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ALKALINE PROTEASE PRODUCTION BY ISOLATED *BACILLUS SP.* IN SUBMERGED & SOLID STATE FERMENTATION

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ABSTRACT

Solid-state fermentations (SSF) are fermentations of solid substrates at low moisture levels or water activitiesSolid-state fermentation has gained renewed attention, not only from researchers but also from industries, due to several advantages over submerged fermentations. This is partly because solid-state fermentation has lower energy requirements, higher yields, produces less wastewater with less risk of bacterial contamination, and partly because of environmental concerns regarding the disposal of solid wastes. This paper reviews The present study objective is to:Study the effect of various parameters on protease production in solid state fermentation by alkaline *Bacillus sp.*Study the effect of various agri wastes as carbon and nitrogen sources.Study the effect of moisture content for optimum production of protease. Study the effect of different pH levels in solid state fermentation Study the protein estimation by Lowry's method.Study the effect of different particle size of the source in solid state fermentation for the protease production.

Keywords: *Solid State Fermentation, Bacillus, Alkaline protease*

Number of Tables : 6 **Number of References: 10**

INTRODUCTION

Solid state (substrate) fermentation (SSF) is generally defined as the growth of microorganisms on (moist) solid material in absence or near – absence of free water (Shuler et al.,2002). OR Solid substrate fermentation is the process in which the substrate itself acts as carbon / energy source, occurring in absence or near – absence of free water. OR It is the process occurring in the absence or near – absence of free water, employing a natural substrate as above or an inert substrate used as solid support.Several authors have reviewed the history, development and scientific elaboration of SSF from time to time. Evidently, food fermentation and production of enzymes were the areas where SSF originated. The recorded history of SSF was described in Asia before the birth of Christ era on cheese making by *Penicilliumrouque fortii*. Egyptians were reported to make bread using SSF process in 2000 BC. The use of Soy Sauce Koji in China was reported in the years 1000 BC and probably in the year 3000 BC (koji process involves fermentation of steamed nice as solid substrate by fungal strain of *Aspergillusoryzae).* Various authors (Macfarlane & Gibson (1993) reported solid fermentation, semi-solid fermentation, surface culture fermentation, Japanese Koji process and bran process for production of enzymes.Bacterial alpha amylase is produced by different fermentation techniques. Solid state fermentation (SSF) has numerous advantages including productivity and may be preferred to submerged fermentation (SmF) due to simple technique, low capital investment, lower levels of catabolite repression and better product recovery. The SSF technique is mainly confined to process involving fungi. It is believed that this technique is not suitable for bacterial cultures because of higher water activity requirements. However, successful bacterial growth by using the SSF technique is known in many natural fermentations.The production of amylase by SSF is limited to the genus *Bacillus* and *B.subtilis, B.polymyxia, B. mesentericus, B,vulgarus, B.licheniformis* have been used for alpha amylase production in SSF. The production of bacterial alpha amylase using the SSFtechnique requires only a fermentation time of 24-48 hours.

Various solid substrates, such as wheat bran, soybean meal etc. have been used in SSF. These substrates were employed individually without any supplementation with other carbon and nitrogen sources. In the SSF process, the solid substrate not only supplies the nutrients to the culture, but also serves as an anchorage for the microbial cells. The moisture content of the medium changes during fermentation as a moisture level of the substrate is therefore most important. Acid-stable alpha amylase and the acid-unstable alpha amylase obtained from *Aspergillusniger*. In both of enzymes acid-stable alpha amylase was more stable in acidic pH. Molecular weights of these two enzymes were calculated from sediment-ation equilibrium, and the molecular shapes were investigated by determining sedimentation coefficient and intrinsic viscosity.

Alkaline proteases production by Solid state fermentation is a better option for economic production of microbial enzymes over classical submerged fermentations because agrowaste material can be used as support as well as substrate. Among all microbes, like *Bacillus* genera are producing good amount of protease in less time.The present study objective is to:Study the effect of various parameters on protease production in solid state fermentation by alkaline *Bacillus sp.*Study the effect of various agri wastes as carbon and nitrogen sources.Study the effect of moisture content for optimum production of protease. Study the effect of different pH levels in solid state fermentation Study the protein estimation by Lowry's method.Study the effect of different particle size of the source in solid state fermentation for the protease production.

MATERIALS AND METHODS

Glucose agar medium was prepared by dissolving 0.75% - Glucose, 1% - peptone, 5 % - salt solution and 2% - agar dissolved in distill water and makeup to 100 ml and adjusted the pH with 1N NaOH. Both medium and empty slant tubes with cotton plugs were autoclaved at 121^0 C, 15 lbs for 15 minutes. The medium was transferred into tubes under sterilized conditions and kept them in slant position and allowed for solidification. They were kept for overnight to check the contamination and the culture was streaked on the slants and was incubated at 32^oC overnight to get to growth. After growth was observed the slants were kept at 4^0C for further use. The culture was grown for 24 hour for using as inoculum.

Fermentation conditions

All the experiments were conducted in 250 ml Erlenmeyer flask containing 10g of Agri residues (wheat bran, green gram, red gram, chick pea and black gram husks) were inoculated with 3 ml of 24 h old culture. The flasks were incubated at 33^0C in an incubator (DK-SI010) for 60 h, samples were withdrawn by suspending in 100ml of glycine NaOH buffer repeated for thrice and were centrifuged at 6000 rpm for 10 minutes and supernatant was used as a crude enzyme for studies.

Protease assay

Alkaline protease activity was determined by the modified method of Kembhavi et.al (1993)1ml of standard stock solution is taken in 10 different test tubes serially and they were make upto 10ml with Glycine-NaOH buffer. In blank 1ml of distill water is taken and made to 10ml with Glycine – NaOH buffer and absorbance of each test tube solution were measured at 280 nm inuv-spectrophotometre (Becman DU 7400).

RESULTS & DISCUSSION

Solid-state culturing of molds has traditionally been limited to polymerized substrates due to their ability to retain water in their porous matrix. Therefore, attempts have been made to grow *Bacillus* sp. on solid supports impregnated with nutritive solutions. In the present investigation, different Agro wastes have been evaluated and based on the results; black gram husk has been chosen as solid medium for protease production by this isolated *Bacillus* sp. Among all agro-wastes sources studied,

black gram waste found to be the best source for protease production. Agro waste type mediated protease production was noticed in this microbial strain. Chick pea husk was found to be inhibitory for protease production. Based on above results further experiments were performed using black gram husk as solid medium for protease production under SSF conditions with this newly isolated *Bacillus* sp (Lee et al., 1991). In terms of thermal stability, this enzyme is superior to the alkaline proteases from an alkilophilic Bacillus sp (Durham et al, 1987) Progressive increase in protease production was noticed with increase in incubation time. Maximum enzyme production was observed at 60 hours of incubation under studied experimental conditions. The enzyme production was found to be growth associated in this bacterial strain. Medium pH found to have regulatory role on protease production. Maximum protease production was noticed at pH 9.0.Variation of pH either sides of this pH regulates negatively on protease production. Relative moisture level found to have an influence on protease production. Parabolic nature of enzyme production curve was observed with increase and decrease in moisture content. The best relative moisture content for enzyme production found to be 100% in this Bacterial strain and solid medium. Particle size of the solid medium influenced the protease production .Maximum protease production was noticed in 14-18# size solid medium. Altering of particle size of above was found to be more detriment production. Initial inoculum level influenced the protease production. Maximum protease production was noticed in 3% inoculum level Increase of inoculum level more than 3% was found to be more detriment production Takashi et al.96 used a new solid state fermentation reactor (SSFR) for the production of Iturin A using Bacillus subtilis from okara as a solid substrate. The new features of this design is that it has two kinds of mixing; they used an ordinary impeller rotating clockwise and the floor of the machine rotates at the same time counter-clockwise, and vice versa, to achieve a higher rate of agitation without needing to use a higher rotation speed. The results show the effectiveness of using SSFR when a comparison was made between the static state and agitation; the production rate in a static condition was low due to the heat generation and temperature rise inside the reactor. The temperature must be maintained within a range of 25–30 °C to have higher production rates. This was achieved through the significant improvement in the manner of agitation. Miron et al.97 used a newly designed bioreactor for the production of glucose oxidase. The bioreactor consisted of a cylindrical tube of glass (16 cm ID and 100 cm L) with a working volume of 20 L, covered with a band of electric resistance to control and maintain the temperature of the bioreactor. Both these sets of data show that immobilized B. mycoides alkaline protease was considerably more stable than the free enzyme, and could be stored for extended periods in both wet and dry forms before use. Various reports confirm that the thermal and storage stability of immobilized protease depend on the applied immobilization methods (Gauthier et al., 1991; Kise & Hayakawa, 1991; Hyndman et al., 1992).

Table 1: *Effect of different Agro wastes as sources on protease production by Bacillus sp. in SSF.*

S. No	Different agro husks	Protease production (ml)
1	Red gram	22,691.34
2	Green gram	43,788.78
3	Wheat bran	31,617.18
4	Black gram	51,584.40
5	Chick pea	13,359.78

Table 2: *Role of incubation time for the production of protease by Bacillus sp. in SS*

S. No	Time (hours)	Protease production (units/ml)
1	12	19,793.34
2	24	20,314.98
3	36	20,894.58
4	48	21,039.48
5	60	21,619.08
6	72	20,981.52
7	84	19,445.58
8	96	5,361.30

Table 3: *Effect of moisture content on protease production by Bacillus sp. in solid state fermentation*

Sno	Water (ml)	Volume of inoculum (ml)	Total moisture content(ml)	Relative moisture content (%)	Protease Activity (units/ml)
1	4	2	6	60	18,634.00
2	6	2	8	80	19,793.34
3	8	2	10	100	22,053.78
4	10	2	12	120	21,474.18
5	12	2	14	140	20,343.96

Table 4: *Effect of different pH on protease production by Bacillus sp. in SSF*

S. No	PH of fermentation Medium	Protease production (ml)
1	8	28,864.08
2	9	30,544.92
3	10	26,342.82
4	11	25,560.36

Table 5: *Effect of different particle size of black gram husk on protease production by Bacillus sp. in SSF*

S. No	Particle size(mm)	Protease production (ml)
1	>7	28,081.62
2	14-18	312, 791.06
3	18-50	29,095.92

Table 6: *Effect of inoculum level on protease production by Bacillus sp. in SSF.*

S. No	Inoculum level (ml)	Protease Activity (ml)
1	1	17,822.70
2	2	20,697.20
3	3	21,329.28
4	4	17,214.12
5	5	16,924.32

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