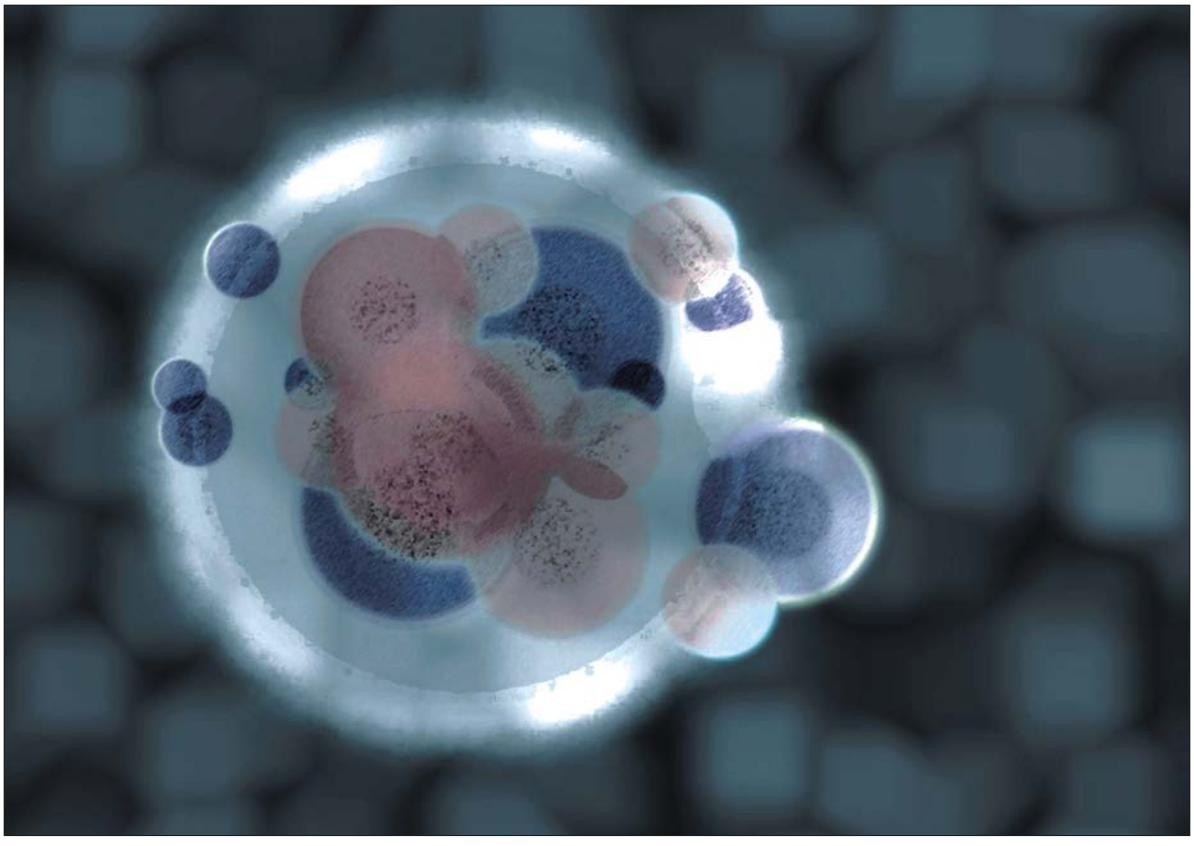

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Isolation and Characterization of a Protease Producing Bacteria *Bacillus amovivorus* and Optimization of Some Factors of Culture Conditions for Protease Production

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Abstract: Following enrichment media technique a bacterial isolate WP was isolated from degraded pulse and identified as a *Bacillus amovivorus* den Dooren de Jong. Primary screening was done by gelatin, skimmed milk casein and egg albumin hydrolysis method. Optimum protease producing incubation period, medium pH and temperature for this isolate were 48 h, 8.5 and 37°C, respectively.

Key words: Isolation, *Bacillus amovivorus*, protease

INTRODUCTION

Proteases are the key enzyme in industrial application. Microbial proteases play important role in biotechnological process with world wide sales representing about 60% of the total enzyme marked[1]. A number of bacteria[2-4], Fungi[5-7] and yeast[8,9] have been reported for protease production. Many of the organisms produce more than one kind of protease. The type of proteolytic enzyme formed may depend on the composition of the medium. Culture conditions play significant role on growth and production of protease by bacteria[2,10,11]. This study deals with the isolation of a protease producing bacteria and some factors affecting on growth and production of protease by this organism.

MATERIALS AND METHODS

Isolate: The bacterial isolate *Bacillus amovivorus* were collected from degraded pulse sample.

Isolation and screening: Proteolytic microbes were isolated following enrichment media technique and the isolates were purified by repeated pour and streak plate method. The isolate WP was screened for protease producing ability by egg albumin, skimmed milk casein and gelatin hydrolysis method. Three screening broth such as peptone-yeast extract-dextrose broth (contained peptone 2%, yeast extract 1%, dextrose 2%)[12], tryptone-yeast extract-dextrose broth (contained tryptone 1%, dextrose 0.1%, yeast extract 0. 5%)[13] and gelatin-yeast extract-glucose broth (contained gelatin 1%, glucose 1%, yeast extract 0.2%, K_2HPO_4 0.3%, KH_2PO_4 0.1%, $MgSO_4.7\ H_20$ trace) [2] were used for the final selection of the isolate.

Identification of selected isolate: Different morphological, cultural and physiological characteristics of the bacterial isolate WP were studied for identification purpose and these were compared with standard description of Bergey's Manual of Determinative Bacteriology[14].

Biomass yield: Bacterial biomass was determined by measuring the absorbance at 600 nm[15].

Measurement of enzyme activity: Enzyme assay was determined by the modified method of Hayashi *et al.*[16] as followed by Meyers and Ahearn[17]. Three milliliter of culture filtrates, 3 mL phosphate buffer and 3 mL 1% casein was taken in a 25 mL test tube. Then the test tube was placed in a water bath at 35^{0}C for 1 h. After reaction, 5mL 20% TCA was added with the solution for stopping the reaction, after one hour, the solution was filtered by Whatman no. 540 (Ashless). From the filtrate solution 1 mL enzyme substrate mixture was taken into a test tube and 2 mL 20% Na_2C0_3 was added to it. To this mixture 1 mL of Folin Ciocalteu Reagent was added and immediately the contents of the tube were mixed well. After 30 min 6 mL distilled water was added to it and the absorbance of the solution was measured at 650 nm in a spectrophotometer and calculated the amounts of amino acids released from a standard curve plotted from known concentration of tyrosine. The enzyme activity was expressed in Unit. One unit of enzyme was defined as the

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amount of enzyme that releases 1 μg of tyrosine mL^{-1} of crude extract h^{-1}.

Optimization of culture conditions

Effect of incubation period: The effect of incubation periods on the growth and protease activity by selected isolate was studied.

For this, 50 mL of selected medium was taken in each 100 mL conical flask. All the flasks were autoclaved at 121°C and 15 lb pressure for 20 min. After cooling the flasks were inoculated with equal quantity of inoculums. The flasks were incubated at 37±2°C. At 24, 48, 72 and 96 h of incubation, the culture filtrates were collected.

Effect of medium pH: To observe the effect of medium pH on enzyme production, 50 mL of selected medium of different pH (such as 5.0, 6.0, 7.0, 8.0 8.5 and 9.0, respectively) was taken in each 100 mL of conical flask. The flasks then inoculated and incubated in optimum incubation periods. The effect of medium pH on biomass characteristics, biomass yield and protease activity was recorded.

Effect of temperature: The culture medium was incubated at different temperature for optimum enzyme production. For this reason, equal quantity of inoculums was added in each conical flask containing 50 mL of selected suitable medium with selected pH. The flasks were then incubated at different temperature (such as 10±2, 30±2, 37±2 and 45±2°C, respectively) for optimum enzyme production. The effect of temperature on biomass characteristic, biomass yield and protease production was recorded.

RESULTS AND DISCUSSION

Seventy two microbial strains were isolated by using enrichment technique. Among these the bacterium isolate, WP exhibited better proteolytic ability by hydrolysis of casein (Fig. 1), egg albumin and gelatin. The isolate WP showed better protease production (activity) in Tryptone-yeast extract-dextrose broth medium and which was finally selected for further studies.

Identification of the isolate: The cultural morphological and physiological characteristics of selected bacterial isolate are shown in Table 1. On the basis of these characteristics the bacterial isolate WP was found to belong to the genus *Bacillus* and closely similar to *Bacillus amovivorus* den Dooren de Jong. Although it showed variable in spore formation with describe species.

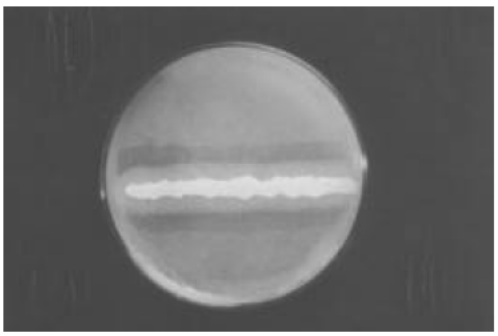

Fig. 1: Skimmed milk casein hydrolysis by *Bacillus amovivorus*

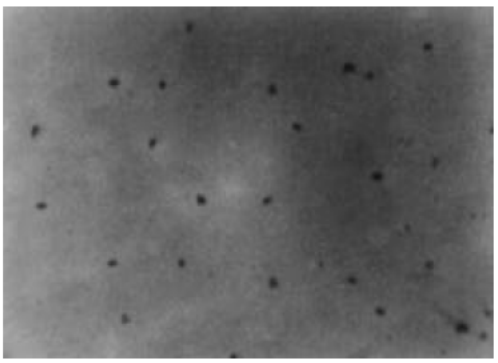

Fig. 2: Vegetative cell of *Bacillus amovivorus* under microscope (40×12x)

Optimization of culture conditions

Effects of incubation period on protease production: Since microorganisms show considerable variation at different incubation period, it is very essential to detect the optimum incubation time at which an organism shows the highest enzyme activity.

When the bacterial isolate WP was grown in a selective media containing tryptone 1%, dextrose 0.1%, yeast extract 0.5%[13] it showed highest protease formation at 48 h of incubation time (Table 2) but highest biomass yield was recorded at 96 h of incubation period. The biomass characteristics of WP was also recorded and it was observed that the isolate showed turbid growth after 24 h of incubation time, but it showed sedimentary growth at 48, 72 and 96 h of incubation. The color of the supernatant changed from golden yellow to golden brown. The pH of the culture filtrates were found higher (6.63 to 7.81).

Table 2 shows that 48 h of incubation for bacterial isolates WP is suitable time for maximum production of proteases. Maximum production of proteases with 48 to 72 h of incubation by bacteria was reported by Hoshino *et al.*[18] and Shumi *et al.*[10]. Present results are in concurrence with their reports.

Table 1: Characteristics of the bacterial isolate WP

Vegetative cells	Short rod. Size: 1 to 2×1 μm (Fig. 2)							
Spore stain	Variable							
Gram stain	Gram positive							
Acid fast stain	Non-acid fast							
Motility test	Positive							
Agar colonies	Irregular, undulate, raised and white in color.							
Nutrient broth	Turbid with membranous growth							
Glucose broth	Very scanty, sedimentary growth							
Asparagine broth	Negative							
Catalase test	Negative							
Oxygen relationship	Aerobic							
Indole test	Negative							
Nitrate reduction test	Positive							
Synthetic medium	Negative							
Inorganic salt medium	Negative							
Growth in citrate medium	No growth							
H_2S production	Negative							
Proteolysis test	Coagulated egg albumin degraded							
Casein hydrolysis	Hydrolyzed the skimmed milk casein (Fig.1)							
Methyl red test	Negative							
Voges- Proskaur test	Negative							
Starch agar	Hydrolyzed							
Growth at different temperature °C	10	27	30	37	45	50		
	-	+++	+++	++++	+++	-		
Growth at different NaCl concentration (%)	0	1	2	3	4	5	6	7
	++++	++++	++++	+++	++	-	-	-
Gelatin hydrolysis	Positive							
Urease test	Negative							
Oxidase test	Positive							
Fermentation of different carbohydrates	Alkali : Starch.							
	No fermentation : Glucose, Fructose, Lactose, Sucrose, Galactose, Arabinose, Raffinose, Glycerol, Rhamnose, Cellobiose, Maltose, Dextrose, Mannitol, Inulin, Xylose							

++++ = Heavy growth, +++ = moderate growth, ++ = Scanty growth

Table 2: Effects of incubation period on the production of protease by the bacterial isolate *Bacillus amovivorus* (WP)

Incubation periods (h)	Color and pH after incubation		Biomass characteristics	Biomass yield (absorbance at 600 um)	Protease activity (U mL $^{-1}$)
	Color	pH			
24	Golden yellow	6.63	Turbid growth	0.830	197
48	Golden brown	7.30	Turbid with sedimentation	1.262	204*
72	,,	7.76	Sedimentary growth	1.720	122
96	,,	7.81	,,	1.947	96

Initial color of the medium: Golden yellow; pH 7.0; temperature 37°C, * Maximum enzyme activity

Table 3: Effects of medium pH on the production of protease by the bacterial isolate *Bacillus amovivorus* (WP)

Medium pH	Color and pH after incubation		Biomass characteristics	Biomass yield (absorbance at 600 um)	Protease activity (U mL $^{-1}$)
	Color	pH			
5.0	Golden yellow	5.57	Turbid growth	0.202	292
6.0	,,	7.30	,,	0.904	327
7.0	Golden brown	7.87	Turbid with sedimentation	1.262	431
8.0	,,	8.08	,,	0.891	510
8.5	,,	8.13	,,	0.675	524*
9.0	,,	8.25	,,	0.186	345

Initial color of the medium: Golden yellow, Incubation period 48 h, temperature 37°C, * Maximum enzyme activity

Table 4: Effects of temperature on the production of protease by the bacterial isolate *Bacillus amovivorus* (WP)

Incubation temperature (°C)	Color and pH after incubation		Biomass characteristics	Biomass yield (absorbance at 600 um)	Protease activity (U mL^{-1})
	Color	pH			
10	Golden yellow	8.09	-	0.134	00
27	,,	7.75	Turbid growth	0.860	407
37	Golden brown	7.86	,,	0.885	549*
45	,,	7.18	Turbid with sedimentation	0.470	150

Initial color of the medium: Golden yellow; pH 8.5; temperature 37°C, * Maximum enzyme activity

Effects of medium pH on production of proteases: Microorganisms are sensitive to the changes in the hydrogen ion concentration of their environment. Therefore, to detect the optimum medium pH, the selected isolates were incubated at different pH and their growth characteristics and production of protease were recorded. The bacterial isolate WP (*Bacillus amovivorus*) exhibited maximum protease production at medium pH 8.5 but the highest biomass yield was recorded at medium pH 7.0 (Table 3). The color of the supernatant were golden yellow at acidic pH and golden brown at neutral to alkaline pH. The pH of the culture filtrate were ranged from 5.57 to 8.25.

Production of proteases at alkaline medium pH was reported by Fermor and Wood[19], Shin *et al.*[20].

Effects of temperature on production of proteases: The growth and enzyme activity of microorganisms is greatly influenced by different incubation temperature. The growth of microorganisms can be inhibited at one temperature but it can be activated at another temperature. So, it is essential to incubate microorganisms at their optimum incubation temperature for their successful growth. The incubation temperature is usually determined by considering the sources from which the organisms have been isolated. For this reason, to detect the optimum incubation temperature, the selected isolates were incubated at different incubation temperature. The highest incubation temperature, growth characteristics, the change of color, pH of the supernatant and biomass production after incubation were recorded (Table 4).

The highest protease activity and the biomass were recorded at 37°C. The color of the supernatant was golden yellow to golden brown and the pH was ranged from 7.18 to 8.09.

The bacterial isolates prefer 37°C for maximum production of protease which are in concurrence with the report of Al-saleh[3] and Shumi *et al.*[10].

In conclusion it can be concluded that the bacterial isolate *Bacillus amovivorus* produce proteases at alkaline culture conditions and different factors greatly regulates the growth and production of proteases. The results in this study on different factors will be useful during further production of protease by this organism.

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