermore, we will able to scale up the solid-state fermentation on Wheat bran. The thermal resistance, ability to function in a broad range of temperature may lead to conclude the future application of *B. subtilis* protease in laundry detergents formulations and in food and pharmaceutical industries which is highly promising.

CONFLICT OF INTEREST

The Authors declare no conflict of interest.

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