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Optimization and partial purification of a protease produced by selected bacterial strains grown on trash fish meal substrate and its antagonistic property against bacterial pathogens

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ABSTRACT

In the present study protease producing bacterial strains such as *Bacillus cereus, Proteus mirabilis, Proteus vulgaris* and *Enterobacter aerogenes* were isolated from spoiled fish and shrimp tissues. The isolated strains were individually inoculated into the formulated culture media supplied with various concentrations (2.5–20%) of trash fish (*Odonus niger*) meat powder as major substrate. Among the tested substrate concentrations, 5% substrate supplied medium favored maximum protease (3.82–5.13 IU ml^{-1}) production. After optimization of substrate concentration, the organisms were tested individually for their protease production capacity at various pHs and temperatures. The results showed that all the tested strains were capable of producing high protease at pH 6 and 50 °C. The crude proteases produced by the tested organisms were individually purified by MLFTPP and ASBP methods. The partially purified enzymes were screened against few pathogenic bacterial strains, and it was observed that the enzyme produced by *P. vulgaris* exhibited the highest antibacterial activity with the inhibition zone ranged between 168.0 ± 0.82 and 204.3 ± 1.25 AU/ml. Further, the partially purified enzyme of *P. vulgaris* alone characterized through SDS-PAGE analysis and determined its molecular mass as 65.0 KDa.

1. Introduction

Protease constitutes one of the most important groups of industrial enzymes, accounting for about 60% of the total world-wide enzymes sales (Schmid et al., 2001). It has a wide range of application in food, meat and leather processing industries as well as pharmaceutical industries (Gupta et al., 2002). Due to its wide application, it has been produced largely in many laboratories by using various sources of microorganisms like bacteria, fungi, protozoa and yeasts. The pervious experimental studies on *Bacillus* sp., had shown the maximum protease production. For e.g., two *Lactobacillus* strains such as *Lactobacillus homohiochii* and *Lactobacillus curvatus* were isolated from a Portuguese traditional dry fermented sausage showing high protease production and also these strains were having tyrene and ornithine decarboxylase activities (Perier et al., 2001; Olajuyigbe and Ajele, 2005).

The protease production by microbes mainly requires the suitable substrates. There were many substrates used for protease production, which include skimmed milk, peptone, casein, raw milk, etc. Some of the agricultural wastes and animal wastes were also used as substrates for the production of protease, because they are readily available, economically very cheap and have high protein content (Prakash et al., 2011). *Bacillus subtilis* Y-108 produced 20.2 U ml^{-1} protease, when grown in crustacean shell waste and this protease is highly active at pH 8 and 50 °C temperature (Yang et al., 1999). Buswell and Shuting (1994) reported that the production of extracellular protease from six mushroom species grown on soybean wastes. Soybean waste is a good substrate for biomass production and also for the expression of proteolytic enzymes. Wheat flour and maltose were found as the most favorable source for the enzyme production. Though, not much favorable, glucose and lactose of the above natural substrates were also constructively utilized for protease production (Mabrouk et al., 1999).

Recognition of the limited biological resources and increasing environmental pollution has emphasized the need for better and more value added utilization of the underutilized fish and the byproducts from the fishing industries. Traditionally, much of this

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material has been converted to powdered fish meal by a combined process of cooking and separation of soluble from insoluble (Ferreira and Hultin, 1994). In view of their high protein content of high quality, fish represents a potential source of industrial peptones for a wide range of applications (Prakash et al., 2011). The fish-processing industry generates considerable quantities of byproducts as waste that includes viscera, shell (from the crustacean and molluscan processing), scales, fins, bone frames and mantles of squids. These wastes are all often high in protein, which can be processed into useful products like fish protein concentrate, fish meal, fish silage, animal feed, etc. (Raa and Gildberg, 1982). However, these fish wastes have been used only to a minor extent as a fermentation substrate for microbial production of bioproducts, despite its availability in large quantities and its low cost (Vecht-Lifshitz et al., 1990). Moreover, fish extracts readily spoil, thereby proving to be an excellent source of nutrients for bacterial growth. Particularly enzymatic hydrolysate from fish waste extract (Jassim et al., 1988) and autolysate of fish vicera (Clausen et al., 1985) have been proved as excellent substrates for bacterial growth. Based on the above literature review, it is understood that the fish waste products are one of the finest substrates for microbial growth and enhancement of metabolic products. Nevertheless, information related to utilization of fish wastes as substrate for enzyme production, in particular protease production is found to be scanty (Triki-Ellouz et al., 2003; Vazquez et al., 2006; Rebah and Miled, 2013). Hence, in the present study, an attempt was made to investigate the microbial protease production by using the Indian trash fish, *Odonus niger* meat powder as a major substrate.

2. Materials and methods

2.1. Sample collection, isolation and identification of protease producing strains

To isolate the bacterial colonies from spoiled food products, fish/shrimp meat samples were aseptically collected from the local fish market. The samples were homogenized individually and serially diluted up to 10^{-5} by using 50% sterilized seawater. Then 100 μl each of diluent samples was plated on 50% aged seawater nutrient agar (Himedia, India) plates, previously prepared and were incubated at 37 °C for 24 h. After incubation, 26 morphologically distinct colonies were selected, purified and maintained as slant culture in the above culture medium. They were designated as MSU-G01 to MSU-G26. Further, the individual colonies were screened for protease activity by using skimmed milk agar medium containing skimmed milk—25.0 g, Casein enzyme hydrolysate—5.0 g, Yeast extract—2.5 g, Dextrose—1.0 g, agar—15.0 g and 50% filter sterilized aged seawater (Uyar et al., 2011) and the plates were incubated for 24 h at 37 °C. After incubation, apparent zone of hydrolysis on the skimmed milk medium was observed around certain bacterial colonies. Among the tested 26 colonies, only four (MSU-G13, MSU-G14, MSU-G17 and MSU-G18) colonies exhibited protease production. These protease producing colonies were characterized by performing various physiological and biochemical tests described by Holt et al. (1996).

2.2. Identification of bacterial strains

The promising protease positive bacterial strains were identified up to species level by using Probabilistic Identification of Bacteria (PIB) software package (Bryant, 1995), an implementation of Bayers theorem by Willcox et al. (1973, 1980). An identification score, as the Willcox probability (P) was calculated for identification thresholds of $P > 0.99$ for all the protease producing bacterial strains. Accordingly, four bacterial strains such as *Bacillus cereus, Proteus mirabilis, Proteus vulgaris* and *Enterobacter aerogenes* were identified. Next to, the utmost protease producing strain MSU-GI7 was confirmed by species level through 16S rRNA sequencing, following the method described by Prakash et al. (2013).

2.3. Media composition and protease activity

The protease activity in the liquid medium was initially assessed, by culturing the individual bacterial strains in an enrichment medium containing beef extract (0.3%), peptone (0.5%), NaCl (0.5%), and glucose (0.5%) at pH 7 for 24 h, and then 10% of enriched cultures were individually inoculated in 250-ml flasks containing 45 ml Basal medium consist of (g/l): $(NH4)_2SO_4$—2 g, K_2HPO_4—1 g, KH_2PO_4—1 g, $MgSO_4$ $7H_2O$—0.4 g, $MnSO_4$ H_2O—0.01 g, $FeSO_4$ $7H_2O$—0.01 g, yeast extract—1 g, and peptone —10 g at pH-7. The cultures were then incubated separately for 2 days by reciprocal shaking at 32 °C. The individual culture broth was centrifuged at 10,000 rpm for 15 min at 4 °C, and the culture free supernatant was used for the further protease assay.

2.3.1. Protease assay

The assay system consists of following ingredients such as 1.25 ml Tris buffer (pH 7.2), 0.5 ml of 1% aqueous casein solution, and 0.25 ml culture supernatant. Approximate controls were also made. The mixture was incubated for 30 min at 30 °C. Then, 3 ml of 5% tricarboxylic acid was added to this mixture. The mixture was allowed to stand for 10 min. After 10 min, it was centrifuged at 10,000 rpm for 10 min. From this, 0.5 ml of supernatant was taken; to this, 2.5 ml of 0.5 M sodium carbonate was added, mixed well, and the mixture was incubated for 20 min. Then, it was added to 0.5 ml of Folin phenol reagent, and the absorbance was read at 660 nm using the UV–vis Spectrophotometer (TECOMP-8500). The amount of protease produced was measured with the help of a tyrosine standard graph. Based on the tyrosine released, the protease activity was expressed in micrograms of tyrosine released under standard assay conditions (Genckal and Tari, 2006).

2.4. Optimization of medium for protease production

The production medium was formulated with five different concentrations (2.5%, 5.0%,10%, 15% and 20% w/v) of sterilized homogenated trash fish (*O. niger*) meat powder with 0.2% yeast extract, 0.3% beef extract, 0.5% peptone and all the components of above basal medium. The media were prepared in sterilized conical flasks. After sterilization, the identified bacterial strains were individually inoculated into the respective medium separately and incubated under shaker incubator at 37 °C for 72 h. The protease production was measured on an interval of every 12 h during fermentation. Accordingly 5% substrate (fish waste) supplied medium showed maximum protease production by a majority of the test organisms. Therefore, 5% substrate added medium was taken and studied further to determine the effect of different pHs (3, 4, 5, 6, 7, 8 and 9) and temperatures (30, 40, 50, 60, 70, and 80 °C) optimization of protease production by individual test organisms.

2.5. Enzyme purification

The protease produced in the optimized culture conditions by the individual organisms was purified by two different methods viz Sodium alginate (or) Macro affinity ligand bound triphase partition (MLFTPP) method (Kalyani et al., 2003) and Ammonium sulfate–butanol precipitation (ASBP) method (Bakare et al., 2005).

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