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Optimized Alkaline Protease Production by *Bacillus thuringiensis*

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In a laboratory study 18 different types of bacteria were isolated from soil showing protease production in 1% casein agar medium. After screening, one potent bacterial culture was identified as *Bacillus thuringiensis* and its protease production was further optimized for various fermentation conditions. Results show that the culture gives maximum alkaline protease production under optimum condition of pH- 11.5 (40.7 U/mL), temperature- 25ºC (60.13 U/mL), substrate concentration- 3% (33.54 U/mL) inoculum size - 1mL (v/v) with optimum activity 60.13 U/mL, incubation time- 24 hr (60.13 U/mL), 0.30% beef extract as nitrogen source (61.23 U/mL), and 1% lactose as carbon source (37.77 U/mL).

Key words: *Bacillus thuringiensis*, alkaline protease, pods of *Cassia fistula*.

Protease is a group of enzymes having wide industrial applications. They are produced from plants, animals and microorganisms but among them proteases from microbial sources are preferred. They possess almost all the characteristics desired for biotechnological applications like rapid growth and less space required for cell cultivation and ease with which the enzyme be genetically manipulated to generate new enzyme for various applications.[1,2] A number of bacteria, fungi and yeast have been reported for the protease production. Many of the organisms produced more than one kind of proteases.[3-5] Culture conditions affects microbial growth and production of protease.[6]

Among bacteria the genus *Bacillus* contains a number of industrially important species and on an estimate it is being said that approximately half of the present commercial production of bulk enzymes derives from the strains of *Bacillus sp*.[7,8] These strains specifically produces extracellular proteases having wide industrial applications and furthermore these strains gives stable production in extreme environmental conditions [9,10]

After species the second most important factor in production process is substrate. Use of easily and abundantly available source will make the production process economic. In present study *Bacillus* strains were isolated from soil, screened and optimized to produce alkaline protease from pods of *Cassia fistula*. Being a leguminous plant pods of *Cassia fistula* are good source of protein. The main objective of present research was to obtain maximum alkaline protease from the source.

MATERIALS AND METHODS

Isolation of alkaline protease producers

Kitchen waste contaminated soil was collected locally, serially diluted in sterilized distilled water and inoculated on casein agar plates then incubated at 37^{0}C for 24 hr. Obtained different colonies were purified on same medium.

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Screening of the potent alkaline protease producing bacteria

On the basis of diameter of zone of clearance and enzyme activity, the potent bacteria was screened. To obtain zone of clearance individual colonies were spot inoculated and were incubated at 37^{0}C for 24 hours. After incubation casein agar plates were flooded with 25% TCA and incubate at 45^{0}C for 15 min then zone of colony was measured. The enzyme activity was estimated as per the standard method.[11]

Quantitative assay for alkaline protease

Preparation of crude enzyme extracts

After complete of incubation, 5mL broth from flask was centrifuged at 5000 rpm for 20 minutes and obtained clear supernatant was used to determine the amount of the alkaline protease released into the assay medium.

Assay procedure

The protease activity was measured by standard method[11] with some modifications. 0.5mL of glycine NaOH buffer (pH 11.5, 0.2 M) was added to 0.5 mL of enzyme in test and was incubated with 1 mL of 1% casein solution as substrate (prepared in glycine NaOH buffer, pH 11.5) for 15 min at 60°C. The reaction was stopped by the addition of 3 mL of 5% (v/v) trichloroacetic acid (TCA). Then 0.5 mL of enzyme was added in blank. The contents were centrifuged at 3000 rpm for 10 min and the supernatant was used for measuring protease activity on the basis of color change. 5 mL of 0.4 M sodium carbonate solutions was added to 1mL of the supernatant. Folin - Ciocalteau Phenol reagent of 1:1 dilution was added and kept in dark for 30 min. The color change was determined at 660 nm and calculated the amounts of aminoacid released from a standard curve plotted from known concentration of tyrosine. The enzyme activity was expressed in unit. 1 Unit=1µg/mL/minute.

Collection of substrate

Different agricultural wastes and pods of *Cassia fistula* was collected from Sadra, Gandhinagar.

Optimization of cultural parameters for alkaline protease production

To find out the optimum fermentation conditions at which the used bacterial species gives maximum alkaline protease production, the selected bacterial species was inoculated and incubated in various substrates (Stems of *Pennisetum glaucum*, *Sorghum vulgare*, *Gossypium spp* and *Ricinus communis* and Pods of *Cassia fistula*) at static or agitatic conditions at various range of pH (10 to 12), temperature (25^{o}C, 35^{o}C and 45^{o}C), inoculum size (1mL to 5mL), incubation time (24 hr, 48 hr and 72 hr), substrate concentration (1% to 5%), nitrogen source (beef extract and yeast extract, each at 0.15, 0.30, 0.45 and 0.60%) and carbon source (glucose, lactose, sucrose, maltose and xylose, each at 1.0%). During optimization of one parameter all other parameters were kept constant. After growth activity of alkaline protease was measured in all the parameters.

RESULTS AND DISCUSSION

Screening and identification of maximum alkaline protease producing bacteria

Screening and selection of potent bacterial species was done on the basis of diameter of zone of clearance and enzyme activity. Although Eighteen bacterial isolates showed zone of clearance but only seven isolates showed measurable amount of alkaline protease (Table 1). Strain M_{16} showing maximum enzyme activity was chosen for further study and identified on the basis of morphological and biochemical tests carried out at Supra-Tech Laboratory, Ahmedabad by VITEK 2 Systems Version: 05.04. This strain was identified as *Bacillus thuringiensis*.

Effect of different substrates on alkaline protease production

Different substrates produces different amount of enzyme activity because it depends on many factors. Being a good source of protein *Bacillus thuringiensis* utilizes pods of *Cassia fistula* efficiently and produces maximum amount of

Table: 1 Alkaline protease activity in different isolated bacterial strains

Culture	Optical density (660nm)	Enzyme activity (U/mL)
M_9	0.021	2.2
M_{12}	0.049	5.13
M_{13}	0.018	1.85
M_{14}	0.059	6.23
M_{15}	0.019	1.95
M_{16}	0.130	14.67
M_{18}	0.041	4.4

enzyme activity in this treatment (Table 2).

Effect of various fermentative conditions on alkaline protease production

After comparisons of results of protease activity of agitating and static condition, we observed that the maximum activity was obtained in agitating condition (40.7 U/mL) than static condition (5.87 U/mL) (Table 3). Cell growth and product formation depends on availability of nutrients and oxygen to cell. In many cases nutrients remains present in medium but if cells can not reach to them then they can't be utilized by cell. This problem can be solved by giving agitation. Due to agitation nutrient availability and oxygen transfer rate to cell increases[12,13]. Higher nutrient and oxygen availability to cells in agitation conditions may result in higher enzyme activity compared to static one.

Table 2. Alkaline protease activity in different substrates

Different substrate	Optical density (660nm)	Enzyme activity (U/mL)
Stems of *Pennisetum glaucum*	0.005	0.51
Stems of *Sorghum vulgare*	0.00	0
Pods of *Cassia fistula*	0.100	11
Stems of *Gossypium spp.*	0.033	3.6
Stems of *Ricinus communis*	0.060	6.6

Table 3. Effect of physical parameter on alkaline protease production by *Bacillus thuringiensis*

Fermentation conditions		Optical density (660nm)	Enzyme activity (U/mL)
AgitationConditions			
	Static	0.054	5.87
	Agitatic	0.368	40.7
pH	10	0.117	12.83
	10.5	0.208	23.46
	11	0.208	23.46
	11.5	0.368	40.7
	12	0.068	7.3
Temp.	25	0.545	60.13
	30	0.398	43.27
	35	0.368	40.33
	40	0.089	9.53
	45	0.072	7.7
Substrate concent-ration	1%	0.162	17.6
	2%	0.171	19.07
	3%	0.284	33.54
	4%	0.118	13.2
	5%	0.034	3.67
Inoculum size	1 mL	0.545	60.13
	2 mL	0.385	42.53
	3 mL	0.266	29.33
	4 mL	0.240	26.4
	5 mL	0.225	24.93
Incubation time	24 hr	0.545	60.13
	48 hr	0.406	44.73
	72 hr	0.021	2.2

Enzymes are amphoteric in nature and contain acid and basic groups of molecules on its surface. The pH of medium in which the enzyme exists affect these groups and with variation in pH charges of these groups also varies which affects their activity[14]. Due to this a variation in enzyme activity was observed with pH range (Table 3) and the optimum pH at which maximum activity was found was 11.5. Maximum alkaline protease activity, produced by *Bacillus sp.* at pH 11.5 has also been reported earlier.[15,16] .

Incubation temperature also affects activity of enzyme. Temperature affects the physical properties of cell membrane and concentration of dissolved oxygen in medium. [17,18] Variation in enzyme synthesis and metabolism with temperature and oxygen uptake has also been reported.[19] Optimum activity of alkaline protease produced by *Bacillus thuringiensis* was found at 25^{o}C temperature and decreased with higher temperature which may be due to denaturation of enzymes at higher temperatures. *Bacillus circulans* has also been reported to give higher alkaline protease activity at 25^{o}C temperature .[20]

Amount of substrate added to the fermentation medium should also be optimum and

Table 4. Effect of nutrient sources on alkaline protease production by *Bacillus thuringiensis*

Chemical parameter		Concentration	Optical density (660nm)	Enzyme activity (U/mL)
Nitrogen source	Beef extract	0.15%	0.346	37.77
		0.30%	0.558	61.23
		0.45%	0.373	40.33
		0.60%	0.145	16.13
	Yeast extract	0.15%	0.346	37.77
		0.30%	0.321	35.20
		0.45%	0.322	35.27
		0.60%	0.292	31.90
Carbon source	Glucose	1.0%	0.068	7.30
	Lactose	1.0%	0.346	37.77
	Sucrose	1.0%	0.215	23.47
	Xylose	1.0%	0.231	25.67
	Maltose	1.0%	0.243	26.40

depends on bacterial uptake. Its concentration should not be less than the requirement of bacteria and higher than it. In some cases it may reduce bacterial growth and activity by feed-back inhibition. The isolated *Bacillus thuringiensis* species would converts a maximum of 3% substrate to product during given incubation time (Table 3).

Similar to substrate, number of bacterial cells added to fermentation medium should also be optimum otherwise the substrate can not be converted fully into product. In present experiment 1mL inoculum amount was found optimum to give maximum enzyme activity (Table 3). Amount of inoculum affects the surface area to volume ratio in medium and higher ratio at lower inoculum volume may give higher activity.[17] Higher inoculum volume may increase competition for nutrients between bacteria and reduce dissolved oxygen which results in lower activity of protease.[21]

Microorganisms should be given optimum time required for growth and to convert substrate into product. Isolated *Bacillus thuringiensis* species in present experiment requires 24hrs to produce maximum alkaline protease from pods of *Cassia fistula* (Table 3). At increased incubation time decreased activity of enzyme show that enzyme may become inactive due to either other constituent proteases or due to exhaustion of nutrient.[22] Further, being an extracellular enzyme alkaline protease acts as growth promoting substance in medium which are generally produced at exponential phase of bacterial growth.[23] Higher alkaline protease activity within 24 hr inoculation period has also been reported previously.[24-26]

Effect of nutrient sources on alkaline protease production

Extracellular proteases production is sensitive to repression by different nitrogen sources.[27,28] It has been found that beef extract may enhance the protease production by *Bacillus cereus* strain 146.[29] Our results are in accordance with many workers.[30,31] Maximum activity of alkaline protease was recorded in treatment receiving 0.30% beef extract (Table 4).

Besides nitrogen the other most important nutrient for bacterial growth and activity is carbon and in present study *Bacillus thuringiensis* showed highest enzyme activity (37.77 U/mL), when lactose was used as carbon source (Table 4). These results show that the addition of sugars in medium which can be readily metabolize was not sufficient to stimulate the protease production from *Bacillus thuringiensis.* Superiority of lactose for maximum alkaline protease production has also been reported previously.[32,33]

CONCLUSION

Isolated *Bacillus thuringiensis* successfully produced alkaline protease from pods of *Cassia fistula* and its activity can be enhanced by optimizing various fermentation conditions.

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