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Use of Mustard Oil Cake for Protease Production by *Bacillus subtilis*

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ABSTRACT

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A Gram positive, motile, mesophilic *Bacillus subtilis* strain capable of producing extracellular protease was isolated from abattoir waste material which was collected from Wadala local market, Mumbai, India. In this work, the efficiency of mustard oil cake as a substrate for protease production by *Bacillus subtilis* strain in submerged fermentation (SmF) has been studied. The mustard oil cake was selected because it is rich in protein, cheap, abundantly available being an agro-industrial waste and can be constructively used as one of the main constituents in medium formulation. Different physical and chemical parameters such as pH, temperature, substrate concentration and incubation time were optimized. The maximum protease activity was found at medium pH of 11 containing 4% mustard oil cake at 40°C, after 70 hours of submerged fermentation. Further, the growth curve studies indicated that the production of extracellular protease was initiated in the early lag phase and was maximum in the late exponential phase.

Introduction

These enzymes mainly function in a narrow range of pH, temperature, and ionic strength. Moreover, the technological application of enzymes under demanding industrial conditions makes the currently known enzymes ineffective. The microorganisms from diverse and exotic environments called as extremophiles, are an important source of enzymes, whose specific properties are expected to result in novel process applications (Kumar and Takagi, 1999).

Enzymes have significant advantages over chemical catalysts of which the most important is specificity, high catalytic activity, ability to work at moderate as well

as extreme temperatures and the ability to be produced in large quantities. Proteases (proteinases, peptidases or proteolytic enzymes) are enzymes that break peptide bonds between amino acids of proteins. Proteases represent one of the three largest groups of industrial enzymes and account for about 60 % of the total worldwide sale of the enzymes. The proteases execute a large variety of functions extending from cellular level to organ and organism level, to produce cascade systems such as homeostasis and inflammation (Rao *et al.*, 1998).

Microbial proteases have tremendous potential to offer to the mankind. Proteases

are widely used in several industrial sectors such as detergent, food, pharmaceutical, chemical, leather, silk, cleaning contact lenses/denatures, dewooling of animal skin, recovery of soluble proteins and amino acids from chrome leather wastes and waste treatment etc (Malathi and Chakraborty, 1991; Rajkumar *et al.*, 2011). Proteases play a critical role in many physiological processes including protein catabolism, blood coagulation, cell growth and migration, tissue arrangement, morphogenesis in development, inflammation, tumor growth and metastasis, activation of zymogens (Delepelaire and Wandersman, 1989), release of hormones and pharmacologically active peptides from precursor proteins and transport of secretory proteins across membranes (Rawlings and Barrett, 1993).

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. They are essential constituents of all forms of life on earth, including prokaryotes, 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.

Enzymes find its application in almost all branches. Today most applications for industrial enzymes are found in food industry i.e. protease for tenderization of meat and improvement to the solubility, emulsification, gelling if foaming properties of various other foods. In the wine industry the use of pectinase preparation is permitted to increase juice yield.

The detergent industry is currently largest user of industrial enzymes and employs protease, lipase, amalyase and cellulases

predominantly in the production of biological washing powder. These enzymes quickly breakdown and release the dirt that normally can be removed at much higher temperatures or by using large quantities of chemical detergents over a longer time period.

The use of enzymes in textile industry is one of the most rapidly growing field in industrial enzymology (Mojsov, 2011). The textile industry has been condemned as being one of the world's worst offenders in terms of pollution because it requires a great amount of chemicals. As many as 2,000 different chemicals are used in the textile industry to aid transfer of colourants and finishing agents using water which is a finite resource and quickly becoming scarce. In every step in textile processing water is not only used to transfer the actives on the textiles but also to wash them out before beginning the next step. The water becomes full of chemical additives and is then expelled as wastewater; which in turn pollutes the environment. Now-a-days enzymes are becoming an integral part of the textile processing due to increased awareness about the environmental concerns and related regulations as the enzymes are biodegradable and work under mild condition, saving the precious energy.

Protease is one such enzyme which can be applied in textile industry to remove stiff and the dull gum layer of sericin from the raw silk to achieve improve luster and softness. Protease treatment can modify the surface of wool and silk fibers to provide new and unique finishes (Doshi and Shelke, 2001).

From environmental as well as economical point of view, there is growing research interest to explore use of waste sources as nutrient for medium formulation in

production of various enzymes. Conversion of wastes into useful biomass by microorganism and their enzymes is a new trend. Crop residues such as bran, husk, bagasse and fruit seeds are utilized as a potential raw material in bioprocesses as they provide an excellent substrate for the growth of microorganism supplying the essential nutrients to them (Ramachandran *et al.*, 2007).

Oil cakes are by-products obtained after oil extraction from the seeds. Many scientists have worked i.e. (Rekha *et al.*, 2012) describing production of lipase enzymes using ground nut oil cake and linseed oil cakes as substrate in solid state fermentation (SSF). Oil cakes are ideally suited nutrient support in SSF rendering both carbon and nitrogen sources, and are reported to be good substrate for enzyme production.

In extracellular protease production, use of solid substrate such as soya bean meal (Miller *et al.*, 1967), rice bran, wheat bran, ground nut oil cake, gingili oil cake, coconut oil cake (Bhaskar *et al.*, 2008) and green gram husk (Kumar *et al.*, 2008) has been reported as culture medium ingredient.

Among the various agro wastes, Mustard oil cake (MOC) contains two major components; protein 38.5% and fiber 3.5%. Amino acid profile of MOC shows them to be rich in leucine, phenylalanine, arginine and glycine (Johnvesly *et al.*, 2002). This solid substrate not only supplies the nutrient to the culture but also serves as an anchorage for the microbial cells, where cost and availability are important considerations (Gattinger *et al.*, 1990).

The majority of the waste in the meat industry is produced during slaughtering. Slaughter house waste consist of the portion of a slaughtered animal that cannot be sold

as meat or used in meat- products. These waste are most of the time dumped to degrade. We have used such degraded waste for our study, as it is available freely and in large amount. Moreover, it is inexpensive source, as it is a waste one (Badhe *et al.*, 2016).

Under this study the optimization for the maximum protease production by *B. subtilis* in solid state flask (SSF) cultures is attempted. The optimization of the SSF system was based on the medium composition and establishment of cultural conditions using mustard oil cake as substrate. Augmenting the productivity of protease in solid state culture may be of great benefit as the lower production cost of protease could lead to new industrial applications.

Materials and Methods

Mustard oil cakes were obtained from a Narayana town in Rajasthan, India and were ground using a mill and then autoclaved at 121°C for 15 minutes. The chemicals viz. gelatine yeast extract, agar and casein were of bacteriological grade and supplied by HiMedia, Mumbai, India. All the other chemicals were procured from S.D. Fine chemicals Ltd., Mumbai, India.

Sample for bacterial isolation

Abattoir waste was collected from local market, Wadala, Mumbai, India in sterile container and was stored at 4° C until use.

Bacteria isolation and screening

For enrichment of protease producing bacteria, collected samples were inoculated in Gelatine Yeast extract (GYE) broth. The broth contained 1% gelatine, 1% glucose, 0.2% yeast extract and 100mL distilled

water. The pH was adjusted to 7.0 and the broth was autoclaved at 121°C, for 15 minutes. Then, 1mL of degraded abattoir waste sample was inoculated in aseptic conditions in the above broth and incubated for 24 h at 40°C.

A volume of 0.5mL from the enrichment broth was serially diluted to 10⁻⁸ dilution and was spread plated on nutrient agar media prepared using 100 mL distilled water. Colonies obtained were further subjected to secondary screening by using Gelatine Yeast Extract (GYE) agar, pH 7.0 and incubated at 40°C, for 24 h.

Slant agar medium in Petri plate was overlaid with 0.25% tannic acid solution for 20 min. Tannic acid solution was then drained off and the slant agar medium was washed with several exchanges of distilled water in order to remove any superficially adhered tannic acid. Diameter (D) of the clear hydrolytic zone around the colonies and the diameter (d) of the colonies were measured. The ratio D:d was calculated and on basis of this value, the proteolytic strains were selected.

The isolate with enzymatic activity were primarily identified by Gram staining and morphological studies. The taxonomic identification was carried out at National Centre for cell science (NCCS), University of Pune Campus, using 16S rRNA method.

Protease production and fermentation condition optimization

Protease production using selected strain *Bacillus subtilis* was carried out by submerged fermentation. A liquid medium containing 1.0% yeast extract, 1.0% glucose and 1.0% mustard oil cake powder with pH adjusted to 7.0 was sterilized by autoclaving at 121°C, 15 psi for 15 min. 1% inoculums

of 24h old *Bacillus subtilis* culture (10⁸ CFU/ml) was then added to 50mL of the medium (in conical flask of 100mL volume). The flasks were incubated at 40°C for different time intervals on a rotary shaker at 150 rpm. The absorbance of the culture was measured by spectrophotometer at wavelength of 660 nm. The culture was then centrifuged at 5000 rpm, 20 min and the cell free supernatant was assayed for protease activity. In order to achieve maximum protease activity, medium parameters such as, incubation period, pH of the medium, incubation temperature, mustard oil cake powder concentration, and yeast extract concentration were optimized. Medium optimization studies were carried out by studying one parameter at a time keeping other parameters constant. Biomass production and protease biosynthesis was determined at interval of 1h. All experiments were done in triplicates.

Protease activity assay

Caseinolytic activity was assayed by the colorimetric method. A volume of 2000 µl of cell free supernatant was incubated with 1000 µl of substrate (casein, 0.65%, w/v, pH 8.0) at temperature of 37°C for 30 minutes under static conditions. After adding 0.5 mL of Folin- ciocalteu reagent, the mixture was kept for 5 min at room temperature. Mixture was finally diluted to 10 mL with deionised water (8 mL). The absorbance of the reaction mixture was measured at 660 nm. One unit of enzyme was defined as the amount of enzyme which catalyses the formation of 1µmol of tyrosine during one minute (Lowery *et al.*, 1951).

Statistical analysis

All determinations were obtained from triplicate measurements and results were expressed as mean ± standard deviation.

Student's T test was used to analyze data and statistical significance was declared at $p < 0.05$.

Results and Discussion

6 bacteria strains were obtained by primary screening. Out of these 3 bacteria strains were found to be protease producers which were detected after flooding GYE agar plates with 0.25% tannic acid solution in secondary screening. These 3 bacterial strains received identification codes PSB1, PSB2 and PSB3. The proteolytic strain showing maximum D/d value is 1.6 i.e. strain PSB2 was selected for further experiments as compare to PSB1 and PSB 3.

Gram staining and morphological examination of the isolate PSB2 revealed that it is Gram positive bacteria; rod shape sporulating microorganism with terminal endospore showing white colonies by growth on GYE agar.

Effect of incubation time

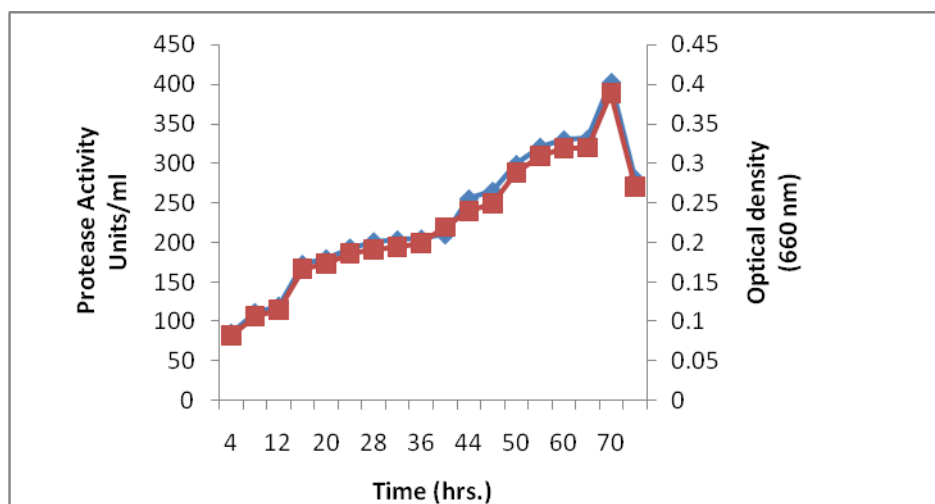
Production of extracellular protease was

evaluated up to 75 h of submerged cultivation. (Figure 1). It was observed that extracellular protease production is directly proportional with growth of *Bacillus subtilis*. Maximum activity was 402.91U/ml and it was found after 70 h of incubation in late exponential phase of the growth of the culture. After that activity decreased as growth of the *Bacillus subtilis* showed decreasing pattern. Similar results were reported in *Bacillus odyssey* with 72 h incubation period (Sandya and Tembeker, 2013).

Effect of pH

Change in pH of the system changes ionization state of the components of the system, due to which accessibility of substrate into bacterial cell changes. Hence enzyme production also varies as pH of the nutrient medium is altered. It was observed from figure 2 that, maximum protease production by *B. subtilis* occurs at pH 11.0. Similar results of protease production were also reported by (Shikha *et al.*, 2006) at pH 11.0.

Fig.1 Effect of incubation time on extracellular *Bacillus subtilis* protease production



Effect of incubation time on extracellular *Bacillus subtilis* protease production. Diamonds represent protease activity in units/mL/min and squares indicate optical density at 660 nm.

Fig.2 Effect of pH on extracellular *Bacillus subtilis* protease production

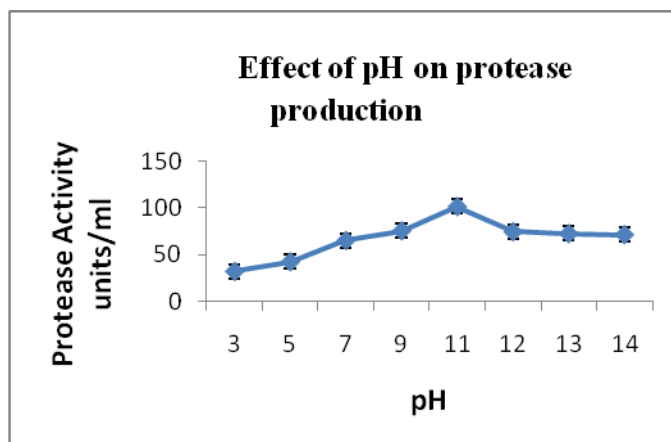


Fig.3 Effect of temperature on extracellular *Bacillus subtilis* protease production

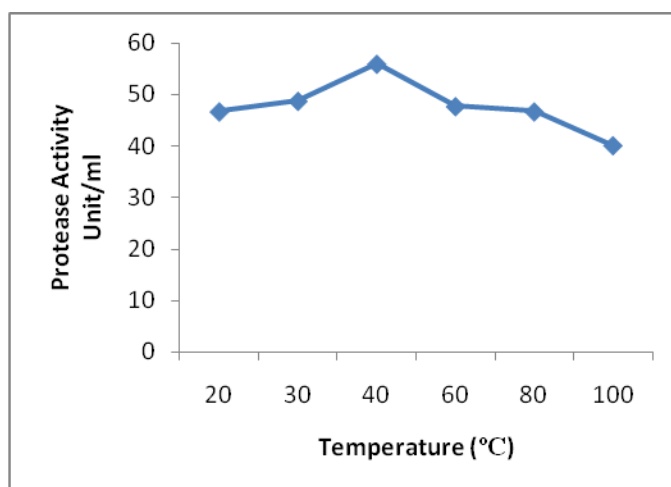


Fig.4 Effect of substrate concentration on extracellular *Bacillus subtilis* protease production

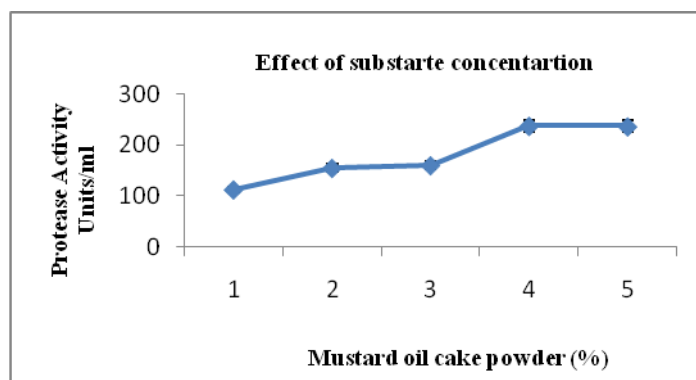
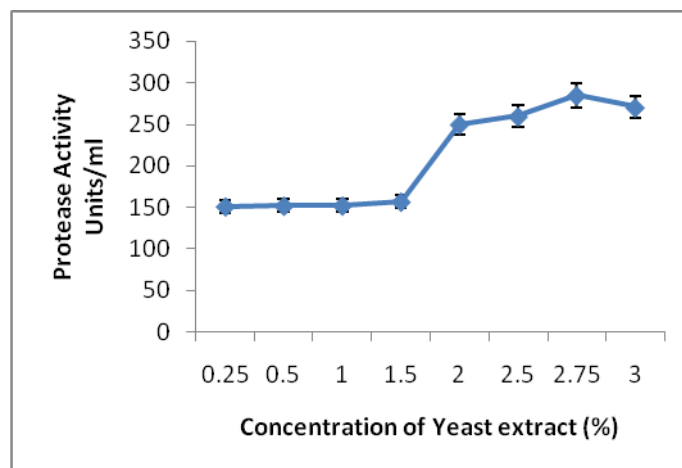


Fig.5 Effect of yeast extract concentration on extracellular *Bacillus subtilis* protease production



Effect of temperature

Temperature is one of the major factors that affect enzyme production by the isolate. Optimum temperature for protease production coincides with optimum growth temperature for the isolate. It has been observed that, generally protease is produced in the temperature range 20–50°C. In this study, optimum temperature for maximum protease production was found to be 40°C as can be observed from Figure 3. *Bacillus* sp. showing protease production at 40°C has been already reported by (Sharma, 2012).

Effect of substrate concentration

Oil cakes have been widely used as substrates for the production of industrial enzymes using fermentation process since they provide both carbon and nitrogen sources in the nutrient medium (Ramchandran *et al.*, 2007).

Figure 4 reveals that increase in the concentration of mustard oil cake powder showed increased production of extracellular protease. However, beyond 4% mustard oil cake powder in production medium, the production of the protease was observed to

decrease. This may be due to increase in the viscosity of the medium beyond 4% of mustard oil cake powder which did not support appreciable growth of *B. subtilis*. Due to this, 4% of mustard oil cake in the medium was considered to be optimum for maximum protease production.

Effect of yeast extract concentration

Figure 5 indicates that the supplementation of the fermentative medium with yeast extract present a positive effect on the protease activity. A concentration of 2.75% yeast extract was found to be optimum for the extracellular protease production. Further increase in yeast extract concentration showed inhibition of enzyme activity. The result revealed that the best nitrogen source was yeast extract for *Bacillus subtilis* which showed highest level of protease activity. N.S.Nisha and J. Divakaran studied the effect of various nitrogen sources on protease production, and they found that yeast extract gives maximum protease activity (Nisha and Divakaran, 2014).

The *Bacillus subtilis* strain isolated from slaughter house waste material was found capable to produce extracellular protease

enzyme. For maximal yield of enzyme production the fermentation parameters were optimized. Use of mustard oil cake as ingredients in fermentative medium for submerged cultivation of bacterium and extracellular protease production makes process economically viable. Based on the present study, it is concluded that *Bacillus subtilis* has wide scope for the industrial production of protease under solid state flask fermentation using mustard oil cake.

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