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Production, purification and immobilization of pectinase from *Aspergillus ibericus* onto functionalized nanoporous activated carbon (FNAC) and its application on treatment of pectin containing wastewater

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ABSTRACT

The fungal strain *Aspergillus ibericus* was isolated from food waste for the production of pectinase. In this study response surface methodology (RSM) was employed to determine optimum conditions for the production of pectinase from citrus pectin. The optimum conditions for the production of pectinase were found to be pH, 4.0; temperature, 40 °C; incubation time, 120 h and substrate concentration (Citrus Pectin), 2% (w/v). The maximum activity of pectinase at optimum conditions was found to be 69.9 U mL^{-1}. The purification by DEAE-Cellulose increased the specific activity of pectinase by about 10.1 fold from 64 to 650 U mg^{-1} protein. The molecular weight of the purified pectinase was found to be 41 kDa and 43 kDa. The purified pectinase was immobilized onto functionalized nanoporous activated carbon (FNAC), the maximum immobilization capacity of pectinase onto FNAC was found to be 3360 U g^{-1} at optimum immobilization conditions; time, 150 min; pH, 5.0; temperature, 35 °C and initial concentration of pectinase, 52×10^3 U (80 mg) L^{-1}. The immobilized pectinase showed better thermal and storage stability than free pectinase. The immobilization of pectinase onto FNAC obeyed the Freundlich isotherm model. The immobilization of pectinase onto FNAC was confirmed by FT-IR, XRD, TGA, DSC and SEM analyses. The pectinase immobilized FNAC packed bed column reactor was used for the treatment of pectin containing wastewater under continuous mode. The maximum treatment efficiency for citrus pectin in synthetic wastewater was observed to be 82% at operating conditions: Hydraulic retention time, 180 min; pH, 5.0 and citrus pectin concentration 1% (w/v). Further, the citrus processing industrial wastewater was treated in pectinase immobilized FNAC packed bed column reactor and the system showed 94% of pectin treatment. The treatment of pectin in wastewater obeyed pseudo second order rate kinetic treatment model. The treatment of pectin in wastewater was confirmed by UV–vis spectroscopy and FT-IR spectrophotometer analyses.

1. Introduction

Citrus fruits constitute an important group of fruit crops that include orange, kinnow, lime, khatta, malta and sweet orange are produced all over the world [1]. During the fruit juice processing the industry produce a huge amount of solid waste and wastewater. Generally, the solid waste generate from orange, lemon and apple peels contain pectin. These raw materials are majorly composed of cellulose, 9%–25%; micro fibrils and hemicelluloses, 20%–50%; pectic substances, 10%–35% and proteins, 10% [2]. The pectin molecules can be degraded through a synergistic and coordinated action of various pectinolytic enzymes such as pectin methylesterase, polygalacturonases (PG), pectatelyases and pectin lyases (PL). The pectinolytic enzymes can be produced in large amount by microorganisms using citrus peels as the substrate [3]. Pectinases constitute a heterogeneous group of enzymes which are widely used to hydrolyse pectin in solid waste from food processing industries [1,4]. These enzymes catalyse depolymerisation and demethoxy-

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lation of pectin containing substrates through enzymatic cleavage of α-1, 4-glycosidic bonds of polygalacturonic and pectic acids [5]. Pectinases are being used in juice and textile industry, vegetable oil extraction, manufacturing of hydrolysed pectin products, poultry feed production, pectin containing wastewater treatment, maceration of tea leaves and cotton fibers [5–7].

The wastewater from citrus processing industry contains pertinaceious materials that are poorly bio-degradable by activated sludge process [8,9]. The pectin containing wastewater generated from citrus processing industries are being treated in multiple steps, including physical dewatering, chemical coagulation, aerobic biological treatment and anaerobic biological treatments are practiced [7,10,11]. The aerobic biologic treatment of pectin containing wastewater showed a slight increase of toxicity level in treated water due to gradual increase in polyaromatic compounds in the treatment of pectin [12]. Similarly, citrus processing wastewater (CPW) contains high organic load with toxic content due to the high concentration of terpene-containing oils and flavonoids [13]. The presence of complex organic and insoluble carbohydrates, proteins, fibers, high nitrogen, and sodium in the wastewater decrease the effective treatment and disposal of the effluent [14]. The proposed study was focused on the isolation of selective pectinase to degrade pectic substances (homogalacturonan and rhamnogalacturonic acid) in wastewater to convert into sugar and other end products of uronic acid [8,9] as an alternative, cost effective method for the treatment of citrus processing wastewater. However, free enzymes are relatively unstable, costly and difficult to recover after industrial process. Hence, the immobilization of enzymes to suitable matrix enhance the stability of the enzyme and the immobilization of enzymes can be easily recoverable and reusable with significantly reduction in operational costs of the process [15–17].

Hence, the focal theme of present investigation was to utilize citrus pectin obtained from citrus peels through microbial biodegradation by *Aspergillus ibericus* for the production of pectinase. The optimization of variables for the pectinase production was carried out by Response surface methodology (RSM). The produced pectinase enzyme was purified and characterized. The purified pectinase was immobilized onto FNAC to increase the stability of enzyme and this may be the first report on immobilization of pectinase on to FNAC. The pectinase immobilized FNAC (PIFNAC) was loaded in to packed bed reactor for the treatment of pectin containing Citrus processing wastewater.

2. Materials and methods

2.1. Chemicals

All the chemical reagents used in the present investigation were of analytical grade and procured from HiMedia chemicals, India.

2.2. Isolation and screening of pectinase producing fungi

The spoiled grapes, rotten oranges, and rotten peaches solid waste were collected from the fruit processing industry located in Chennai, Tamil Nadu, India. The fungi were isolated by serial dilution method using sterile distilled water. The fungi were isolated by pour plating method using potato dextrose agar as the substrate and the petri plates were incubated at 37 °C for 5 days. After the incubation period the pure cultures were collected and stored in potato dextrose agar (PDA) slants. The isolated fungi were inoculated into a sterile culture liquid medium with composition: Citrus pectin, 10 g L^{-1}; $(NH_4)_2$ SO_4, 1.4 g L^{-1}; K_2HPO_4, 2 g L^{-1}; $MgSO_4 \cdot 7H_2O$, 0.2 g L^{-1}; $FeSO_4 \cdot 7H_2O$, 0.005 g L^{-1}; $MnSO_4 \cdot 7H_2O$, 0.0016 g L^{-1}; Zn $SO_4 \cdot 7H_2O$, 0.0014 g L^{-1}; $CoCl_2$, 0.002 g L^{-1} and agar agar, 20 g L^{-1}. The medium pH was adjusted to 5.0 using 0.1 M HCl and 0.1 M NaOH and the plates were incubated at 37 °C for 5 days. The plates with matured cultures were rinsed with hexadecyltrimethyl ammonium bromide solution 1% (w/v). The production of pectinase from the matured cultures was confirmed by the zone of clearance around the colonies in plates. Based on size of the zones in plates, the fungi species were selected and used in further experiments.

2.3. Preparation of inoculum

The screened isolates from pour plate were inoculated in potato dextrose agar slants and allowed to grow at 37 °C for 3 days. The spores were harvested by the addition of 5 mL of Tween 80 (0.02% (v/v)). The harvested spores in suspension were stored at 4 °C in sterile falcon tube until any experimental study. The initial concentration of spores in suspension was determined to be 1×10^5 spores mL^{-1}, using visible spectrophotometer (Model: HALO-VIS-10, Japan).

2.4. Optimization of pectinase production by response surface methodology (RSM)

The production of pectinase was carried out with the same medium composition as was used for the isolation of fungi. The optimum conditions for the production of pectinase was carried out by varying the process parameters such as incubation time, 96–144 h; pH, 3.0–5.0; temperature, 30–50 °C and substrate concentration (Citrus pectin), 1.0-3.0% (w/v). The most significant factors were optimized by Central composite design as statistical optimization tool in response surface methodology (RSM). The selected process parameters and the design of experiments with regression values are presented in Table S1. In this present investigation four independent factors were selected and studied at three different levels with a set of 30 experiments. The general mathematical equation used for the analysis is presented in Eq. (1).

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{j} \sum_{<i=2}^{K} \beta_{ij} X_i X_j + e_i \quad (1)$$

where, Y is predicted response; X_i and X_j are factors (i and j = 1 to k), β_0 is the constant, β_i, β_{ii} and β_{ij} are the interaction coefficients of linear, quadratic (second order) and interaction terms respectively, k is the number of independent parameters and e_i is error.

2.5. Pectinase assay

The activity of pectinase in aqueous solution was determined by measuring the reduced sugars released from hydrolysis of pectin in citrus pectin using 3,5-dinitrosalicylic acid (DNSA) method [18]. The reaction mixture consisted of 0.5 mL of 1% citrus pectin (w/v), 0.4 mL of 0.1 M acetate buffer (pH, 5.0) and 0.1 mL of crude enzyme extract. The reaction mixture was incubated at 37 °C for 10 min and the reaction was terminated by adding 2 mL of 3,5-dinitrosalicylic acid reagent. Reduced sugars liberated in the reaction were estimated calorimetrically using galacturonic acid as standard at λ_{540nm}. One unit of pectinase activity (U) is defined as 1 μmol of galacturonic acid released per minute.

2.6. Extraction, purification and characterisation of pectinase

The produced pectinase in the culture medium was centrifuged at 10,000 rpm for 10 min to get clear supernatant and mixed with ice cold acetone (99%) at 1:3 in volumetric ratio and kept under undisturbed condition for overnight at 4 °C. The settled precipitate was collected by cooling centrifugation at 9000 rpm for 30 min. The resulted precipitate of crude pectinase was dissolved in 10 mL of