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Utilization of Vegetable Wastes for Production of Protease by Solid State Fermentation Using *Aspergillus niger*

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Abstract: Globally over 50 million tons of vegetable wastes are generated every year. Microbial technology proves to be a critical tool to combat the problem of organic waste management. The present study was taken up to utilize different vegetable wastes as input for protease production using *Aspergillus niger*. Wastes like potato, pumpkin, cauliflower, cabbage and brinjal procured from local market served as substrates for the solid state fermentation. Various parameters like pH, temperature and incubation time were optimized. Among the various substrates examined, it was inferred that cauliflower and cabbage yielded the maximum enzyme activity 1.082 U g^{-1} of substrate and 0.886 U g^{-1} of substrate after 96 hours, respectively. The temperature optimum for the substrates were recorded at 30°C for cauliflower and 35°C for cabbage at an optimum of pH 6 for both the substrates. Highest total protein content of 291.54 µg ml^{-1} was recorded for cauliflower among all other substrates. Estimation of specific activities for individual substrates conferred the highest upon pumpkin with13.44 U mg $protein^{-1}$ ml^{-1}, with comparable results obtained for cauliflower and cabbage. This study presents a novel - economical approach for the bioconversion of vegetable wastes for the production of protease that is industrially significant.

Key words: Waste management · Protease production · Aspergillus niger · Solid state fermentation

INTRODUCTION

Earlier, every technological advancement or innovation focused on enhancement of supplies to meet the then growing demands. In today's world, there has been a shift in focus to regeneration of supplies from wastes, which are found in abundance as a by-product of any process. India's population is widely expanding. In lieu of this, it is appropriate to conceive about the fact of waste generation. Koyambedu Market, Chennai, India is an Asia's biggest vegetable, fruit and flower market spread over an area of 60 acres and generates a total waste of 80 tons per day [1]. Approximately 700 million tons of organic waste such as food waste, livestock manure, vegetable waste and wastewater sludge is produced in India each year [2]. Owing to the inherent biodegradable characteristics of fruits and vegetables, the total wastes generated from them accounts for about 50 million tons per annum. Only 0.5% of these wastes are appropriated as inputs for various processes [3] while the rest is dumped or regarded as landfills. Thus the present scenario demands effective utilization of waste as a resource and microbial technology significantly suffices the demand.

From time, immemorial enzymes have proved their worth to mankind. Naturally synthesized enzymes are a treasure within the human system; commercially produced enzymes are industrially authentic with regard to their applications. Proteases account for nearly 60% of the total enzyme sales [4] in lieu of their diversified applications in dry cleaning, detergents [5], meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds [6] and waste management [7]. However, the industrial production of enzymes is not economical owing to the cost of materialistic inputs.

Booming industrialization presents to the world a perennial need for raw materials for the produce. With regard to this, if the generated waste can satisfy the

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requirements, it shall substantially result in reduction in the cost of production. The present study aimed at evaluating the utilization of vegetable wastes for the production of protease by *Aspergillus niger* to provide a positive stimulus for the production of enzyme economically.

MATERIALS AND METHODS

Substrate: The vegetable wastes of potato, pumpkin, brinjal, cauliflower and cabbage were obtained from Koyambedu market, Chennai, Tamil Nadu, India.

Microbial Culture and Inoculum Preparation: *Aspergillus niger* culture maintained on potato dextrose agar slants was used for inoculum preparations. The inoculum was prepared by dispersing two 5 mm mycelial discs cut from 7 day old culture in 0.1 % Tween-80 solution [8, 9].

Protease Production

Protease Production Process: Five grams of each individual vegetable waste was taken in 250 ml Erlenmeyer flask separately, moistened with 10 ml of the salt solution (Composition of salt solution, g per 100 ml) : ammonium nitrate 0.5 g, potassium dihydrogen orthophosphate 0.2g, sodium chloride 0.1g and magnesium sulphate 0.1g). The flasks were sterilized at 121°C for 15 min, cooled at room temperature and inoculated with 2 ml of the fungal spore suspension. The flasks were incubated at 37°C. Sampling was done after 120 h of incubation and assayed for protease activity [9].

Preparation of Crude Enzyme Extract: Ten ml of 0.1% Tween-80 solution was added to 2 g of the sampled substrate and the mixture was homogenized on a rotary shaker at 180 rpm for 1 h. Centrifugation was performed on the samples at 8000 rpm for 15 min at 4°C. The supernatant was collected and assayed for protease activity [9].

Assays

Determination of Proteolytic Activity: Protease activity was measured using the caseinolytic assay [9]. To 200 µl of crude enzyme extract, 500 µl of casein (1 %) and 300 µl of 0.2 mol l^{-1} phosphate buffer (pH 7.0) were added and the mixture was incubated at 60°C for 10 min. The reaction was terminated by the addition of 1 ml of 10% (w/v) trichloroacetic acid [10]. The reaction tubes were centrifuged at 8000 rpm for 15 min to remove the precipitate. To the supernatant collected, 5 ml of 0.4 mol l^{-1} of Na_2CO_3, 1 ml of Folin and Ciocalteau's phenol reagent (1:3 dilutions) were added. The reaction mixture was incubated for 30 min at room temperature for the development of blue color. The absorbance of the solution was read at 660 nm using a tyrosine standard [11]. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze casein to produce color equivalent to 1.0 mmole (181 mg) of tyrosine per minute at pH 7.5 at 37 °C.

Estimation of Total Protein Content and Specific Activity of Enzyme: Total protein contents of the enzyme solution were measured according to the method described by Lowry *et al.* [11]; using bovine serum albumin (BSA) as a standard. The specific protease activity was expressed in terms of units per mg protein per ml.

Optimization of Culture Parameters

Effect of Incubation Period: The effect of incubation period on protease was determined by incubating production medium for different incubation periods viz. 24, 48, 72, 96 and 120 h.

Effect of Incubation Temperature: The inoculated substrates were incubated at different temperatures viz. 25, 30, 35 and 40°C to find the effect of temperature on protease production.

Effect of pH: Different levels of pH; 4.0, 5.0, 6.0, 7.0 and 8.0 were evaluated for protease production.

RESULTS

Protease Activity: Maximum enzyme production was observed in case of cauliflower substrate with an activity of 1.082 U g^{-1} of substrate and a minimum production of 0.43 U g^{-1} of substrate was in case of potato as observed in Fig. 1. Pumpkin, brinjal and cabbage yielded 0.787 U, 0.627 U and 0.886 U of Protease per gram of substrate, respectively.

Total Protein Content and Specific Enzyme Activity: The total protein contents for the various substrates were e stimated as follows: 117.08 µg ml^{-1} for pumpkin, 198.43 µg ml^{-1} for potato, 203.60 µg ml^{-1} for brinjal, 239.34 µg ml^{-1} in case of cabbage and the highest was found with cauliflower as 291.54 µg ml^{-1}.

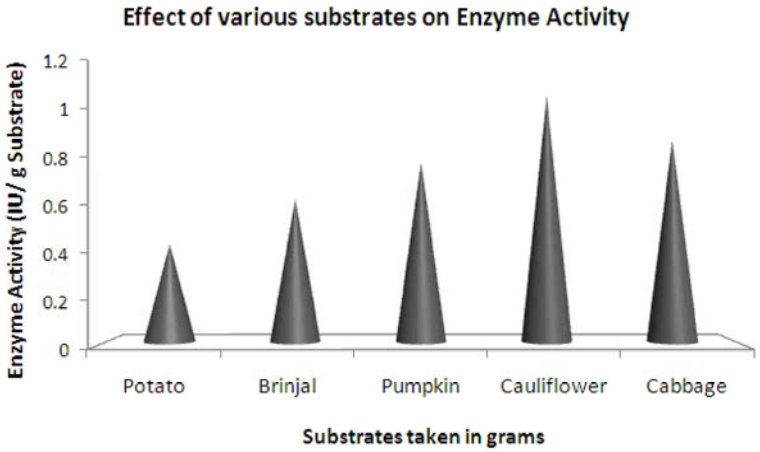

Fig. 1: Effect of various substrates on enzyme activity

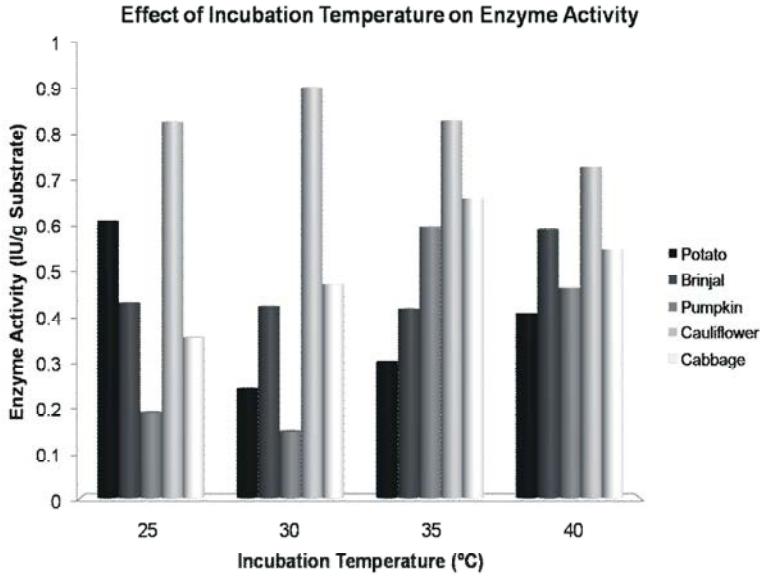

Fig. 2: Effect of incubation temperature on protease activity

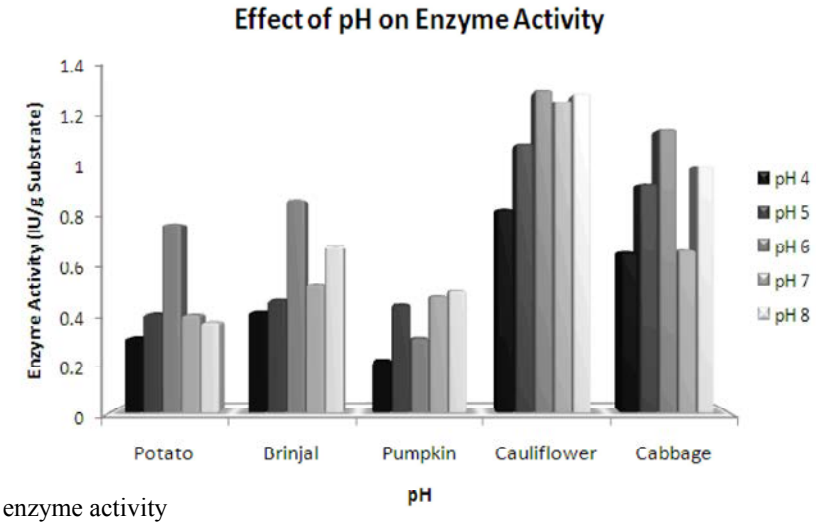

Fig. 3: Effect of pH on enzyme activity

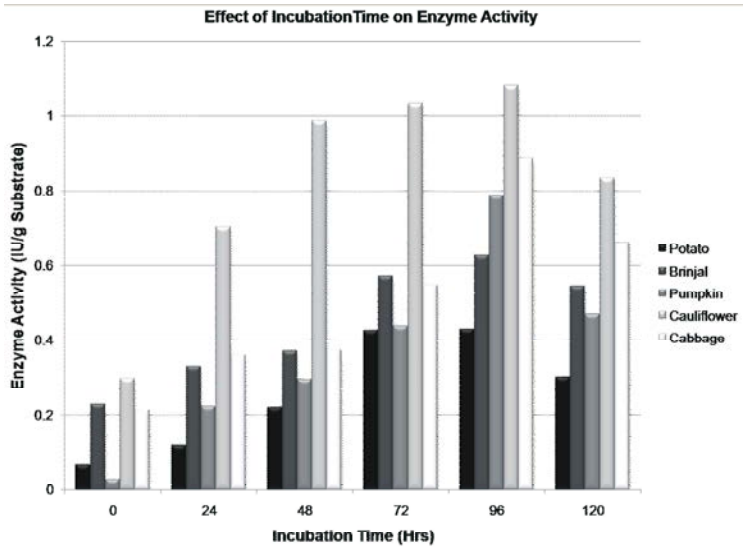

Fig. 4: Effect of incubation time on enzyme activity

The specific activity was determined for different substrates amongst which the highest (13.44 U mg protein^{-1} ml^{-1}) was calculated for pumpkin. The specific activities of cabbage and cauliflower were comparable each with 7.4 U mg protein $^{-1}$ ml^{-1}. Brinjal and potato recorded low specific activities of about 6.16 U mg protein $^{-1}$ ml^{-1} and 4.33 U mg protein $^{-1}$ ml^{-1} respectively.

Effect of Culture Parameters

Effect of Temperature: Optimum temperature for maximum production of enzyme varied for different substrates as depicted in the Fig. 2. Maximum protease activity for pumpkin and cabbage substrates was obtained at 35°C while it was 30°C for cauliflower. Potato gave maximum activity at 25°C; to the contrary brinjal had an optimum activity at 40°C. However, when cauliflower was used as the substrate, the production exceeded much beyond the yields obtained from all other substrates.

Effect of pH: Cauliflower demonstrated very high enzyme activities in a wide pH ranges such as 4, 5, 6, 7 and 8 with cabbage being the second to yield maximum protease at almost all pH ranges. However, both the substrates demonstrated their optimum to be at pH 6 as in the case of most substrates but pumpkin displayed maximum activity at pH 8, the data for which are represented in Fig. 3.

Effect of Incubation Time: At the end of 96 hours, all the substrates delivered maximum enzyme activity. The highest of 1.1 U g^{-1} of substrate was estimated for cauliflower and lowest of 0.43 U g^{-1} of substrate for potato. Cabbage was effective enough to yield 0.89 U g^{-1} of substrate. Other substrates yielded intermediate activities as observed in Fig. 4.

DISCUSSION

Effect of Substrate: Microbes can produce amino acids from inorganic nitrogen sources, but a pool of amino acids provided as protein can stimulate enzyme production [12]. Cauliflower proved to be a superior substrate for fungal growth and protease production at all conditions during the course of optimization of culture parameters in lieu of its nutritional characteristics. Cauliflower is a rich source of protein with essential amino acids [13] that is made available to the organism for growth and metabolism in the form of crude proteins [14]. This contributed to the high yield of enzyme when cauliflower was used as a substrate. Cabbage yielded comparable results.

Initial moisture contents of the substrate critically influence microbial growth and enzyme production [15, 16]. However, as too much water adversely affects oxygen diffusion in substrates, pumpkin may have contributed to its low enzyme content than cauliflower and cabbage owing to its high moisture content. Potato and brinjal showed lesser activity.

Total Protein Content and Specific Activity: The total protein contents were recorded to be the highest in cauliflower and cabbage which may be due to the release of proteins by the high levels of protease associated with both the substrates in the medium as suggested by

Caineab *et al*. [17]. Brinjal and potato demonstrated comparable results while pumpkin yielded a lower total protein content. With regard to the specific activity of protease in different substrates, pumpkin yielded the maximum specific activity. Both cauliflower and cabbage have high phenolic content nutritionally. They gave comparably low value which could be associated with the inhibition of protease by their equivalent phenolic contents [18, 19].

Effect of Temperature: Maximum protease activity for pumpkin and cabbage substrates was obtained at 35°C while it was 30°C for cauliflower. Similar results were demonstrated earlier [20-22]. Brinjal had an optimum activity at 40°C, proving to produce thermostable protease similar to that demonstrated by El-Safey and Abdul Raouf [23]. In general, it is inferred that for most of the substrates, activity increases with initial raise in incubation temperature and a reduction in activity is observed at temperatures beyond optimum indicating the loss of enzyme activity at higher temperatures due to denaturation effects as reported by Haq *et al.* [24] and the active site evolution constrained by temperature dependence according to Daniel *et al*. [25].

Effect of pH: Substrates like cabbage, cauliflower, potato and brinjal gave maximum activity at pH 6 indicating acid protease production. Pumpkin gave maximum activity at pH 8 indicating alkaline protease production that is in agreement with the results recorded previously [20, 26]. Any further rise in pH lead to decrease in productivity, which may be due to the alterations in the three-dimensional structure of enzyme or ionization state of amino acids by disturbing the electrostatic interactions among the charged amino acids. It could be inferred that filamentous fungi *Aspergillus niger* displays both acidophilic and alkanophilic nature based on substrates used.

Effect of Incubation Time: In the study, maximum fungal growth using these substrates was obtained in 96 hours, which can be attributed to the fact that maximum enzyme production was during the growth phase of fungi. Our results are in agreement with that demonstrated by Kamath *et al*. and Olajuyigbe *et al.* [27, 28] that reported time optimum of 96h for fungal sp. In general, maximum yield in fungi are obtained after 6-12 days of fermentation [29]. This signified that these substrates were effectively utilized for faster growth and multiplication of fungi to give maximum protease activity.

In the light of the results obtained, it is deciphered that fermentation technology can be applied to effectively utilize vegetable wastes for production of industrially significant enzymes.

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