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Alkaline Protease Production from *Sorghum vulgare* by *Staphylococcus sciuri*

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Nine bacterial strains were isolated from the waste water collected from slaughter house of Sadra village, Gujarat, India. Among the isolates, one potent strain was selected on the basis of diameter of zone of clearance and enzyme activity. The strain was identified on the basis of microscopic, cultural and biochemical tests as *Staphylococcus sciuri*. Biochemical tests were performed by VITEK® 2 Systems Version: 05.04 at Supra Tech laboratory, Ahmedabad. Some fundamental parameters like effect of raw materials, substrate concentration, pH, temperature, incubation time and inoculum size, nitrogen and carbon sources for maximum protease production were also studied. Maximum yield of enzyme was obtained at pH of 9.0 with 3 mL inoculum size in the media containing 2% stem of *Sorghum vulgare* (jowar) as substrate, after 24 h of incubation in environmental shaker maintained at a temperature of 25ºC along with maltose as carbon source and beef extract as nitrogen source. The study show that the *Staphylococcus sciuri* can efficiently produce alkaline protease using stem of *Sorghum vulgare*.

Key words: Enzyme production, Plant waste, Bacterial isolate, Optimization of enzyme.

Proteases are biocatalysts used in a variety of industrial processes and on an estimate they share 60% of the total enzyme market.[1-3] These proteases hydrolyze peptide bonds and hence they are classified as hydrolases and categorized in the subclasses peptide hydrolases or peptidases.[4] Proteases are found in a variety of plants, animals and microorganisms.[5] Production of proteases from plant and animal sources is not economic because their growth depends on a number of uncontrollable environmental factors. On the other hand use of microorganisms is a cheaper one because we can control and optimize the production of protease here. A number of bacterial and fungal species are being used in many industrial processes to produce proteases. Proteases may be neutral, acid or alkaline in nature and among them alkaline proteases (E.C.3.4.21-24,99) are widely used in industries like detergent producing, tanning, textile and dairy industries, organic synthesis, peptide synthesis, instant recovery of silver from photographic plates and waste water treatment.[6] On an estimate alkaline proteases shares 60 to 65% of the global industrial enzyme market.[7-9] Present study is an attempt to use plant source *Sorghum vulgare*, commonly known as jowar in India for production of alkaline protease using isolated strain of *Staphylococcus sciuri*.

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MATERIALS AND METHOD

Sample collection and isolation of proteolytic bacteria

The waste water sample was collected from slaughter house of Sadra, Gujarat, India and processed immediately in the laboratory for screening of alkalophilic microorganisms. Sample collected was serially diluted (up to 10^{-3}) in sterile

distilled water and an aliquot of 0.1 mL was spread on casein agar plates (nutrient agar with 1% casein having pH adjusted to 8.0). The inoculated casein agar plates were then incubated at 25°C and 37°C for 24 hours. The isolates were identified based on their microscopic, colonial and biochemical characteristics.

Screening of proteolytic bacteria

Individual bacterial colonies isolated above were further screened for proteolytic activity on casein agar medium containing ingredients (g/L)- nutrient broth- 13.0; agar powder- 30.0; casein powder- 10.0. The pH of the medium was adjusted at 8.0 using 1N KOH. Individual colonies were spot inoculated and were incubated at 25°C for 24 hours. The plates were flooded with 25% TCA solution and incubated for 15 min at 45°C to observe zone of clearance properly.

Quantitative assay for alkaline protease

Preparation of crude enzyme extracts

Each isolate was inoculated in 100 mL of assay medium (Nutrient broth + 1% Casein) having pH 8.0 and incubated at 25°C for 24 hrs in environmental shaker at 100 rpm. After incubation the broth cultures were subjected to centrifugation at 5000 rpm for 20 minutes in a Remi centrifuge (R-23). The supernatant obtained was used to determine the amount of the extracellular protease released into the assay medium.

Enzyme assay method

Proteases activity was determined by a modified method.[10] The reaction mixture containing 1 mL of 1.0% casein solution in 0.05 M Glycine-NaOH buffer having pH 10.0 and 0.5 mL of a given enzyme solution were incubated at 60°C for 15 minutes and the reaction was then stopped with 3.0 mL of 5% trichloroacetic acid. The mixture was allowed to stand for some time and was then centrifuged at 3000 rpm for 10 min. 1.0 mL of the filtrate was then diluted with 5.0 mL of 0.4 M Na_2CO_3 followed by addition of 1.0 mL of FCR and 30 min incubation in dark, respectively. After incubation, the absorbance of the liberated tyrosine in the filtrate was measured against a blank (non-incubated sample) at 660 nm. One proteolytic unit was defined as the amount of the enzyme that released 1µg of tyrosine under the assay conditions. Standard curve of tyrosine was prepared using 20, 40, 60, 80 and 100 µg/mL tyrosine.

Optimization for production of alkaline protease

Effect of substrates on enzyme activity

To find out the suitable substrate for protease production, fermentation medium was supplemented with different substrates such as stem of mango (*Mangifera indica*), stem of jowar (*Sorghum vulgare*), stem of millet (*Pennisetum glaucum*), stem of babul tree (*Acacia nilotica*), stem of cotton (*Gossypium spp.*), pod of golden shower (*Cassia fistula*) and stem of eranda (*Ricinus communis*). The media were autoclaved, inoculated and incubated at 25°C in environmental shaker at 100 rpm for 24h.

Effect of substrate concentration on enzyme activity

The most suitable concentration of substrate was determined by addition of substrate at varying concentration in the culture medium. In the experiment, fermentation was carried out with 1% to 5% substrate concentration and other optimized parameters were kept constant and enzyme activity measured after fermentation by performing enzymatic assay.

Effect of pH on enzyme activity

The pH of fermentation medium is one of the important parameter affecting protease production. In present experiment protease production was checked at different pH values of 7.0, 8.0, 9.0, 10.0 and 11.0. The flasks were incubated at 25°C for 24 h at 100 rpm on environmental shaker. After fermentation, protease activity was estimated and optimum pH value was determined.

Effect of temperature on enzyme activity

Temperature is the key factor which regulates the protease production. To determine the effect of various temperatures on protease production, the optimized parameters were kept constant and the flasks were incubated at temperature 25, 30, 35, 40 and 45°C at 100 rpm for 24 h in shaker. After fermentation, enzyme assay was done for each fermentation medium incubated at different temperatures.

Effect of inoculum size on enzyme activity

As described earlier, inoculum was prepared. All optimized parameters were kept constant. Inoculum size ranges from 1% to 5% were inoculated in N-broth containing substrate and incubated for 24 h in environmental shaker at 100rpm at 25°C. Then samples were analyzed for

protease production and selected the proper inoculum size.

Effect of incubation time on enzyme activity

Optimization of incubation time was performed to determine the time required for the maximum protease productions. So, the fermentation medium was incubated at 25°C temperature at 100 rpm in environmental shaker for 24 h. At regular time interval (24 h) that was starting from 24 h to 72 h, 5.0 mL of medium withdrawn and analyzed for protease production by protease assay method. Optimum incubation time was found out.

Effect of nitrogen source on enzyme activity

To obtain the best nitrogen source for protease production, different nitrogen sources used were beef extract and yeast extract at different concentration. The other components were remained constant. Here, nitrogen sources were added at different concentrations i.e. 0.15 %, 0.30 %, 0.45 %, and 0.60 %. Inoculated culture media were incubated in environmental shaker at 100 rpm. The enzyme activity was checked and one nitrogen source which gives highest enzyme production was selected.

Effect of carbon source on enzyme activity

Carbon source like glucose, lactose, sucrose, xylose and maltose at 1% concentration were used. Fermentation media prepared, inoculated and incubated in environmental shaker at 100 rpm. The samples were analyzed for enzyme activity.

RESULTS AND DISCUSSION

Screening and selection of protease producers

On the basis of diameter of zone of clearance on casein agar plate containing 1% casein, nine different isolates were selected. Enzyme activity of each of nine isolates was measured and the isolate exhibited highest activity was screened out. Further this alkaline protease positive isolate was maintained as pure culture in slants in refrigerated conditions at 4°C.

Quantitative assay tests conducted for protease production resulted in highest activity from *Staphylococcus sciuri* out of all the positively screened alkaline proteolytic bacteria (Table 1). Microscopic and colonial characterization revealed that the isolate was a gram +ve *cocci* which was further identified to be *Staphylococcus sciuri* on the basis of biochemical tests performed by VITEK® 2 Systems Version: 05.04 at Supra Tech laboratory, Ahmedabad.

Effect of substrates

Among different experimental substrates used stem of *Sorghum vulgare* were found suitable for microbial action and with this substrate highest enzyme activity was obtained (Table 2). Suitability of substrates for microbial action depends on a number of factors including composition and

Table 1. Protease activities of isolates

Culture	Optical density at 660 nm	Enzyme activity (U/mL)
K_1	0.017	1.75
K_2	0.008	0.82
K_3	0.009	0.92
K_4	0.005	0.51
K_5	0.011	1.13
K_6	0.021	2.16
K_7	0.018	1.85
K_8	0.019	1.95
K_9	0.041	4.21

Table 2. Effect of different substrates on protease production by *Staphylococcus sciuri*

Name of substrate	Optical density at 660 nm	Enzyme activity (U/mL)
Stem of *Mangifera indica*	0.124	13.2
Stem of *Pennisetum glaucum*	0.078	8.07
Stem of *Sorghum vulgare*	0.227	24.93
Stem of *Acacia nilotica*	0.079	8.43
Stem of *Gossypium spp.*	0.008	0.82
Stem of *Cassia fistula*	0.026	2.57
Stem of *Ricinus communis*	0.028	2.93

nature of substrate, its physical properties, bonding etc.

Effect of substrate concentration

For the maximum production of extracellular protease, organism requires proper concentration of substrate. So, to obtain highest protease production, different concentration of the grinded stem of *Sorghum vulgare* was used. It was observed that enzyme activity was increased up to **5.13 U/ml**, at 2% as shown in Table 3. This study showed that application of stem of *Sorghum vulgare* as crude protein source influence the yield of protease. Sometime protein may inhibit enzyme synthesis because of feedback inhibition. Maximum protease production by *Bacillus subtilis* at 2.09 % (w/v) substrate concentration was also obtained previously.[11]

Effect of pH

The pH of fermentation medium affect the production of enzyme by *Staphylococcus sciuri*. As the pH increases from neutral to alkaline range the enzyme production increases, reaches maximum at pH 9.0 and then decreased (Table 4). Previously highest protease production at pH 9.0 was also reported using *Staphylococcus aureus* [12] and *Bacillus cereus* MCM B-326.[13]

Effect of temperature

Temperature is a critical parameter which affects microbial enzyme production. Temperature of 25^{0}C was found optimum for the production of protease by *Staphylococcus sciuri* (Table 5) and the activity of enzyme decreased with increased fermentation temperature. Activities of extracellular enzyme depends on its secretion from microbial cell. Permeability of cell membrane is being affected by temperature.[14] Our results reveal that highest cell permeability of *Staphylococcus sciuri* cells may be at 25^{0}C temperature. Conformation changes in enzyme may take place at higher temperature which may result in their decreased activity.[15] High protease activity at optimum temperature of 25^{0}C in case of *Bacillus circulans* has been reported previously.[16]

Effect of inoculum size

Nutrient absorption by microbial cells depends on the ratio of their density to the amount of available nutrients. As this ratio disturbs, it affect the activity of microorganisms. Present study show that for given amount of fermentation broth 3ml inoculum size was optimum. Increase or decrease in inoculum size reduces the activity of enzyme (Table 6). Importance of inoculum size was also

Table 3. Effect of different concentrations of *Sorghum vulgare* on protease production by *Staphylococcus sciuri*

Substrate concentration (%)	Optical density at 660 nm	Enzyme activity (U/mL)
1	0.032	2.93
2	0.051	5.13
3	0.049	4.95
4	0.045	4.77
5	0.013	0.73

Table 4. Effect of pH on protease production by *Staphylococcus sciuri*

pH	Optical density at 660 nm	Enzyme activity (U/mL)
7	0.001	0.10
8	0.127	13.04
9	0.138	14.17
10	0.134	13.78
11	0.002	0.21

Table 5. Effect of temperature on protease production by *Staphylococcus sciuri*

Temp (^{0}C)	Optical density at 660 nm	Enzyme activity (U/mL)
25	0.138	14.17
30	0.061	6.26
35	0.024	2.2
40	0.008	0.82
45	0.00	0.00

Table 6. Effect of different inoculum sizes on protease production by *Staphylococcus sciuri*

Inoculum size (mL)	Optical density at 660 nm	Enzyme activity (U/mL)
1	0.020	2.05
2	0.026	2.2
3	0.267	29.33
4	0.054	5.13
5	0.01	0.73

reported previously.[17] At lower percentage of inoculum size, insufficient number would lead to reduced amount of secreted protease. At higher percentage of inoculum size, protease activity was found to be decrease even though luxurious growth was observed and it may be due to reduced dissolved oxygen and increased competition towards nutrient. Our results are in accordance with previous work.[18]

Effect of incubation time

Protease production was monitored up to 72h of incubation. Samples were withdrawn at regular intervals of 24h for analysis of enzyme activity. The highest protease activity was observed at 24h with optimum activity **15.03 U/mL** (Table 7). A gradual decrease in enzyme activity was observed with increased incubation time. The duration needed for incubation might depends on the growth rate of microbes and its enzyme production might be due to depletion of nutrient of fermentation medium with time. Maximum protease activity at 24h incubation by *Staphylococcus aureus*[12] and *Pseudomonas fluorescens* were reported previously.[19]

Table 7. Effect of incubation time on protease production by *Staphylococcus sciuri*

Incubation time (h)	Optical density at 660 nm	Enzyme activity (U/mL)
24	0.138	15.03
48	0.081	8.43
72	0.081	8.43

Table 8. Effect of different nitrogen sources on protease production by *Staphylococcus sciuri*

Nitrogen source	Concentration (gm)	Optical density at 660 nm	Enzyme activity (U/mL)
Beef extract	0.15	0.032	2.93
	0.30	0.031	2.93
	0.45	**0.131**	**13.93**
	0.60	0.035	3.67
Yeast extract	0.15	0.032	2.93
	0.30	0.008	0.82
	0.45	0.015	0.73
	0.60	0.005	0.36

Table 9. Effect of different carbon sources on protease production by *Staphylococcus sciuri*

Carbon source	Optical density at 660 nm	Enzyme activity (U/mL)
Glucose	0.040	4.4
Lactose	0.046	4.77
Sucrose	0.031	3.18
Xylose	0.029	2.98
Maltose	0.092	9.5

Effect of nitrogen sources

Nitrogen is required for growth of microbial cells. It can be supplied in medium through various sources. Uptake of nitrogen from these sources by microbial cells depends on a number of factors among which the most important is the nature of source. Present study show that cells of *Staphylococcus sciuri* prefers beef extract over yeast extract and gives maximum enzyme activity at 0.45g concentration of beef extract (Table 8). Maximum enzyme activity under beef extract was also reported previously.[20,21]

Effect of different carbon sources

The carbon sources were supplemented to the medium at 1% concentration. To determine the effect of different carbon source on protease production, we have supplemented five different carbon sources i.e., glucose, lactose, sucrose, xylose and maltose. Then the protease activity was measured. Results showed that maximum protease activity **(9.5 U/mL)** obtained, when maltose was used as source of carbon (Table 9). Moderate protease activity was observed in case of lactose (4.77 U/mL) and glucose (4.4 U/mL). Xylose and sucrose were very less effective as carbon source.

Superiority of maltose over other carbon sources for maximum enzyme activity was also concluded in earlier works.[18,22,23]

CONCLUSION

The present study investigated the suitable substrate and the optimum cultural conditions i.e., substrate concentration, temperature, pH, inoculum size and time, suitable carbon source and nitrogen source for maximum protease production. Protease producing bacteria *Staphylococcus sciuri*, isolated from slaughter house waste water of Sadra, Gandhinagar, preferably utilizes stem of jowar (*Sorghum vulgare*) and produces maximum protease in optimum conditions of pH 9.0, temperature 25°C, incubation time 24 h, inoculum size 3 mL, substrate concentration 2%, beef extract as nitrogen source and maltose as carbon source.

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