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Short communication

Cow dung as a novel, inexpensive substrate for the production of a halo-tolerant alkaline protease by *Halomonas* sp. PV1 for eco-friendly applications

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ABSTRACT

The production of a halo-tolerant-alkaline protease by *Halomonas* sp. PV1 under solid state fermentation was optimized. Among the substrates evaluated, cow dung supported the maximum protease production (1351 U/g) when compared with wheat bran (1013 U/g). Process parameters such as, the fermentation period (72 h), pH (8.0), initial moisture (140%, v/w) and the inoculum level (15%, v/w) were optimized. The optimum enzyme production was achieved with 1.5% (w/w) xylose and a 1.25% (w/w) yeast extract. The partially purified protease enzyme was active over a temperature range of 30–50 °C and a pH range of 7–10. The protease was remarkably stable on sodium dodecyl sulfate and on various commercial detergents. The enzyme effectively dehaired goat hides, and makes it a potential source of alkaline protease. This substrate may have wide spread applications in enzyme bioprocesses like wheat bran.

1. Introduction

Proteases constitute one of the most important groups of enzymes and their annual sales account for 60% of the total world enzyme market [1]. Among proteases, alkaline proteases are widely used in many industrial processes. The use of enzyme-based products is currently being explored in many areas of the leather making process, with increasing importance in the dehairing process, thus eliminating the use of hazardous sodium sulfide [2]. Due to the increasing demand of enzyme in the leather industry, there arises a need for new proteases [3]. Extremophiles, the microbes dwelling in unusual habitats, can potentially serve in a variety of industrial applications. Although proteases from many organisms have been widely studied, only few reports have been made on the extracellular protease of *Halomonas* sp. [4]. The microbial strain is unique in their molecular, biochemical, metabolic and enzyme production properties. Hence, an in-depth knowledge of kinetics and the catalytic behavior during protease production from any new strain is a pre-requisite for the evaluation of its biotechnological potential [5].

From an industrial point of view, around 30–40% of the production cost of enzymes is estimated to be accounted for the cost of the growth medium [6]. The cost of the production medium is comparatively less in solid state fermentation (SSF). Utilization of agro-industrial residues as substrates in the SSF processes provides an alternative avenue and value-addition to these otherwise-or non-utilized residues [7]. There are several reports describing the use of agro-industrial residues for the production of proteases. These agro-industrial residues are considered as waste but, it is also to be noted that these can be used as feed/ingredients for the formulations of ruminants feed, aqua-feed or poultry feed. In this decade, more attention has been paid to evaluate suitable agro-wastes for the production of proteases in solid state culture: tannery solid waste [8], coffee pulp and coffee husk [9] and shrimp shell waste [10]. However, reports on the utilization of cow dung for the production of alkaline protease may be little or perhaps nil. Cattle manure consists of ash (13.3–13.4%), nitrogen (1.2–1.6%), cellulose (35.4%) and hemicelluloses (32.6%). As waste, cow dung still contains high amounts of nutrients [11]. Considering its economic importance and availability, the present study was attempted to effectively utilize cow dung as a solid substrate for the enzyme bioprocess.

2. Materials and methods

2.1. Screening of proteolytic bacteria and identification of a potent strain

Twenty seven bacterial isolates were isolated from soil sediments (pH 7.8, temperature 32 °C) of a solar salt pan, Tamilnadu (8°11′N Latitude and 77°28′4″ Longitude), India, using nutrient agar medium ((gram per liter): peptone 5; yeast extract 5, KH_2PO_4

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10; $MgSO_4$ 0.2 and agar 15) containing 5% (w/v) NaCl. All isolates were further screened on nutrient agar medium containing 1% (w/v) skimmed milk and 5% (w/v) NaCl. The plates were incubated at 37 °C for 48 h and the single isolate showing the largest halo zone was selected. The selected bacterial isolate was identified as *Halomonas* sp. PV1 and 1403 bp sequence has been deposited in the NCBI database (accession number: JF499686).

2.2. Solid-state fermentation

Agro-industrial residues such as apple peel, cotton seed oil cake, rice bran, wheat bran, pineapple peel, saw dust, banana peel, paddy straw and cow dung were collected. All of the substrates were dried, ground in a mixer grinder and used. SSF was carried out separately in a 250 ml Erlenmeyer conical flask containing 5.0 g (w/w) of the substrate moistened with 5.0 ml of *tris*–HCl buffer (pH 8.0, 0.05 M). The contents were sterilized and inoculated with 0.5 ml of 18 h grown (0.613 OD at 600 nm) culture broth under sterile conditions. The contents were incubated at 37 °C for 72 h for initial experiments. The process parameters such as fermentation period (12–96 h), pH (5.0–10.0), particle size (0.5–4.0 mm), moisture content (60–200%, v/w), inoculum size (5–40%, v/w), carbon source (1%, w/w) (glucose, lactose, trehalose, maltose, xylose, and starch), nitrogen source (1%, w/w) (gelatin, ammonium nitrate, peptone, yeast extract, urea, and casein) and the combination of xylose and yeast extract (0.25–1.5%) were evaluated. All experiments were carried out in triplicate, and average values are presented.

2.3. Enzyme extraction and measurement of protease activity

The enzyme was extracted from agro-residues and cow-dung using 50 ml of *tris*–HCl buffer (pH 8.0, 0.05 M) by shaking on a rotary shaker and passing through cotton for rapid filtration. This was further centrifuged at 10,000 × *g* for 20 min at 4 °C and the clear supernatant was used as the crude enzyme. The protease activity was assayed according to the method of Kim et al. [12]. One unit of the alkaline protease activity was defined as 1 μg of tyrosine liberated min^{-1} under assay conditions.

2.4. Partial purification of enzyme

The crude enzyme was precipitated by ammonium sulfate salting-out (25–70% saturation) procedure and the resulting pellet was dissolved in a small amount of 0.05 M *tris*–HCl buffer (pH 8.0). This enzyme solution was loaded for gel-filtration chromatography using a sephadex G-75 column (1.5 cm × 50 cm) equilibrated with the same buffer. The fractions containing protease were pooled and concentrated using solid ammonium sulfate salting-out procedures. This partially purified enzyme was used for characterization studies.

2.5. Enzyme characterization

The optimum temperature for protease activity was determined at selected temperatures ranging from 30 °C to 70 °C. The temperature stability of the enzyme was evaluated by incubating the enzyme at 50 °C for 120 min at pH 8.0 and the residual activity was determined. The enzyme activity was also tested for pH optima ranging from 4.0 to 10.0 using 0.05 M buffer systems (succinate buffer, pH 4.0–5.0; phosphate buffer, pH 6.0–7.0; *tris*–HCl buffer, pH 8.0 and glycine–NaOH buffer, pH 9.0–10.0). In order to determine the enzyme pH stability, the protease was incubated in buffer at 37 °C for 1 h after which the residual activity was determined. The impact of various metal ions (0.005 M) (Ca^{2+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Na^{+}, Fe^{2+} and Zn^{2+}) and sodium chloride (0–5.0%, w/v) were evaluated.

2.6. Evaluation of alkaline protease for eco-friendly applications

The effect of alkaline protease on solvent, surfactants, detergents and goat hide was evaluated. The enzyme was incubated with organic solvent (1%, v/v) (ethanol, methanol, acetone, acetonitrile, benzene, toluene, and butanol), surfactants (1%) (SDS, Brij-35, Tween-20, Tween-80, and Triton X-100) and commercial detergents (1%) (Sunlight, Mr. White, Henko, Ujala, Tide+, Aircel, and Surf excel) for 1 h at 30 °C and the residual enzyme activity was assayed. Dehairing of goat-skin was performed using the dip method. The skin pieces (2 cm × 2 cm) were dipped in buffer (*tris*–HCl, pH 8.0, 0.05 M) containing 1.0 ml of crude enzyme (1:10) and incubated at room temperature (30 °C) for 24 h.

3. Results and discussion

3.1. Screening of different agro-industrial residues and cow dung for alkaline protease production

The selection of an ideal agro-biotech waste for enzyme production in an SSF process depends upon several factors, mainly related to the cost and availability of the substrate material [9]. The results presented in Fig. 1 showed that protease production by *Halomonas* sp. PV1 varied with the type of substrate. Maximum enzyme production (1351 U/g material) was observed with cow dung. The result presented here is highly significant because no reports so far evidenced on the use of cow dung as the substrate for the production of alkaline protease. Based on this fact, cow dung can be effectively utilized as an ideal substrate for the production of alkaline protease because of its low cost and availability.

3.2. Optimization of enzyme bioprocess

Enzyme production increased linearly with increase in fermentation period up to 72 h (1836 U/g biomass) and decreased there after. Enzyme productions were 4.8, 18, 213, 329, 1128, 1409 and 477 U/g biomass at 12, 24, 36, 48, 60, 84 and 96 h, respectively. The reduction in enzyme yield after the optimum period was probably due to the depletion of nutrients that are available to the microorganism. These results are in accordance with observations made by Rajkumar et al. [13]. Alkaline protease production by microbial strains strongly depends on the extra-cellular pH [14]. Results showed that the enzyme production was maximum at pH 8.0 and drastically decreased thereafter (Fig. 2). The maximum enzyme production (1630 U/g material) was observed with approximately 1.0 mm particle size cow dung material and decreased to 65% for other particle sizes. In the SSF process, the availability of surface area plays a vital role for microbial attachment, mass transfer of various nutrients and substrates and for the subsequent growth of microbial strains and product production [5]. Enzyme production increased linearly up to 140% moisture and decreased there after

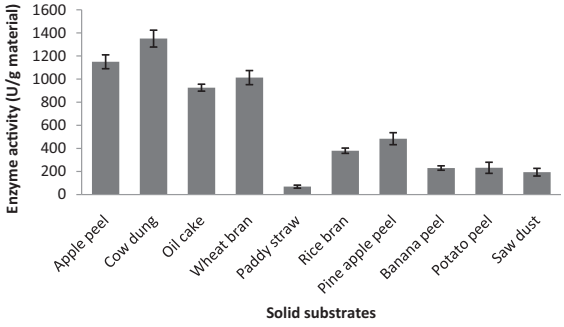

Fig. 1. Screening of different agro-wastes for the production of alkaline protease.