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Process Assessment for the Purification of *Aspergillus carbonarius* Polygalacturonase Produced by Submerged and Solid-State Fermentations

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Abstract—**Integrated membrane process (IMP) and alginate affinity precipitation (AAP) were assessed for the purification of *Aspergillus carbonarius* polygalacturonase (PG) obtained after submerged (SmF) and solid-state fermentations (SSF). IMP enhanced the purity (4.69 fold) and recovery (76%) of SmF-PG, but did not improve the purity of SSF and commercial PG due to the presence of other similar molecular mass proteins. AAP enhanced the PG purity and recovery in all the PG samples (SmF, SSF and commercial PG). IMP offers several process advantages over AAP but not suitable for purification of all types of PGs. Our studies on SmF- and SSF-PG had shown that downstream process needs are specific for individual cases and warrants careful considerations.**

Index Terms—**Alginate affinity precipitation, integrated membrane process, polygalacturonase, submerged and solid-state fermentations.**

I. INTRODUCTION

Microorganisms produce a mixture of metabolites along with target protein during fermentation, and downstream processing of the fermented medium requires purification and concentration of the target molecule for application. Pectinases have several applications in food, paper and textile industries. Microbial pectinases contribute to almost 25% of the global food enzyme sales and is estimated to increase further [1].

Membrane processing offers several advantages over conventional downstream processing methods as they are convenient and easy to scale-up. Porous membranes have been employed generally for the concentration and there are only a few attempts towards purification of microbial pectinases [2]-[4].

Alginate affinity precipitation (AAP) is a simple and scalable separation process, which exploits the affinity of a smart macroaffinity ligand with the target protein present in the broth and involves selective precipitation of target protein-macroaffinity ligand complex by the application of suitable stimulus. This method employed predominantly for the purification of commercial enzyme preparations [5], has not been attempted for processing crude microbial broths.

We had been focussing on integrated membrane process (IMP) and AAP for the purification of polygalacturonase (PG) in the past few years. A concise performance assessment of these two processes for submerged (SmF) and solid-state fermentation (SSF) PG purification is presented in this paper.

II. MATERIALS AND METHODS

Chemicals

Galacturonic acid, bovine serum albumin and sodium alginate were procured from M/s Sigma Chemicals, USA. Commercial pectinase was obtained from M/s SPIC Biotechnology Division, India. Other analytical and laboratory grade chemicals used were procured from reputed companies in the country. Corn flour and wheat bran were purchased from the local market.

Organism

A mutant A. carbonarius, deposited at the culture collection centre of the Food Microbiology Department, CFTRI under the accession number UV 10046, was used.

For submerged fermentation, the fungus was grown at 30°C for 48 h in a medium made of 2.5% corn flour and 0.313% di-ammonium hydrogen phosphate and ammonium dihydrogen phosphate. For aeration, the flasks were kept on a rotary platform shaker (200 rpm).

For solid-state fermentation, the fungus was grown on solid-state wheat bran medium (20 g wheat bran; 47 mg NaCl; 32 mg yeast extract; 1.25 ml 2 N HCl; 6.75 ml water) and incubated at 30oC for 60 h for PG production. The medium was mixed once in 24 h. For obtaining the enzyme after solid-state growth of the fungus, 10 g of moldy bran was shaken with 100 ml of 0.1 M acetate buffer (pH 4.3) in an orbital shaker (200 rpm) for 30 min.

Prior to processing, the debris and spores were removed by filtration through Whatman No.1 filter paper.

Membranes

Hydrophilic nylon 6,6 microfiltration (MF) 450 nm membrane (Pall Pharmalab, India) and ultrafiltration (UF) 50 kDa membrane with polysulphone as active layer and polypropylene as support layer (Danish Separation Systems AS, Denmark) were used in the study. For use with the self-stirred flat membrane cell MF and UF membranes were cut into circular discs (4.7 cm diameter and 15 cm^2 effective area) and fitted in such a way that the active surface was in contact with the feed material.

Membrane filtration system

Experiments were conducted in batch mode at room temperature under nitrogen atmosphere by charging the cell with 50 ml culture broth or extract. Pressure applied

depended on the type of membrane process (MF: 0.1 MPa; UF: 0.5 MPa) and was adjusted using the regulator of the nitrogen cylinder. To minimize concentration polarization effect, the contents in the cell were stirred on a magnetic stirrer (800 rpm). The experimental run was stopped upon achieving the desired volume concentration ratio (VCR). Flux measurement, membrane cleaning and diafiltration operation were followed as described elsewhere [4].

Performance parameters

The performance parameters for MF and UF were determined as follows:

Microfiltration

$$R\ (\%) = \left(\frac{C_P \times V_P}{C_F \times V_F}\right) \times 100 \qquad (1)$$

$$E\ (\%) = \left(\frac{(C_F \times V_F) - (C_P \times V_P)}{C_F \times V_F}\right) \times 100 \qquad (2)$$

Ultrafiltration

$$R\ (\%) = \left(\frac{C_R \times V_R}{C_F \times V_F}\right) \times 100 \qquad (3)$$

$$E\ (\%) = \left(\frac{(C_F \times V_F) - (C_R \times V_R)}{C_F \times V_F}\right) \times 100 \qquad (4)$$

Where R and E are recovery and elimination. C_F, C_P and C_R are the enzyme activity (U/ml) or protein content (µg/ml) or carbohydrate content (mg/ml) in feed, permeate and retentate. V_F, V_P and V_R are the volume of feed, permeate and retentate (ml), respectively.

Overall enzyme and protein recovery and elimination of carbohydrates in the multi-step process were estimated with reference to the crude value.

The above equations were also used for estimating the recovery of enzyme and protein as well as the elimination of carbohydrates during alginate affinity precipitation by appropriate substitution of feed, process and reject stream values.

Alginate affinity precipitation

AAP was followed as described by Mondal et al. [6]. While the incubation time (2 h) and temperature (30°C) were modified.

Polygalacturonase assay

PG activity was determined using 0.5% polygalacturonic acid (sodium salt) prepared in 0.1 M sodium acetate buffer (ph 4.3) as substrate. Assays were carried out for 10 min at 50°C and the reducing sugars were quantified as galacturonic acid equivalents according to Nelson–Somogyi method [7]. Activity corresponded to µmole galacturonic acid released min-1ml-1.

Protein estimation

Protein content was determined by the dye binding method [8] using Coomassie Brilliant Blue G 250. Bovine serum albumin was used as standard.

Total carbohydrate estimation

Total carbohydrate was determined by the phenol-sulphuric acid method using dextrose as standard [9].

Electrophoresis

SDS-PAGE under reducing conditions was performed in 10% gels and protein bands were visualized by silver staining [10].

III. Results and Discussion

Integrated membrane processing

SmF culture broth containing PG was prefiltered and processed by IMP employing MF 450 nm and UF 50 kDa membranes (Fig. 1). During MF, the specific activity of SmF-PG increased to 1.50 fold purity by eliminating 45% larger contaminating proteins and resulted in a recovery of 85% PG. Subsequently, UF 50 kDa with diafiltration enhanced the specific activity of SmF-PG to 5590 U/mg (4.69 fold) by eliminating 65% smaller contaminating proteins with an overall recovery of 76% PG (Fig. 1). IMP was also effective in eliminating the carbohydrates (96%).

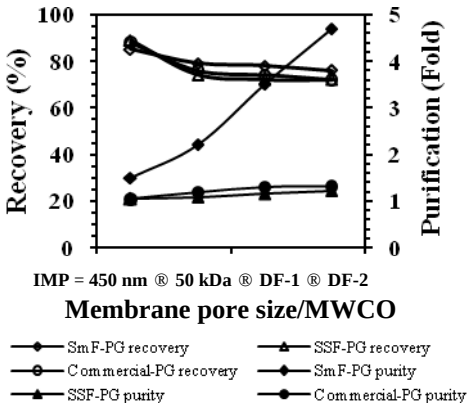

Fig. 1: IMP for PG purification

The purity of SmF-PG was also confirmed by silver stained SDS-PAGE (Fig. 2). However, IMP did not improve the specific activity of SSF-PG (1.2 fold) due to the presence of other similar molecular mass proteins, although enzyme recovery (72%) and carbohydrates elimination (98%) were unaffected. Even IMP of commercial PG preparation behaved similar to that of SSF-PG (97% carbohydrates elimination; 72% PG recovery; 1.28 fold increase in PG specific activity) (Fig. 1).

The presence of other similar molecular mass proteins in the SSF culture extract and commercial PG were confirmed by SDS-PAGE (Fig. 2) which was responsible for low membrane selectivity restricting the improvement in purity. Therefore affinity purification was attempted as an alternate process approach considering its reported success with many other commercial enzyme preparations.

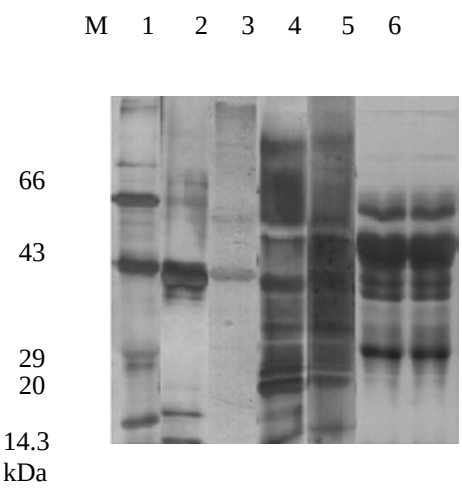

Fig. 2: SDS-PAGE pattern for IMP

M – Markers;
1 – SmF feed; 2 – SmF retentate;
3 – SSF feed; 4 – SSF retentate;
5 – Commercial feed;
6 – Commercial retentate

Alginate affinity precipitation

For PG purification, eluent (NaCl) concentration for the separation of bound PG from the alginate precipitate was optimized. At 1 M NaCl concentration, PG recovery was reasonably higher (54%) with only a small loss in purity.

The alginate precipitate containing bound PG was separated from enzyme alginate solution by centrifugation. The supernatant contained 81% of carbohydrates along with 17% PG. The precipitate containing bound PG was washed with 0.02 M $CaCl_2$ (pH 3.8) for three times to remove the unbound proteins. This step resulted in the removal of additional 16% carbohydrates; however, 2% of PG was also lost in the wash.

Table I: Affinity precipitation of PG[1]

Description/ Steps	E (%)	Sp. activity (U/mg)	R (%)
Submerged fermentation			
Crude broth	-	1130	-
Elution	99	9770	74
Solid-state fermentation			
Crude extract	-	620	-
Elution	97	2450	61
Commercial PG preparation			
Com. PG	-	330	-
Elution	97	1480	64

[1]Pretreatment: Filtered through Whatman No.1 filter paper.

E – CHO elimination; R – PG recovery; Feed volume: 10 ml; Elution condition: 1 M NaCl in 0.1 M acetate buffer (pH 4.3) at 30°C for 2 h.

The PG bound to the cross-linked alginate beads was eluted using a 0.1 M acetate buffer containing 1 M NaCl. Under optimum conditions of AAP the specific activity of SSF-PG enhanced to 2450 U/mg (4 fold) with a recovery of 60% PG and resulted in almost complete elimination of carbohydrates (Table I).

The results showed evidence of alginate binding to SSF-PG, significantly improving its purity in the process and the same was confirmed by SDS-PAGE which exhibited a single band for the purified PG (Fig. 3).

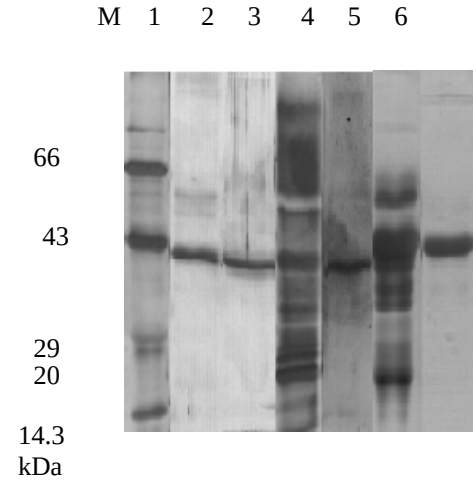

Fig. 3: SDS-PAGE pattern for AAP

M – Markers;
1– SmF feed; 2 – SmF purified;
3 – SSF feed; 4 – SSF purified;
5 – Commercial feed;
6 – Commercial purified

The process efficacy of AAP was tested for the purification of SmF and commercial PG. The specific activity of SmF and commercial PG enhanced to 9770 U/mg (8.7 fold) and 1480 U/mg (4.4 fold) with a PG recovery of 74% and 64%, respectively (Table I). AAP also exhibited complete elimination of carbohydrates (Table I). The purity of SmF and commercial PG were also confirmed by SDS-PAGE (Fig. 3). The result revealed that AAP is suitable for the purification of PG from various sources.

IV. Conclusions

AAP exhibited excellent separation ability between the enzyme and other impurities including similar molecular mass proteins owing to the selective affinity of alginate towards any particular protein (enzyme) under appropriate process conditions. On the other hand IMP worked reasonably well in a process when there was a significant molecular size difference between target proteins (enzymes) and contaminant proteins/impurities such as purification of SmF-PG. Besides, IMP offers several advantages over other downstream processes. From process viewpoint, IMP with appropriate membranes matching the separation requirements should be given due consideration in a downstream process before looking at other alternate approaches.

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