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Studies on extracellular protease production by *Bacillus* sp. Isolated from soil

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Abstract

The collected soil sample was serially diluted by serial dilution method and 10^{-5th} tube was used for the isolation of protease producing bacterial strain. The zone of clearance was observed on SMA plate after 24hrs of incubation at 37°c temperature. The zone of clearance was recorded as 5 mm on SMA plate. The significant protease producing bacterial colony was used for biochemical analysis and it tentatively identified the bacterial strain as *Bacillus* strain. The various physico-chemical parameters were used to protease production such as incubation period, carbon, nitrogen source, temperature and pH. The incubation period for maximum production of protease at 24 hrs. It was 0.72 U/ml and minimum at 72 hrs. (0.40 U/ml). The maximum production of protease when fructose was used as carbon source (1.23 U/ml) and minimum at lactose and maltose that was 0.75 U/ml. The best nitrogen source for protease production was peptone (1.55 U/ml) and minimum at ammonium chloride (0.10 U/ml). The maximum production of protease was recorded in a medium at pH 8 that was 1.34 U/ml. The maximum biosynthesis of protease was recorded at 40°C that was 1.31 U/ml.

Keywords: Soil sample, Protease, physical and chemical components, *Bacillus* sp.

1. Introduction

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology [1]. They are essential constituents of all forms of life on earth, including prokoryates, fungi, plants and animals.

They can be cultured in large quantities in relatively short time by established fermentation methods and produce an abundant, regular supply of the desired product. In recent years there has been a phenomenal increase in the use of alkaline protease as industrial catalysts [2].

2. Materials and Method

Sample Collection:
The soil sample was collected in sterilized polythene bags from garden of Digambarrao Bindu college, Bhokar. The collected soil sample was used to make the serial dilutions and used for isolation of potent strain for higher protease production. The isolated bacterial strain was tentatively identified by culture dependent method (Biochemical method) [3].

Screening of Protease production from isolated strain:
The isolated bacterial colonies were used to screen the higher protease producing strain on plate assay method. The plate assay method was takes place by using Skimmed milk agar (SMA) plate. The media was punched by borer and made a holes on the SMA plate and add one bacterial culture aseptically and incubate the plate at 40°C for 24 hrs and observe the result [4].

Enzymes assay
Protease activity was assessed in triplicate using casein (1%) in 50 mM NaOH buffer (pH 7.5) at 37°C for 30 min. The 1-mL reaction was terminated by the addition of 0.5 mL of 15% trichloroacetic acid and then centrifuged at 8000 g for 10 min at 4-8°C in cooling centrifuge. One enzyme activity unit (U) was defined as the amount of enzyme required to produce an increase in absorbance at 520nm. Protein was measured by the method of the Lowry method [3, 5].

Protease production.
Protease production was carried out at shake flask level. *Bacillus* species were inoculated in fermentation media [6]. The medium was incubated at 40° C on a rotary shaker (200 rpm) for 24 hrs. After incubation centrifuged the liquid medium at 8000 rpm for 15 minutes. After centrifugation the supernatant was collected and it was used as a crude protease enzyme source [7].

Physico-chemical Characterization for protease production.
Effect of Incubation period on Protease production:
The effect of incubation period on the protease production was determined by performing the standard assay procedure at pH 7.5 within an incubation period range of 18, 22, 24, 48, 72 hrs. After treatment the residual proteolytic activity was assayed. Proteolytic activity was measured under standard assay conditions [8, 9].

Effect of carbon & nitrogen source on Protease production:
The effect of carbon and nitrogen source on the protease production was determined by performing the standard assay procedure at pH 7.5 by using glucose, fructose, starch, lactose, sucrose maltose & glycerol as a carbon source and peptone, glutamic acid, ammonium sulphate, ammonium chloride, urea, ammonium nitrate, ammonium citrate and sodium nitrate as a nitrogen source used for protease production medium. After treatment the residual proteolytic activity was assayed. Proteolytic activity was measured under standard assay conditions [8].

Effect of pH and Temperature on Protease production
The effect of pH and temperature on the protease production was determined by performing the standard assay procedure by using various pH range such as 5 to 10 and Temperature were 25, 30, 35, 40, 45 and 50°C. After treatment the residual proteolytic activity was assayed. Proteolytic activity was measured under standard assay conditions [8, 10].

3. Result

The extracellular protease enzyme was synthesized by *Bacillus* species previous isolated from soil. The results obtained in this work revealed the ability of Bacillus species to produce extracellular protease. Different

culture conditions were used to obtain the maximum levels of protease productivity by *Bacillus* species. The effect of different factors on enzyme activity was observed.

Isolation of the microorganism

Soil sample was used for the isolation of protease producing microorganism. The serial dilution plate technique used for isolation. The isolated colorless colonies of Bacillus species were observed, on the basis of morphological, cultural and biochemical characters microorganism were isolated.

Colony characteristics of *Bacillus* species.

The isolated colonies was picked and checked its morphological characters as presented in table 1.

Table 1: morphological characters of isolated *Bacillus spp.*

Sr. No.	Characters	Nature
1.	Size	1mm
2.	Shape	Circular
3.	Color	White
4.	Margin	Entire
5.	Elevation	Flat
6.	Opacity	Opaque
7.	Consistency	Butyrious
8.	Grams Nature	Gram Positive
9.	Motility	Motile

Biochemical characteristics.

The biochemical characteristics were carried out of the isolate and obtained result tabulated in table 2.

Table 2: Biochemical characteristics of isolate.

Sr. No	Tests	Result
1.	Catalase	+
2.	Oxidase	+
3.	Indole	+
4.	MR Test	-
5.	VP Test	+
6.	Citrate	+
7.	Starch Hydrolysis	+
8.	Gelatin Hydrolysis	+

Screening test of protease producing bacteria.

The isolated *Bacillus* species produced protease enzyme was confirmed by the screening test on gelatin agar medium. The bacterial cultures were streaked on a gelatin agar plate. The plate put in incubator for 37^{o}c for 24 hrs. After incubation the flood Hgcl2 solution on gelatin agar medium. The clearing zone around bacterial growth were on gelatin agar medium i.e. indicated that the ability of microorganism to hydrolyze gelatin.

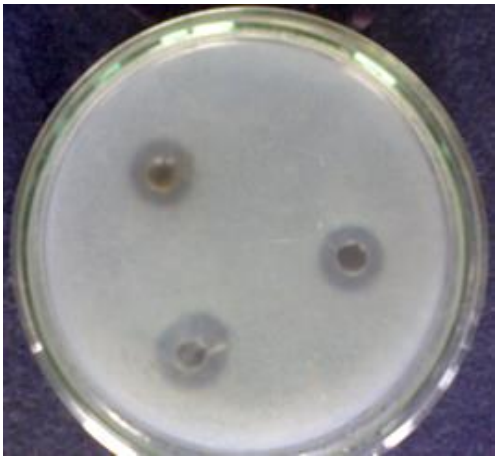

Fig 1: the clearance of zone around the well shows the protease activity.

4.3 Protease production.

Protease production was carried out at shake flask level. *Bacillus* species were inoculated in fermentation media. The medium was incubated at 40^{o} C on a rotary shaker (200 rpm) for 24 hrs. After incubation centrifuged the liquid medium at 8000 rpm for 15 minutes. After centrifugation the supernatant was collected and it was used as a crude protease enzyme source.

Assay of proteolytic activity

The activity of protease enzyme was checked by plate assay method. The zones of clearance on the plate were observed indicates proteolytic activity.

Effect of physico-chemical parameter on protease production.

The impacts of some physical and chemical parameters on the production of protease which influence its synthesis some of them are checked for the present investigation are as follows.

Incubation period:

The maximum production of protease at 24 hrs, that was 0.72 U/ml and minimum at 72 hrs. (0.40 U/ml). The production of protease was proportionally increased with the incubation time within the time ranged from 16 hrs to 24 hrs. Whereas after 24 hrs incubation, the production of protease decreased. The obtained result summarized in table 3.

Carbon sources:

The result showed the ability of *Bacillus* species to utilizing fructose as a carbon source and energy material to produce protease enzyme. The maximum production of protease when fructose was used as carbon source (1.23 U/ml) and minimum at lactose and maltose that was 0.75 U/ml. An experiment was designed to investigate the effect of different carbon sources on protease production by Bacillus species. The result in fig. Shows that the best carbon source for protease production was fructose. When the *Bacillus* species used fructose as a carbon source, the protease production reaches to the maximum. While the other carbon sources gave weak or loss protease production.

Table 3 Effect of incubation period on protease production

Sr.No.	Incubation period (hrs)	Absorbance at 520 nm	Enzyme activity (U/ml)
1	18	0.054	0.29
2	22	0.092	0.49
3	24	0.135	0.72
4	48	0.085	0.45
5	72	0.075	0.40

Table 4: Effect of carbon sources on protease production.

Carbon sources	Absorbance at 520 nm	Enzyme activity (U/ml)
Glucose	0.182	0.97
Fructose	0.229	1.23
Starch	0.19	1.02
Lactose	0.115	0.61
Sucrose	0.16	0.86
Maltose	0.14	0.75
Glycerol	0.19	1.02

Table 5: Effect of nitrogen sources on protease production.

Sr.No.	Nitrogen sources	Absorbance at 520 nm	Enzyme activity (U/ml)
1	Peptone	0.289	1.55
2	Glutamic acid	0.190	1.02
3	Ammonium sulphate	0.040	0.21
4	Ammonium chloride	0.02	0.10
5	Urea	0.225	1.20
6	Ammonium nitrate	0.145	0.77
7	Ammonium citrate	0.092	0.49
8	Sodium nitrate	0.179	0.96

Nitrogen sources:

Table 5 Showed the results of different nitrogen sources in relation to protease production by Bacillus species. Different organic and inorganic nitrogen sources were used. The best nitrogen source for protease production was peptone (1.55 U/ml) and minimum at ammonium chloride (0.10 U/ml).

Effect of pH :

The result represented in fig. Indicated that the maximum production of protease was recorded in a medium at pH 8 that was 1.34 U/ml.

Incubation temperature:

The effect of different incubation temperatures on protease production by Bacillus species was carried out by incubating asset of inoculated flasks at 20, 25, 30, 35, 40, 45, 50ºc. The results of the effect of different incubation temperatures on production of protease represented in fig. The maximum biosynthesis of protease was recorded at 40°C that was 1.31 U/ml.

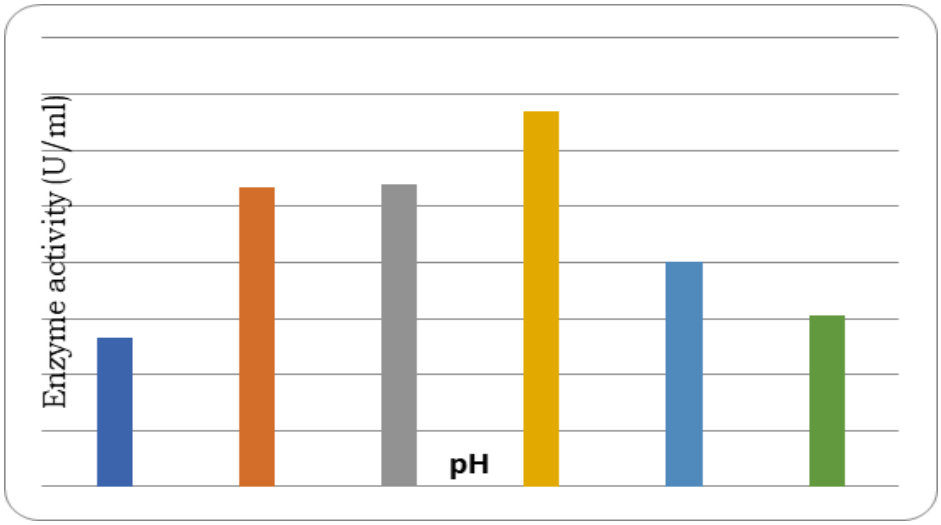

Fig 2: Effect of pH on protease production

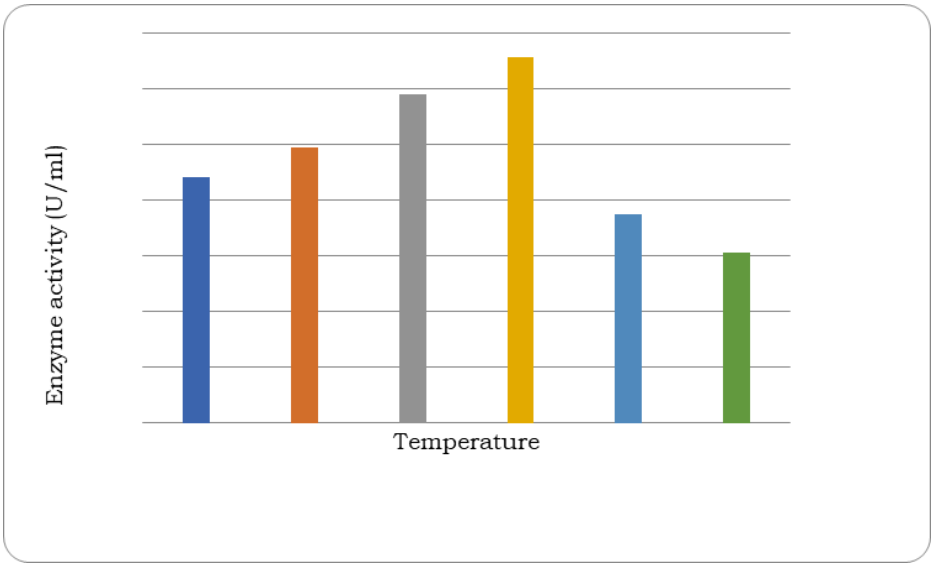

Fig 3 : Effect of temperature on protease productio

4. Discussion:

The number of enzymes secreted by various strains of *Bacillus* species includes amylase, proteases, levansucrase, alkaline phosphotase. Data presented here show that Bacillus species produces an extracellular protease. The optimal conditions for protease production have been fully detrmined under bench scale fermentation conditions.

Our results indicated that the optimum incubation period for protease production was 24 hrs. This result is in complete with finding of many investigators [11]. However, Abdul-raouf reported that Bacillus exhibited their maximum ability to biosynthesize protease within 24 hrs incubation period since the productivity reached up to 126.09 U/ml [12]. Moreover, Johnvesly et al., found that found that protease activity was observed after 24 hrs [13].

Different carbon sources were used for optimization of protease production. The maximum protease productivity was in the presence of fructose as a carbon sources. Yan, et al. studied the effect of carbon sources on the production of protease by Bacillus subtilis growing in shrimp and crab cell powder medium containing one of the additional carbon sources; glucose, lactose, or arabinose and rice bran [14]. They found that protease production was greatly enhanced by the addition of lactose or arabinose in to the medium and that 1% (w/v) arabinose was the most effective substrate and concentration for protease production. Moreover, Aderibigbe et al., found that the protease production reached to the maximum when added D-glucose to the medium especially when used at low concentration (30g/l) [15]. However, Beg et al., recorded that the sucrose was good substrate for production of extracellular proteases. Actually, the production of two extracellular proteases, an endopeptidase and aminopeptidase, by the marine bacterium. Different nitrogen sources were used for protease production. The maximum protease production was in the in the presence of peptone as a nitrogen source [16].

Our results indicated that the optimum temperature for protease productivity by Bacillus species was 40°C. Many investigators study the relation of temperatures and protease production the temperature ranging from 2-70°C or more all depends on the type of organism, the medium conditions and the type of enzyme. In addition to that, the optimum temperature for protease production was between 30 and 45°C [17].

The production medium was adjusted it different pH values of different buffers. Results indicated that the best pH for production of protease was at pH 8.0 with protease productivity 5 U/ml. In view of the data of the other investigators, John vesly et al, reported that, a high level of extracellular protease production by isolated bacterial species at various physico-chemical parameters [18].

5. Conclusion

The present investigation was focused on the protease producing bacterial strain from the dry climatic situations because most of the research were completed on the optimum growth conditions and climate. Hence the isolated *Bacillus* species from the Bhokar region was a better for showing the proterolytic activity in the waste management process.

Conflict of interest

No conflict of interest influenced in this research.

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