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Production of amylase and protease from fruit peels using *Bacillus subtilis* by solid-state fermentation

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ABSTRACT

In this study, the agro wastes like fruit peels (Banana, pomegranate and grapes) used as substrate for the production of amylase and protease by *Bacillus subtilis* using solid state fermentation (SSF). The microorganisms were isolated from agro waste soils and the organism was identified as bacteria *Bacillus subtilis* by Gram's staining, spore staining and biochemical techniques. The process parameters were optimized and the enzyme concentrations were assayed by spectrophotometry. The enzyme production were found to be at the rate of 74.5 mg per 0.3g of agro waste fruit peels for amylase enzyme and 11.0 mg per 0.3g for protease enzyme under optimal experimental conditions. From the study it may be concluded that fruit peels can be effectively used a as substrates in solid state fermentation for amylase and protease production by *B.subtilis* using optimum pH, temperature, inoculum and fruit peels concentration for beneficial industrial applications.

KEYWORDS: Amylases, *Bacillus subtilis*, Fruit peels, Solid state fermentation, Proteases

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INTRODUCTION

Numerous of enzymes are extensively used in various industrial processes among those amylases and proteases are widely used. Amylases are essential for all living organisms and which is applied in biotechnology, food, detergents, waste management and pharmaceutical products[1]. Similarly, proteases are also industrially useful enzyme, which is catalyse the hydrolysis of peptide bond from protein molecule. It is constitute 50-65% of the global industrial enzyme market[2] and have been widely used in pharmaceutical, leather, laundry, food and waste processing industries[3].

Microbial proteases and amylases are extracellular enzymes which are directly secreted into the fermentation medium by the producer, therefore simplifying downstream processing as compared to proteases obtained from plants and animals[4]. Numerous of microbial strains have been reported to produce proteases and amylases including bacteria (*Bacillus licheniformis, Bacillus firmus, Bacillus alcalo, Bacillus subtilis* and *Bacillus thuringiensis*) and fungi (*Aspergillus flavus, Aspergilus miller, Aspergillus niger* and *Penicillium griseofulvin*) [5,6].

Among the bacteria, *B.subtilis* is a specific producer of extracellular enzymes, due to its excellent fermentation properties with high product yields (20 to 25 g /L), therefore, it has been used for production of amylase and proteases[7]. Cheap and readily available agricultural waste like peels of grapes, banana and pomegranate are used as substrate for the growth of microorganism in solid state fermentation and production of enzymes[8,9].

The overall cost of enzyme production is very high due to high cost of substrate and medium used and therefore, development of novel processes to increase the yield of proteases with increasing the production cost is highly appreciable from the commercial point of view[10]. To achieve these goals, effects have been directed to expose the means to reduce the protease production costs through improving the yield and the use of either cost free of low cost feed stocks or agriculture by products as substrates for protease production[11]. Hence, the present study aimed to isolate and screen the amylase and protease producing bacteria from agro waste soil and optimizing the physiochemical parameters for the maximum production of protease and amylase present in agro waste fruit peels of banana, pomegranate and black grape by using solid state fermentation.

MATERIALS AND METHODS

Solid substrate fermentation (SSF) system was selected for the study due it's superior productivity, simpler technique, lower capital investment, lower energy requirement and less water output, better product recovery and lack of foam build up, besides it is reported to be the most appropriate process for developing countries. Recently, researchers evaluated whether SSF is the best system for producing enzymes and found to be very effective[12].

Preparation of the substrate

In to a series of 250 ml conical flask fruit peels such as banana, pomegranate and grapes were chopped into pieces (0.3 g), 1.0% of the inoculums was added and was incubated at 37°C for 24 h. The cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates can be used as carbon and energy source. It holds tremendous potential for the production of amylase and protease[13].

Isolation, identification and growth of B.subtilis from agro waste soil sample

Soil samples were collected in sterile bottles from various fruit wastes from dumping market site and fruit store in and around Erode, Tamil Nadu, India and stored till use. Fruit dumping site soil were subjected to serial dilution. These wastes can be as a substrate of amylase and protease enzymes production in culture medium. About 0.1 ml of the supernatant of each tube containing suspension of soil and culture media were inoculated in nutrient agar plates by streaking at 37°C incubated for 24 h. Morphologically similar colonies were taken and sub-cultured. After that, the plates were examined. The purified colonies were identified by using Gram staining and endospore staining techniques and various biochemical tests to find out the bacterial cell according to the bergey's manual.

Inoculum preparation

The spores of *B.subtilis* were transferred aseptically to a 500 ml conical flask containing 100 ml of pre-sterilized inoculum medium in laminar air flow. The flask was then kept on shaker (120 rpm) at 37^{o}C for 24 h. The homogenous spore suspension (106-107 spores/mL) was used as inoculum.

Production of enzymes in B.subtilis by using solid state fermentation medium

Production medium containing (g/l) 0.5 g glucose, 0.15 g peptone, 0.1 g ferrous sulfate, 0.5 g potassium dihydrogen phosphate, 0.5 g magnesium sulphate. 20 ml of medium was taken in a 100 ml conical flask. The flask was sterilized in an autoclave at 121°C for 15 min and after cooling, the flask was inoculated with overnight grown bacterial culture. The inoculated medium was incubated at 37°C in shaker incubator for 24 h. At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 15 min to obtain the crude extract, which served as enzyme source.

Assay of amylase

Amylase activity was determined by the colorimetric method[14]. A reaction mixture containing 0.5 ml of 1% soluble starch solution prepared in 0.2 M acetate buffer and 0.5 ml of diluted enzyme solution was incubated at 50°C. After 10 min incubation the reaction was terminated by adding 1.0 ml of DNS solution (1 g of 3,5-Dinitrosalicylic acid dissolved in 20 ml of 2 M sodium hydroxide, to which 30 g of sodium potassium tartarate and water were added to make it 100 ml). Reaction mixtures were boiled for 15 min and after cooling 18 ml water was added. Absorbance was measured at 540 nm. The analysis was performed in triplicate and enzyme concentration was calculated in µg/ml.

Assay of protease

Protease was determined by Folin Lowry's method[15]. Protease activity was assayed by using 1 ml of 1% casein in 0.05 M Tris HCl buffer (pH 7.8) as substrate. Casein solution was incubated with 0.5 ml of enzyme at 50ºC for 30 min. After 30 min, the reaction was terminated by the addition of 2 ml of 10% TCA. Mixture was centrifuged and 1 ml of supernatant was added to 5 ml alkaline reagent. This was preceded by the addition of 0.5 ml Folin *Ciocalteau* reagent. After 25-30 min, the color developed was read at 660 nm against a reagent blank prepared in the same manner. The analysis was performed in triplicate and enzyme concentration was expressed in terms of µg/ml.

Optimization of fermentation parameters for amylase and protease production by B.subtilis

Different fermentation parameters were optimized for enzymes production by conducting a series of experiments.

Effect of pH of the medium on enzymes production

In to a series of flasks broth containing optimum concentrations of substrate and carbon source were taken and the pH of the broth was adjusted to 6.0, 7.0 and 8.0 in different flasks using 1N HCl and 1N NaOH and sterilized. The cultures were inoculated and incubated at particular pH. At the end of incubation period the cell free culture filtrate was obtained. The concentration of the enzymes was measured. The results values were expressed as µg/ml.

Effect of incubation temperature of the medium on enzymes production

The present study was carried out to check the effect of temperature on amylase and protease activity at 25°C, 35°C and 45°C respectively keeping the other experimental conditions at optimum

level. The concentrations of the enzymes were measured at the end of the incubation period and the results were expressed as µg/ml.

Effect of inoculum of the medium on enzymes production

Production medium was inoculated with overnight grown selected bacterial strain. The broth was incubated with different volume of strain i.e.1.0%, 1.5% and 2.0%. At the end of incubation period the cell free culture is obtained. The amount of the enzymes was measured. The results were expressed in µg/ml.

Effect of agro based waste material as substrate for amylase and protease production

To find out the suitability of agro based different waste materials such as fruit peels of banana, pomegranate and black grape were taken as substrates for the enzymes production. They were added in various concentrations 0.1 g, 0.2 g and 0.3 g to the growth medium under submerged condition. The enzyme activity was measured after 24 h of incubation period under optimal enzymatic conditions by spectrophotometer and the results were expressed as µg/ml.

Production of amylase in B.subtilis using peels of banana, pomegranate and grape

Amylase production in *B.Subtilis* using different fruit peels such as banana, pomegranate and grape were taken separately in SSF under optimum pH 6.0, temperature 35°C and inoculum 1% and incubated for 24 h. The concentrations of the enzyme production were measured and the values were expressed in terms of µg/ml.

Production of protease in B.Subtilis using peels of banana, pomegranate and grape

Protease production in *B.Subtilis* using fruit peels such as banana, pomegranate and grape were taken separately in SSF under optimum pH 8.0, temperature 45°C and inoculum 1% and incubated for 24 h. The concentrations of the enzyme production were measured and the results were expressed as µg/ml.

Enzymes production in B.Subtilis using optimal conditions using combined peels of banana, pomegranate and grape

The production of amylase and protease enzymes from *B.Subtilis* were determined using optimum parameters such as pH, temperature and inoculum and equally 0.3 g of banana, pomegranate and grape (combined fruit peels) by SSF. The broth was incubated for 24 h. The concentrations of the enzymes produced were expressed in µg/ml.

RESULTS AND DISCUSSION

Selection of a suitable solid substrate and its level are important factors for solid state fermentation. Fruit peels added to medium for alpha-amylase production has an important role in providing nutrients for microbial growth. The ultimate benefit of utilizing agro industrial waste is to reduce pollution problems for human beings, which otherwise need to be disposed off thus adding to environmental pollution. In the present study, amylase and protease producing bacteria were isolated from agro waste soil. Microbial fermentation was carried out using *B.Subtilis* by solid state fermentation. Effect of varying pH, temperature, innoculum and fruit peels as substrates on the production of both amylase and protease enzymes by *B.Subtilis* were studied.

Isolation and identification of bacteria from the agro waste soil

Bacteria were isolated from the agro waste soil i.e., soils from the vegetable and fruit dumping sites. The bacterial strains were isolated by serial dilution method and identified by Gram staining and endospore staining techniques and various biochemical tests as shown in Table 1.

Table No.1: Identification of bacterial strains by morphological and biochemical characters

Test	Result
Morphological characters	
Gram staining	Gram positive (Small size rod)
spore staining	Endospores
Colony formation	Present
Colour of the colony	Whitish cream colour
Biochemical test	
Indole	-
Methyl red (MR)	-
Voges-Proskauer (VP)	+
Citrate	+
Catalase	+
Nitrate reduction	+

'+' = Positive; '-' = Negative

The bacteria were rod shaped, respond to Gram positive test and spores formed Bacillus. The VP test and catalase test for these bacteria were positive, while indole test was negative and also showed their behavior by colony characterization with light whitish cream colour. They are aerobic or facultative anaerobic. Based on the result the isolated organism was identified as *Bacillus subtilis.*

Bacillus strains such as *B.Subtilis, B.Stearothermophilus, B.licheniformis* and *B.amyloliquefaciens* are known as good producers of alpha-amylase for various applications[16]. Many *Bacillus* species are of remarkable importance because they construct antibiotics. The potential of *Bacillus* species to synthesize a wide variety of metabolites with antimicrobial activity has been widely used in medicine and pharmaceutical industry [17]. One of its abilities is to control various diseases in animals, humans and plants when applied as a biological control agent. Many investigations have utilized the antimicrobial properties of *Bacillus* strains [18].

Bacillus are widely used, for members of the *Bacillus* genus are generally found in soil and represent a wide range of physiological abilities, allowing the organism to grow in every environment and compete desirably with other organisms within the environment due to its capability to form extremely resistant spores and produce metabolites that have antagonistic effects on other microorganisms[19]. For SSF fermentation different substances are used as substrate [20].

Effect of pH, temperature and inoculums on enzymes production

In the present study, *B.Subtilis* produced high concentration of amylase and protease enzymes under optimal pH, temperature and inoculums conditions (Fig. 1).

The amylase production was found to be higher than the protease production. These results indicated that enzyme production may be influenced by various parameters like pH, temperature and inoculums. The effect of pH on amylase and protease production media was studied over a wide range of pH 6.0, 7.0 and 8.0. The maximum amylase activity was 650 µg/ml observed at pH 6.0. Similarly, the maximum protease activity was 210 µg/ml noticed at pH 8.0. However, the protease activity was decreased at pH 6.0. The broth was incubated at different temperatures range from 25°C, 35°C and 45°Cfor 24 h. At the end of incubation period the cell free culture was obtained and the enzymes concentration were assayed.

Production media was inoculated with different volume of selected bacterial strain (1.0%, 1.5% and 2.0%) using optimal experimental parameters (Fig. 1). The yield of amylase and protease by *B.Subtilis* in SSF, which were found to be 550, 525 and 500 µg/ml for amylase and 240, 200 and 170 µg/ml for protease for inoculum size of 1.0%, 1.5% and 2.0% respectively. Similar result also reported [21].

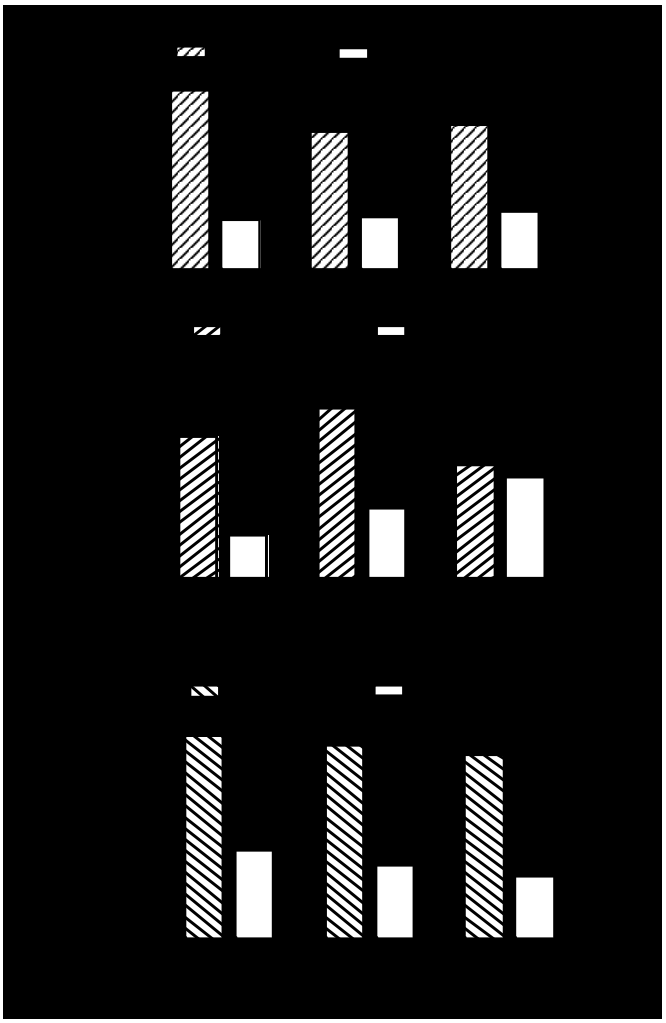

Figure 1: Effects of pH, temperature and inoculums on amylase and protease production by *Bacillus subtilis*

Bacillus subtilis was found to be exhibit maximum activity of amylase at temperature 35°C and protease at temperature 45°C the enzymes were incubated for 24 h using SSF at optimum process conditions. A decrease or increase in incubation temperature caused a decrease in enzyme production by *B.Subtilis* [22]. The result revealed that maximum activity of enzyme amylase was 775µg/ml observed in the fermentation medium adjusted at temperature 35°C but for same temperature the protease activity was reduced. The maximum protease activity was 460 µg/ml observed at 45°C. This shows that protease enzyme requires high temperature for high yield of enzymes by the organism.

In the fermentation medium, adjusted lower inoculum size at 1.0% showed a maximum production of both enzymes whereas the higher inoculums size of 2.0% was found to reduce the production of enzymes. Therefore, high inoculum sizes do not necessarily give higher enzymes yield.

The increase in the production of enzymes using small inoculum size was suggested to be due to the higher surface area to volume ratio, which resulted in the increased production of enzymes. In addition, an improved distribution of dissolve oxygen and more effective uptake of nutrient also contributed to a higher amylase and protease production [23].

Effect of agro based waste on amylase and protease production by Bacillus subtilis

The effect of agro based by- products are alternative substrate for bacterial enzymes production using SSF were studied by several research workers. In the present study, banana, pomegranate and grape fruit peels were taken as substrates and were found to be the best inducer of amylase and protease production by the bacterial isolate. It is obvious from the study that as the concentration of fruit peels as substrates increases, the production of enzymes also increases. Among these fruit peels, pomegranate peel showed highest yield of amylase production (5100 µg/ml) and protease production (820 µg/ml) due its nutritional values (Fig. 2 and 3). It has been reported that increased production of enzymes were achieved by the use of fruit peels [24].

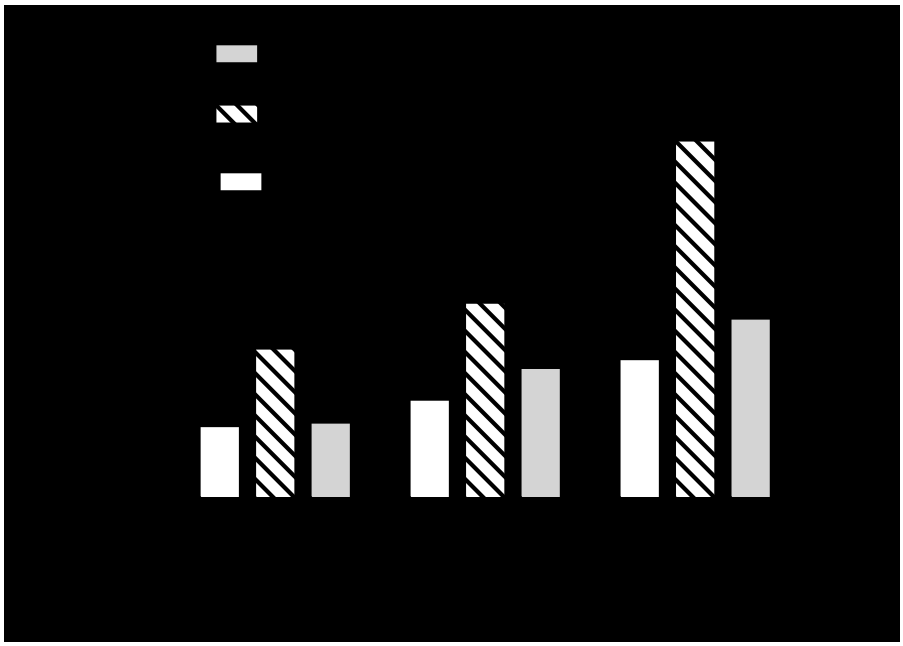

Figure 2: Amylase production in *B.Subtilis* using fruit peels as substrate

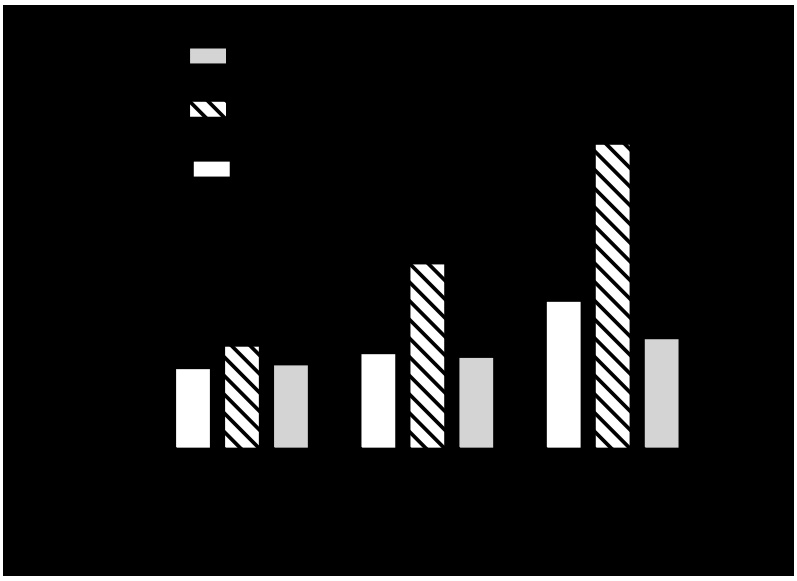

Figure 3: Protease production in *B.Subtilis* using fruit peels as substrate

Optimum source were subjected to enzymes production by *B.Subtilis*. In the present study the maximum production of amylase was about 7450 µg/ml and maximum production of protease was about 1100 µg/ml under optimal experimental conditions. It has also been reported that increased production of enzymes were achieved by the use of optimum experimental conditions. It may be concluded that fruits wastes could be utilized for the production of amylase and protease enzymes which might be beneficial for various industrial applications High concentration of combined fruit peels indicated high yield of amylase and protease production under optimal process parameters by *B.Subtilis* using *SSF*. These results indicated that fruits wastes could be utilized for the production of amylase and protease enzymes which might be beneficial for various industrial applications [25]. High concentration of combined fruit peels of banana, pomegranate and grapes represented high amount of enzymes production obtained by *B.subtilis*.

CONCLUSION

From the current study it may be concluded that fruit peels effectively used in solid state fermentation for amylase and protease enzymes production by *B.subtilis*. The enzymes showed optimum pH, temperature, inoculum and fruit peels which indicated its suitability for various beneficial industrial applications. The study recommended following suggestion for future research to identify the suitable environment for the production of amylase and protease enzymes from different vegetable and fruit peel wastes by using microorganisms and analyzing the best parameter for the large production of enzymes using agro wastes as commercial sources by solid state fermentation.

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