Membrane Processing of Polygalacturonase from *Aspergillus carbonarius*

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Certificate

I **E. Nakkeeran**, certify that this thesis is the result of research work done by me under the supervision of **Dr. R. Subramanian** at Central Food Technological Research Institute. I am submitting this thesis for possible award of **Doctor of Philosophy** (Ph.D.) degree in **Biotechnology** of the University of Mysore.

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Dedicated to my beloved Parents and Teachers…

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Abstract

The investigation was focused on assessing membrane technology for the purification of *Aspergillus carbonarius* polygalacturonase (PG) produced by submerged (SmF) and solid-state fermentations (SSF) considering its due merits while comparing the performance with alginate affinity purification (AAP) process. Primary assessment of upstream processes revealed that SmF yielded 2.7 fold higher PG production over SSF in terms of starch present in the substrate. Initial attempts on processing SmF culture broth employing a set of microfiltration (MF) and ultrafiltration (UF) membranes showed that diafiltration with 10 kDa UF membrane effectively eliminated carbohydrates that otherwise interfered with the purification process, along with other impurities. Although PG was almost completely retained in the process, there was very little improvement in specific activity. Therefore, further attempts were made to develop an integrated membrane process (IMP) to improve the specific activity by eliminating the contaminant proteins along with other non-protein impurities. The IMP thus developed employing 450 nm MF followed by 50 kDa UF membranes improved the specific activity of SmF-PG to 5590 U/mg protein (4.7 fold) with a greater recovery (76%). However, IMP did not improve the specific activity of SSF-PG due to the presence of other similar molecular mass proteins/enzymes while the PG recovery and carbohydrates elimination were similar to the level achieved processing SmF-PG. SDS-PAGE analysis confirmed the presence of other similar molecular mass contaminant proteins in the SSF culture extract and lesser contaminant proteins in the SmF culture broth, which explained the difference in membrane selectivity.

Attempts with AAP revealed its suitability for the purification of PG irrespective of its type of production while displaying better process performance than IMP. In the light of the above, economic analyses were

carried out for three potential combinations, SmF-IMP, SmF-AAP and SSF-AAP processes for PG production. The analyses showed that the unitary product cost of purified PG by SmF-IMP process was 24-36% lower than SmF-AAP and SSF-AAP processes.

Further studies were made with SmF-PG to establish the performance of IMP in a cross-flow system that offers several advantages over stirred membrane cell. Assessing the shear sensitive nature of PG revealed that there is a critical speed of stirring corresponding to a shear stress of 2.1 Pa which should not be exceeded in self-stirred membrane cells. Likewise, peristaltic pump showed its suitability for processing PG over shear intensive gear pump. The specific activity improvement (4.1 fold) attained in cross-flow unit was comparable with the stirred membrane cell (4.7 fold) while achieving greater normalized productivity. In pilot scale system, the specific activity of PG obtained with 200 nm MF followed by 20 kDa UF membranes was comparable with self-stirred system which provided evidence that the performance could be replicated in the scale up. Resistance-in-series model analysis of IMP showed that the fouling resistance was predominant with MF while it was the cake resistance in UF. The efficacy of SmF- and SSF-PG tested for the extraction and clarification of apple juice in terms of sugar yield and clarity suggested the suitability of SmF-PG processed by IMP for formulating as a commercial enzyme.

The results obtained in the present study on production of SmF- and SSF-PG had shown that the process selection needs careful considerations backed up with a techno-economic analysis. In the present case, SmF followed by IMP revealed its suitability for the production of purified PG from *A. carbonarius* in terms of process performance, economics and product application.

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List of Abbreviations

AAP	Alginate affinity precipitation
AIS	Alcohol-insoluble-solids
C_F	Contents of each component in the feed
C_P	Contents of each component in the permeate
C_R	Contents of each component in the retentate
CE	Cumulative elimination
CHO	Carbohydrates
CR	Cumulative recovery
D_i	Stirrer diameter
D_t	Stirred cell diameter
DF	Diafiltration
FCI	Fixed capital investment
h	Stirrer height
IMP	Integrated membrane process
J	Permeate flux
MF	Microfiltration
MWCO	Molecular weight cut off
n_b	Number of stirrer blades
N_{Re}	Reynolds number
NTU	Nephelometric turbidity unit
PE	Polyethylene
PEG	Polyethylene glycol
PES	Polyethersulphone
PG	Polygalacturonase
PS	Polysulphone
r	Radius
r_c	Critical radius
R^2	Correlation coefficient
R_c	Cake layer resistance
R_f	Fouling resistance
R_m	Membrane resistance
R_t	Total resistance

R_o	Percentage rejection
Re_i	Impeller Reynolds number
RO	Reverse osmosis
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SmF	Submerged fermentation
SSF	Solid-state fermentation
TCI	Total capital investment
TPC	Total product cost
TSS	Total soluble solids
UF	Ultrafiltration
UV	Ultraviolet
V_F	Volume of feed
V_P	Volume of permeate
V_R	Volume of retentate
VCR	Volume concentration ratio
wt	Weight
w/v	Weight/Volume
ΔP	Transmembrane pressure
%T	Percent transmission

Greek letters

δ	Momentum boundary layer thickness
μ	Dynamic viscosity
ω	Angular velocity
ρ	Density
τ	Shear stress

Units

cm	Centimetre
cm^2	Square centimeter
g	Gram
h	Hour
INR	Indian rupees
kDa	Kilodalton
kg	Kilogram

kl	Kilolitre
kU	Kilounits
l	Litre
$l/m^2 \cdot h$	Litre per square metre per hour
m	Metre
m^2	Square metre
m^3	Cubic metre
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
mPa	Millipascal
M	Molar
MPa	Megapascal
µg	Microgram
µl	Microlitre
µm	Micrometre
µmole	Micromole
nm	Nanometre
N	Normality
Pa	Pascal
rad	Radian
rpm	Revolutions per minute
s	Second
U	Units (enzyme activity)

1.1 Pectinases

Pectinases are a group of enzymes that catalyze the degradation of pectic polymers in plant cell walls. Polygalacturonases (PG; EC 3.2.1.15) constitute a major part of pectinases in commercial preparations. PG is a depolymerizing pectinase, which catalyzes the hydrolysis of α-1,4 glycosidic linkages in homopolygalacturonan backbone. PGs have been found in higher plants, fungi, yeasts and bacteria. However, they are industrially produced from fungal sources, either by submerged fermentation (SmF) or solid-state fermentation (SSF) especially from *Aspergillus niger* (Naidu and Panda, 1998; Semenova *et al.,* 2006). Pectinases have several applications for processing food, paper and textiles, and are extensively used in fruit and vegetable industries (Kashyap *et al.*, 2001). In the industrial market, microbial pectinases contribute to almost 25% of the global food enzyme sales (Singh *et al.*, 1999) which is estimated to increase further (Tari *et al.*, 2007).

1.2 Enzyme production by submerged and solid-state fermentations

Microbial enzymes are industrially produced by SmF and SSF processes. Though both the processes have claimed merits, about 90% of all industrial enzymes are produced by SmF using genetically manipulated microorganisms owing to several process advantages over SSF process (Holker *et al.,* 2004). The major problems encountered with SSF are difficulties in scale-up and control of process parameters such as pH, temperature, oxygen transfer and moisture. Besides, SSF process suffers from low mixing efficiency and higher impurity product increasing the product recovery costs (Couto and Sanroman, 2006). Despite difficulties and

drawbacks, SSF gained more attention from the researchers and industries alike, in the last three decades due to the possibility of using cheap agro-industrial wastes as substrates.

There are only a few of studies that compared pectinase productivity by SmF and SSF. Solis-Pereira *et al.* (1993) compared the production of endo- and exo-pectinases by *A. niger* CH4 in SmF and SSF and reported that the overall productivities in SSF were correspondingly 18.8 and 4.9 times higher than in SmF. The lower productivity obtained in SmF could be mainly due to the catabolic repression at higher concentrations of free sugars and longer fermentation time. The pectinase production by *A. niger* DMF 27 and DMF 45 in SmF and SSF, respectively were carried out using deseeded sunflower head as a substrate and reported that the maximum production of endo-pectinase (12.6 U/ml) occurred in SmF and exo-pectinase (34.2 U/g) in SSF (Patil and Dayanand, 2006a). These researchers further demonstrated that the pectinase production increased much higher in both SmF and SSF by supplementing the deseeded sunflower head with green gram husk. The maximum production of endo- (19.8 U/g) and exo-pectinases (45.9 U/g) was obtained with SSF compared to endo- (18.9 U/ml) and exo-pectinases (31 U/ml) obtained by SmF (Patil and Dayanand, 2006b).

Microorganisms, substrates and conditions employed in the fermentation processes can affect enzyme production. Higher productivity claims in SSF based on enzyme units per gram dry substrate as against units per millilitre broth in SmF cannot reflect enzyme yields *per se*. Hence there is a need for realistic comparisons, probably by normalizing the enzyme activity in terms of starch present in the substrate, for process assessments. Ghildyal

et al. (1985) while reporting the economics of amyloglucosidase production by *A. niger* by SmF and SSF showed similar enzyme yields in terms of starch, even though the enzyme titre obtained in SSF (extract of moldy bran) was 10 times more than that obtained by SmF (culture broth).

1.3 Downstream processing

Enzyme production by microbial cultivation generally involves use of low cost starchy substrates in growth media. Since the organisms for industrial enzyme production are chosen after the rigors of screening and improved for efficiency, the fermented broths usually contain high concentrations of the target protein along with a mixture of media components, especially carbohydrates and salts. Although downstream processing methods like centrifugation, evaporation, crystallization, solvent extraction, membrane filtration, chromatographic separation, differential freezing, precipitation and aqueous two-phase extraction have been described to eliminate impurities (Dechow, 1989; Singh *et al.,* 1999; Gummadi and Panda, 2003; Shimizu *et al.,* 2005) no single method has been defined for the removal of a particular component from the fermented medium. In downstream processing of enzymes, choice of the technique has to provide preservation of enzymes' fragile structure that is associated with their biological function (Krstic *et al.,* 2007). Membrane process, as one of them, allows both concentration and primary purification of enzymes at ambient temperature under pressure conditions.

1.3.1 Membrane processing of enzymes

Membrane processing that involves the principles of separation by size and shape of molecules or particles is a simple procedure for concentration and purification of enzymes during downstream processing. It offers several advantages over conventional downstream processing methods as they are convenient and easy to scale-up. Even though microfiltration (MF) and ultrafiltration (UF) are pressure-driven, they are attractive and cost effective, since the absence of phase change and inter-phase mass transfer necessitates less energy (Raman *et al.*, 1994). Hence, membrane processes using MF and UF have been in vogue on a commercial scale since 1970s and have largely replaced or complemented some of the traditional methods. Use of membrane processing has also resulted in the production of new products (Hwang and Hwang, 2006; O'Sullivan *et al.*, 1984).

In an earlier study by this laboratory, Singh *et al*. (1999) recognized the need to remove polysaccharides (carbohydrates) which interfere with the purification processes and colour during the concentration of PG, extracted from *Aspergillus carbonarius* fermented wheat bran. They reported loss in enzyme activity following ethanol precipitation and only ~38% carbohydrate removal by sodium chloride precipitation. They obtained 91% recovery with 50 fold concentration by employing 9 kDa followed 30 kDa molecular weight cut-off (MWCO) UF membranes but did not address the removal of polysaccharides. Therefore, research efforts are required towards elimination of carbohydrates present in the fermented broth containing enzymes.

Porous membranes (MF/UF) have been employed generally for the concentration of enzymes (Lee *et al.*, 2009; Shimokawa *et al.*, 2009) and

there are only a few attempts towards purification of microbial pectinases in conjunction with other known methods (Bohdziewicz and Bodzek, 1994; Spagna and Pifferi, 1994; Sathish-Kumar and Palanivelu, 1999; Gainvors *et al.*, 2000; Silva *et al.*, 2007).

Sathish-Kumar and Palanivelu (1999) employed a 450 nm MF membrane for the removal of particulate materials from the culture filtrate of *Thermomyces lanuginosus* containing an extracellular PG. With subsequent acetone precipitation and ion-exchange chromatography, the authors achieved a specific activity of 131 U/mg protein with an overall recovery of 13%. The reduced specific activity (16 U/mg protein; 0.69-fold purity) and recovery (44%) obtained in the membrane process could be reasoned out to lack of process optimization. Gainvors *et al.* (2000) reportedly purified an acidic endo-PG from *Saccharomyces cerevisiae* by UF (30 kDa; 4.8-fold, specific activity 15.5 U/mg protein, recovery 83%) followed by acetone precipitation and anion exchange chromatography (specific activity 2534 U/mg protein, overall recovery 6%). Silva *et al.* (2007) included a pre-treatment step with kaolin while purifying *Penicillium viridicatum* exo-PG for improving UF performance (10 kDa; 7.7-fold, specific activity 1000 U/mg protein, recovery 28%). Further size exclusion chromatography improved the specific activity (1400 U/mg protein) while resulting in reduced recovery (7%). Although there have been contributions to the use of membranes for enzyme purification, its potential has not been completely exploited to achieve greater purity as well as recovery. Therefore, it is necessary to focus on intensive membrane-based approaches towards purification of desired enzymes from the application viewpoint for the industrial scale production

(www.novozymes.com).

1.3.2 Alginate affinity precipitation

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. Alginate is a copolymer of guluronic and mannuronic acid residues and precipitates with Ca^{2+} ions and it was found to bind to pectinase present in the broth (Mondal *et al.*, 2004).

Gupta *et al.* (1993) employed AAP for the purification of commercially obtained endo-PG from *A. niger* and reported improved specific activity (2202 U/mg protein) with ~10 fold purity and ~91% enzyme recovery. Researchers from the same group also attempted affinity precipitation using untreated and microwave treated alginate for the purification of commercial pectinase obtained from *A. niger* and reported enhanced specific activity of 2945 U/mg protein with 10 fold purity and 81% enzyme recovery using untreated alginate under optimum conditions. While microwave-treated alginate resulted in much higher specific activity (5946 U/mg protein), purity (20 fold) and recovery of 83% pectinase compared to untreated alginate (Mondal *et al.*, 2004). This method, employed predominantly for the purification of pectinases and other commercial enzyme preparations such as α- and β-amylases, glucoamylase, lipase, and phospholipase D (Teotia *et al.,* 2001; Jain *et al.,* 2006), has not been attempted for the purification of enzymes from crude microbial broths.

The recovery of target-enzyme with high specific activity or purity by eliminating the contaminant proteins including other enzymes would help maintaining a high specificity of target-enzyme and increase its storage stability. The PG with high specific activity is recommended for industrial application purposes since only a small quantity of the enzyme is needed to perform the desired function. Besides, a small quantity of enzyme addition also helps to retain the natural characteristics of the product. In specific applications, an enzyme preparation is employed by formulating the principal enzyme with other enzymes to improve the overall performance as in the case of enzymatic extraction and clarification of fruit juices. However, such formulations should be prepared ideally from relatively pure enzymes free from contaminants that would otherwise affect the process performance and product quality.

1.4 Economic analysis

Economic analyses are an essential tool not only to guide a rational selection of process type, but also a way to disclose the aspects of a given process that need further attention and optimization (Evangelista *et al.,* 1998). In spite of this, only a few earlier works have compared economic aspects of SmF and SSF (Ghildyal *et al.,* 1985; Castilho *et al.,* 2000) and brought out the great advantage of the SSF process utilizing extremely cheap raw material as the main substrate.

Ghildyal *et al.* (1985) reported the economics of SmF and SSF using corn flour and wheat bran, respectively as main substrate for the production of amyloglucosidase by *A. niger* for three different plant capacities of 9, 30 and

150 m^3 enzyme concentrate per annum. Amyloglucosidase production by SSF was shown to be economical especially for higher capacity plants while the return on investment was either negative or low with SmF. Castilho *et al.* (2000) carried out an economic analysis of lipase production by *Penicillium restrictum* in both SmF and SSF for a production scale of 100 m^3 lipase concentrate per year. The SSF provided unitary product cost 47% lower than the lipase market price. The great advantage gained in the SSF process was due to the use of a cheap agro-industrial residue babassu cake as substrate, while in the SmF case the main component was meat peptone, which is very expensive. The above studies compared only the economics of upstream processing costs and it would be desirable to include the downstream processing costs for a comprehensive analysis. However, such a detailed economic analysis has not been carried out on PG, although it is a major food enzyme contributing to almost 25% of the global food enzyme sales (Singh *et al.*, 1999).

1.5 Scale up studies of membrane process

Scale up of membrane processes is rather easy, nevertheless, need to be done in a systematic manner to replicate the performance of laboratory scale operations in the scaled-up process. Stirred membrane cells offered operational convenience and are commonly used in the laboratory for membrane selection by studying their separation behaviour in the intended process. Cross-flow filtration systems offer several advantages over dead end filtration systems. Therefore, it is necessary to focus on employing a cross-flow filtration system which is more relevant to the industrial scale

operation for up-scaling any potential protocol with a view to further improve the laboratory scale performance.

1.5.1 Effect of stirring on enzyme activity in dead end filtration

Stirring plays a vital role, during processing in a stirred membrane cell, by minimizing concentration polarization effect. Although there are numerous reports on the role of stirring in improving reaction rates in reactors (Caussette *et al.,* 1997), adequate attention has not been paid on the negative aspects of stirring such as activity loss of enzymes in stirred systems. It is imperative that effect of stirring should be carefully considered while processing enzyme solutions in stirred membrane cells and other similar applications.

1.5.2 Pumping effect on enzyme activity in cross-flow filtration

Cross-flow filtration system has been the natural choice in industrial scale operation. Even these systems encounter some major problems while processing enzyme feed stock owing to its adsorption on the membranes and inactivation by shear stress occurring during the operation of the system. Cross-flow membrane filtration systems fitted with various types of pumps, such as peristaltic, centrifugal, diaphragm, lobe and gear pumps have been used for processing microbial suspensions containing enzymes/proteins (Caridis and Papathanasiou, 1997; Conrad and Lee, 1998; Sur and Cui, 2005; Mohammadi *et al.,* 2005; Beier and Jonsson, 2007; Becht *et al.,* 2008). Pumping and turbulent flow through devices often induce enzyme damage leading to loss of enzyme activity (Krstic *et al.,* 2007). O'Sullivan *et al.* (1984) attributed shear inactivation associated with pumping, adsorption losses at the membrane surface and inactivation due to changes in the ionic environment

to the reduced enzyme recovery. However, quantitative assessments on product loss have not been made in the above studies. Denis and Boyaval (1991) tested the shear resistance of *Erwinia chrysanthemi* pectate lyase in a bioreactor, coupled with an UF system fitted with a centrifugal pump and reported that the activity was not affected up to 7.5 h of operation due to shear caused by pumping (Denis and Boyaval, 1991). Under exaggerated conditions, an activity loss of 36% was observed after 25000 passes (144 h of operation) through the pump. They concluded that pectate lyase possesses sufficient resistance to shear damage to withstand the process duty of 2500 passes (equivalent to ~14 h of operation). There are no attempts reported on the selection of pumping devices for processing enzyme solutions.

1.5.3 Comparison of dead end and cross-flow filtration systems

There are only a few comparative studies between dead end filtration and cross flow filtration systems with microbial suspensions/broth. Keskinler *et al.* (2004) reported that the classical cake filtration was the dominant filtration mechanism during MF of low concentration non-living yeast suspension. In the cross-flow system, steady-state permeate fluxes increased with membrane pore size and cross-flow velocity, and decreased with increasing yeast concentration. The specific cake resistance increased with increasing transmembrane pressure drop indicating that the cake was compressible in both cross-flow and dead end filtration systems. Higher the cross-flow velocity, lower was the specific cake resistance and vice-versa. In that study, specific cake resistance of cross-flow filtration system was in fact higher compared to dead end filtration system, which reminded that the comparison

depended on the actual cross-flow velocity employed in the system. Becht *et al.* (2008) compared the rejection behaviour of single protein solutions (bovine serum albumin and lysozyme) in stirred cell and cross-flow filtration systems using a polyethersulphone (PES) membrane with a MWCO of 50 kDa. Concentration polarization was more pronounced in the cross-flow filtration system compared to the stirred cell owing to the significant differences in the mixing conditions/operating dynamics, which led to differences in the observed solute rejection behaviour. Therefore, it is appropriate to compare the performances of two systems only under identical mixing conditions, typically with a similar magnitude of wall shear stress.

1.5.4 Decline of process flux

The flux decline with MF and UF biological suspensions has been studied by many researchers. Bailey and Meagher (2000) observed flux and protein transmission decline over processing time with MF 0.1 μm membrane during separation of soluble protein from inclusion bodies in *Escherichia coli* lysate in a cross-flow filtration system. Choi *et al.* (2005) studied the flux decline during processing biological suspension in a cross-flow filtration system with MF (0.3 μm) and UF (30 kDa) membranes. The flux decrease was sharp in UF compared to MF. The permeate flux of UF reached a pseudo-steady state within 1 h while MF took 4 h to reach a steady state. In spite of this difference, MF was selected due to issues associated with preservation of the biological suspension and excessive destruction of flocculated microorganisms by pump shear at higher operating pressures. The above studies were carried out with single step processing using either MF or UF

and such attempts have not been made on multi-step membrane processes, like IMP.

1.5.5 Resistance-in-series model

The process productivity is one of the important parameters in any membrane process. The reasons for flux decline have been explored in terms of various filtration resistances namely, membrane, adsorption, concentration polarization and fouling resistances during MF and UF of biological suspensions (Choi *et al.,* 2005). Rai *et al.* (2007) analysed the decline of permeate flux using a resistance-in-series model during UF of mosambi juice (sweet orange) using a 50 kDa membrane in a self-stirred filtration system and reported that the cake resistance was predominant. These studies suggest that every potential membrane process should be analysed for the various types of resistances involved to achieve greater productivity.

1.5.6 Pilot scale studies

Concentrations of enzyme solutions to maximize enzyme activity using pilot scale membrane systems have been reported. Pilot scale studies is an intermediary and important step between laboratory and industrial scale operations, to generate necessary and valuable data needed for the scale up of the process. Singh *et al.* (1999) employed a 9 kDa UF membrane in a pilot scale filtration system for processing PG extracts of *A. carbonarius* fermented wheat bran and achieved a 10-fold concentration starting with an initial feed volume of 100 l. Further concentration to 50 fold using a laboratory stirred cell system with a 30 kDa UF membrane ended up in an overall enzyme recovery of 91%. Goklen *et al.* (1994) employed cross-flow MF and UF systems for

separation and concentration of lipase produced by *Pseudomonas aeruginosa.* Initial screening with various MF membranes revealed that flux and enzyme permeation were affected owing to a soluble broth component forming a gel layer. Reformulation of fermentation medium enhanced the flux (40 $l/m^2 \cdot h$) and enzyme permeation with hydrophilically-modified polyvinylidenedifluoride (PVDF) membranes. Subsequent concentration of microfiltered enzyme using a 30 kDa UF membrane resulted in a 130 fold concentration with complete enzyme retention and higher flux (57 $l/m^2 \cdot h$). Similar filtration performances were obtained with 100 and 1000 l scales of operation with both MF and UF systems.

1.6 Application of pectinase for fruit juice extraction and clarification

Pectinases are extensively used for the extraction and clarification of fruit juices (Kashyap *et al.,* 2001). Therefore, it is desirable to test the efficacy of any potential pectinase for the above application from the viewpoint of commercial utilization. Apple juice is most popular worldwide next only to orange juice and contains high amount of pectin. Accordingly, the efficacy of purified *A. carbonarius* PGs was tested for the extraction and clarification of apple juice. Apple juice contains high concentrations of polyphenolic constituents that are responsible for its beneficial health effects (Kahle *et al.*, 2005). It is widely consumed as clear juice, produced from its concentrate. Enzymatic treatment of crushed apple mash with pectinase followed by pomace extraction is widely employed in the production of apple juice concentrate to improve the juice yield. Enzymes are mainly used to develop mashes with lower viscosities and lower water binding capacities, thus

resulting in an easier juice extraction and a better working capacity of the presses or decanters (Will *et al.,* 2002).

Several studies have been reported on the enzymatic extraction and clarification of apple juices using commercial pectinases (Faigh, 1995; Will *et al*., 2002; Sorrivas *et al.,* 2006; Oszmianski *et al.,* 2009). Oszmianski *et al.* (2009) studied the effect of different commercial pectolytic enzymes on apple mash treatment and reported that it had a positive effect on the production of cloudy apple juices by improving its yield and polyphenolic contents. The influence of enzymatic mash treatment at various temperatures (30-60°C) on the analytical composition of apple juice revealed that the highest values of juice yield, colloid concentration and polyphenol content were obtained at 50°C, which is the optimum temperature for the commercial pectinase used in the study. However, the influence of temperature on other juice parameters like total titratable acidity, density, sugar and mineral concentrations was low (Will *et al.,* 2002). The studies on pectinase produced from *A. niger* van Tieghem on clarification of commercial apple juice revealed that a most effective clarification ($\%T_{650}$, 85%) along with a viscosity drop of 35.5% was achieved, using an enzyme dosage of 15 U/ml of juice in the presence of 0.01% gelatin, at 45°C for 6 h (Singh and Gupta, 2004). Kaur *et al.* (2004) reported increase in juice yield while treating apple, banana and grapes with *Sporotrichum thermophile* PG. Semenova *et al.* (2006) tested the efficacy of chromatographically purified *Penicillium canescens* pectin lyase for the clarification of commercial non-clarified apple juice, and reported that its performance was comparable with commercial pectinase preparations. *Bacillus pumilus* pectin lyase has also been attempted for the extraction of

various fruit juices (Nadaroğlu *et al.*, 2010).

In addition to pectinases, other enzymes such as hemicellulases and cellulases are used in the production of fruit juices. While there are no restrictions on the use of microbial pectinases, the principles of the council of the European Union, forbids hemicellulases and cellulases in Fruit Juice Directive (Riberio *et al.*, 2010). In fruit juice production enzymes that have novel applications have also been described. Enzymes such as laccases, naringinases and limoninases have been studied in order to improve organoleptic, physical and chemical characteristics of juices. These studies indicated their potential applications for the removal of bitterness in citrus juices and for the extraction/removal of phenolic compounds and pigments (Riberio *et al.*, 2010).

1.7 Scope of the present investigation

Enzymes are industrially produced by SmF and SSF. Since microorganisms produce a mixture of metabolites along with the target protein (enzyme) during fermentation, purification and concentration are important for downstream processing. In order to meet the increasing demand for pectinase, membrane purification of enzymes appears both cost effective and efficient over other alternate processes.

Although PG is the major food enzyme, there are only a few of studies that compared pectinase productivity by SmF and SSF. In this study, SmF and SSF processes were assessed for the production of PG from *A. carbonarius*, normalizing the productivity claims in terms of starch present in the substrate.

The fermented broths usually contain high concentrations of target protein along with a mixture of media components, especially carbohydrates and salts. Elimination of carbohydrates from culture broth is essential that would otherwise trap the enzymes and interfere with the purification processes leading to enzyme loss. The difference in molecular weight of PG and carbohydrates suggested the application of porous membranes for the elimination of impurities. In the present work, a set of MF (70-450 nm) and UF (10-500 kDa) membranes were examined to eliminate carbohydrates and other non-protein impurities from SmF culture broth containing PG.

Conventional downstream processing methods employed for the purification of enzymes results in higher enzyme purity, but generally with low overall recovery. Although membrane based approaches have contributed towards improving methodologies for enzyme purification, the potential of membrane technology has not been completely exploited. In this study, a protocol for an integrated membrane process (IMP) was developed after screening various MF and UF membranes for their separation abilities, to obtain greater enzyme-specific activity and recovery of PG from crude culture broth/extracts.

AAP 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, has not been attempted for the purification of enzymes from crude microbial broths. In the present work, AAP was evaluated for the purification of PG to obtain greater PG specific activity and recovery by

eliminating similar molecular mass contaminant proteins from the crude culture extract/broth of *A. carbonarius*.

SmF and SSF employed for microbial production of enzymes claim relative merits, so also the downstream processes, IMP and AAP. This scenario suggested the need for a techno-economic analysis for configuring an appropriate process combination. In this study, a comparative techno-economic analysis was performed on upstream and downstream processes for a base-case plant capacity of 30 kl purified PG concentrate per year.

Cross-flow membrane systems are preferred for industrial scale operation as they offered several advantages over the dead end filtration systems. Scale-up studies were carried out in cross-flow membrane systems to improve the process performance of IMP protocol developed earlier with a stirred flat membrane test cell for the purification of PG from culture broth and estimated the various resistances that are responsible for the process flux decline. Also the effect of stirring and pump performance was evaluated to assess the shear sensitive nature of PG and select appropriate operating conditions and devices for processing.

PG is a major food enzyme extensively used for the extraction and clarification of fruit juices. Commercial enzyme preparations generally employed contain other cell wall digesting enzymes in addition to PG to obtain greater juice yield and clarity. In the present work, the efficacy of *A. carbonarius* PGs (SmF-IMP-PG, SSF-IMP-PG, SmF-AAP-PG and SSF-AAP-PG) obtained in the laboratory was tested and compared with commercial PGs for the extraction and clarification of apple juice in terms of sugar yield and clarity.

2.1 Materials

2.1.1 Organism

The mutant *A. carbonarius* isolated by UV mutagenesis (Venkatesh, 2004), deposited at the culture collection centre of the Food Microbiology Department, CFTRI under the accession number UV 10046, was used in this study.

2.1.2 Membranes

Flat sheet membranes - Hydrophobic polyethylene MF membranes of pore size 70 and 150 nm (M/s Tonen Chemical Corporation, Tokyo, Japan) were obtained from the National Food Research Institute, Tsukuba, Japan. Hydrophilic amphoteric nylon 6,6 MF membranes of pore size 200 and 450 nm were procured from M/s Pall Pharmalab Filtration Pvt. Ltd., Bangalore, India. Hydrophilic UF membranes with polysulphone (MWCO 20, 25, 50, 100 and 500 kDa) and polyethersulphone (MWCO 10 kDa) as active layer/coating and polypropylene as support layer (M/s Danish Separation Systems AS, Nakskov, Denmark) were obtained from the National Food Research Institute, Japan. Both MF and UF membranes were cut into circular discs (4.7 cm diameter and 15 cm^2 effective area) for use with the self-stirred flat membrane cell. For use with laboratory scale cross-flow membrane unit, the membranes were cut into rectangular pieces of 46 mm x 180 mm (60 cm^2 effective area). These membranes were fitted in the membrane units in such a way that active surface was in contact with the feed material.

Tubular membranes - Hydrophilic tubular ceramic (α-alumina) MF membrane of pore size 200 nm (0.3 m^2 effective area; 19-channels of 0.85 m length) was

purchased from M/s Pall France SAS, Cedex, France. Hydrophilic tubular polysulphone UF membrane of MWCO 20 kDa (1.2 m long) was procured from M/s PCI Membrane Systems Ltd., Hampshire, England. For use with pilot scale UF system, 18 tubular UF membranes (0.9 m^2 total effective area) were fitted into a stainless steel membrane module.

2.1.3 Enzymes

Commercial pectinase preparations were obtained from M/s SPIC Biotechnology Division, Chennai, India (commercial PG-1; 1620 U/ml; *A. niger*) and M/s Sigma Chemical Company, St. Louis, USA (commercial PG-2; 570 U/ml; *A. niger*).

2.1.4 Chemicals

Galacturonic acid, tyrosine and bovine serum albumin (BSA), used as standards in the enzyme assays of PG and protease, and protein estimation were procured from M/s Sigma Chemical Company. Potassium dihydrogen phosphate, dextrose and maltose used in the estimation of inorganic phosphate, carbohydrates and amylase assay were purchased from M/s Hi-Media Laboratories Pvt. Ltd., Mumbai, India. Molecular mass standards were obtained from M/s Genei Ltd., Bangalore, India.

Polygalacturonic acid and sodium alginate were procured from M/s Sigma Chemical Company. Casein (Hammarsten) and starch were purchased from M/s Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Detection reagents like Coomassie Brilliant Blue G 250, R 250 and silver nitrate were obtained from M/s Loba Chemie Pvt. Ltd., Mumbai, India.

Reagents required for SDS-PAGE like Acrylamide, N, N'-methylene-bis-acrylamide, ammonium per sulphate, N, N, N', N'-tetramethylethylenediamine (TEMED), sodium dodecyl sulphate, dithiothreitol and β-mercaptoethanol were purchased from either M/s Hi-Media Laboratories or M/s Sigma Chemical Company. Polyethylene glycol (PEG)-20000 was procured from M/s Sisco Research Laboratories.

Laboratory grade chemicals were used for media preparation. Media components such as peptone, yeast extract and agar-agar were purchased from M/s Hi-Media Laboratories. Bulk media components like corn flour and wheat bran were procured from local market.

Detergent P3-Ultrasil 10 for cleaning membrane was purchased from M/s Henkel Hygiene GmbH, Dusseldrof, Germany. All other laboratory and analytical grade chemicals were procured from reputed companies in the country.

Apples (Ambri Kashmir variety) of proper maturity and ripeness were purchased from the local market.

2.2 Methods

2.2.1 Cultivation of fungus

2.2.1.1 Shake-flask cultivation

Inoculum for shake-flask cultivation was developed in corn flour broth using spores from one-week-old slants. After growth at 30^{o}C for 48 h on an orbital shaker (200 rpm), the inoculum was transferred to corn flour salt medium and incubated at 30^{o}C for 24 h on an orbital shaker, as above. After harvesting, the culture broth was filtered through muslin cloth to separate the mycelia and

the pH was adjusted to 4.0 using 2 M sodium hydroxide. Prior to membrane processing, the debris and spores were removed by filtration through Whatman No. 1 filter paper.

Corn flour broth

Corn flour	-	40 g/l
Peptone	-	10 g/l
Yeast extract	-	6 g/l
pH	-	5.5

Corn flour salt medium

Corn flour	-	25 g/l
Ammonium dihydrogen phosphate	-	3.13 g/l
di-ammonium hydrogen phosphate	-	3.13 g/l
pH	-	5.5

2.2.1.2 Solid-state cultivation

Inoculum for solid-state cultivation was developed in corn flour broth using spores from one-week-old slants. After growth at 30^{o}C for 48 h on an orbital shaker (200 rpm), the inoculum was mixed with solid-state wheat bran medium and incubated at 30^{o}C 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, typically 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. The extract obtained after filtration through ordinary filter paper followed by Whatman No. 1 filter

paper constituted the crude enzyme.

Corn flour broth

Corn flour	-	40 g/l
Peptone	-	10 g/l
Yeast extract	-	6 g/l
pH	-	5.5

Solid-state wheat bran medium

Wheat bran	-	20 g
NaCl	-	47 mg
Yeast extract	-	32 mg
2 N HCl	-	1.25 ml
Distilled water	-	6.75 ml

The above components were mixed thoroughly and autoclaved at 121°C (0.1 MPa pressure) for 45 min (Kavitha, 2001).

2.2.1.3 Pilot scale submerged fermentation

The mutant *A. carbonarius* was grown in a medium made of 2.5% (w/v) corn flour and 0.313% (w/v) each of di-ammonium hydrogen phosphate and ammonium di-hydrogen phosphate. SmF was carried out in a 75 l fermentor (M/s Bioengineering AG, Wald, Switzerland) using an inoculum developed in the medium in shake flasks (200 rpm) at 30^{o}C for 48 h with spores from one-week-old slants. The fungus was grown in the fermentor by maintaining the following process conditions: Working volume 50 l; Temperature 30°C; Initial

pH 5.5; Stirrer speed 300 – 400 rpm; Air flow rate 300 (first 4 h) – 375 l/min. After 24 h fermentation, the culture broth was filtered through muslin cloth to separate the mycelia and the pH was adjusted to 4.0 using 2 M sodium hydroxide. Prior to processing, the debris and spores were removed by filtration through an ordinary filter paper.

2.2.2 Membrane filtration systems

2.2.2.1 Self-stirred flat membrane test cell

Experiments were conducted in batch mode at room temperature (28±2°C) under nitrogen atmosphere by charging the cell (M/s Millipore Pvt. Ltd., Bangalore, India) with 50 ml culture 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 at 800 rpm (Fig. 2.1). The experimental run was stopped upon achieving the desired volume concentration ratio (VCR). Flux corresponded to the actual measurement or average of the measurements of permeates collected every 30 min. Pure water flux was also measured before processing the culture broth. Membranes were cleaned with 0.25% (w/v) P3- Ultrasil 10 (detergent) followed by 0.1 N sodium hydroxide and 400 mg/kg sodium hypochlorite solutions for 30 min each. The recovery of original water flux was the criterion followed for membrane reuse after cleaning.

The effect of stirring on enzyme activity was studied in a glass beaker (Volume 50 ml; Diameter 3.6 cm; Height 6 cm) as a model system for stirred membrane cell to circumvent unaccounted enzyme/protein losses due to

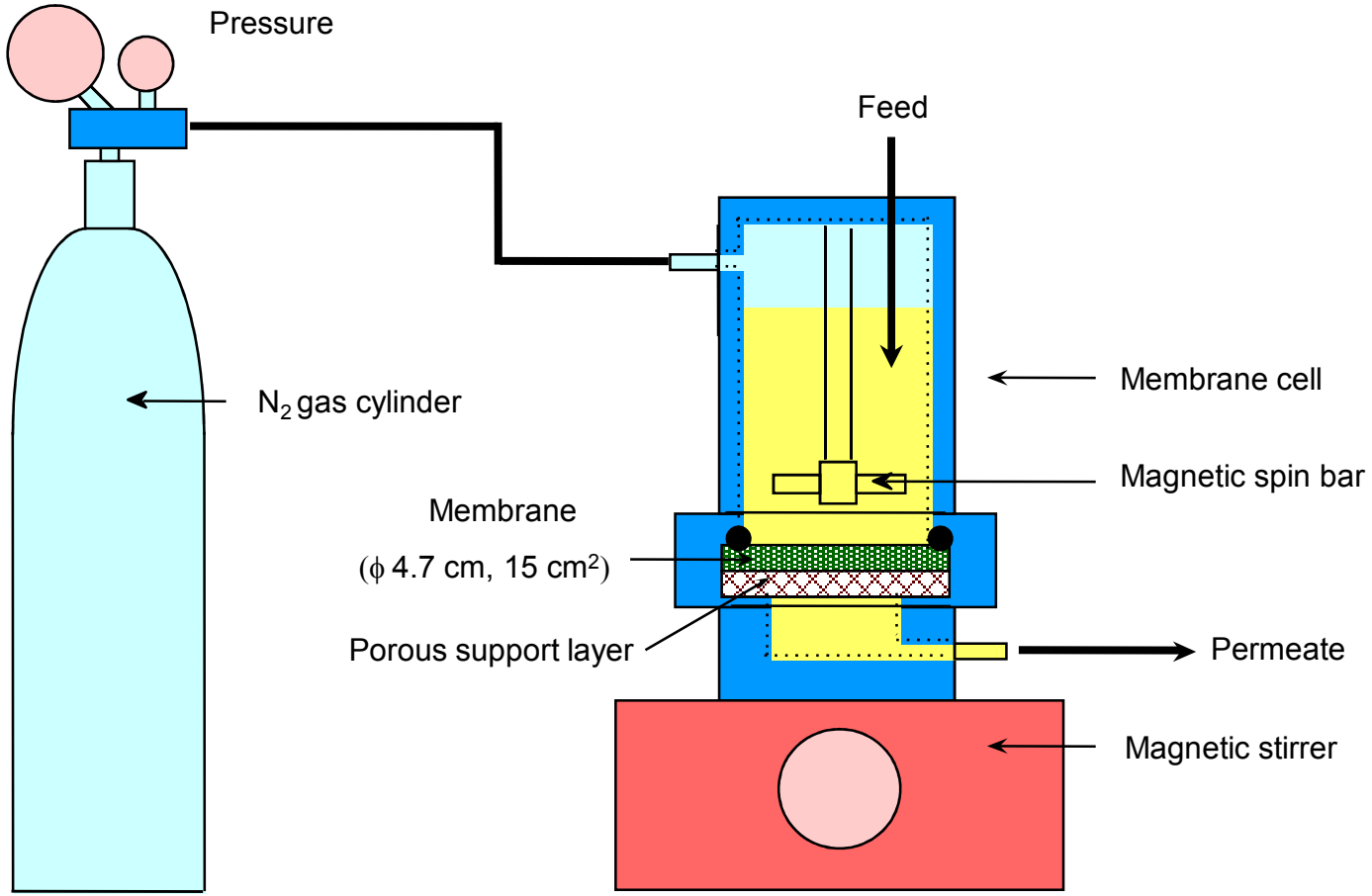


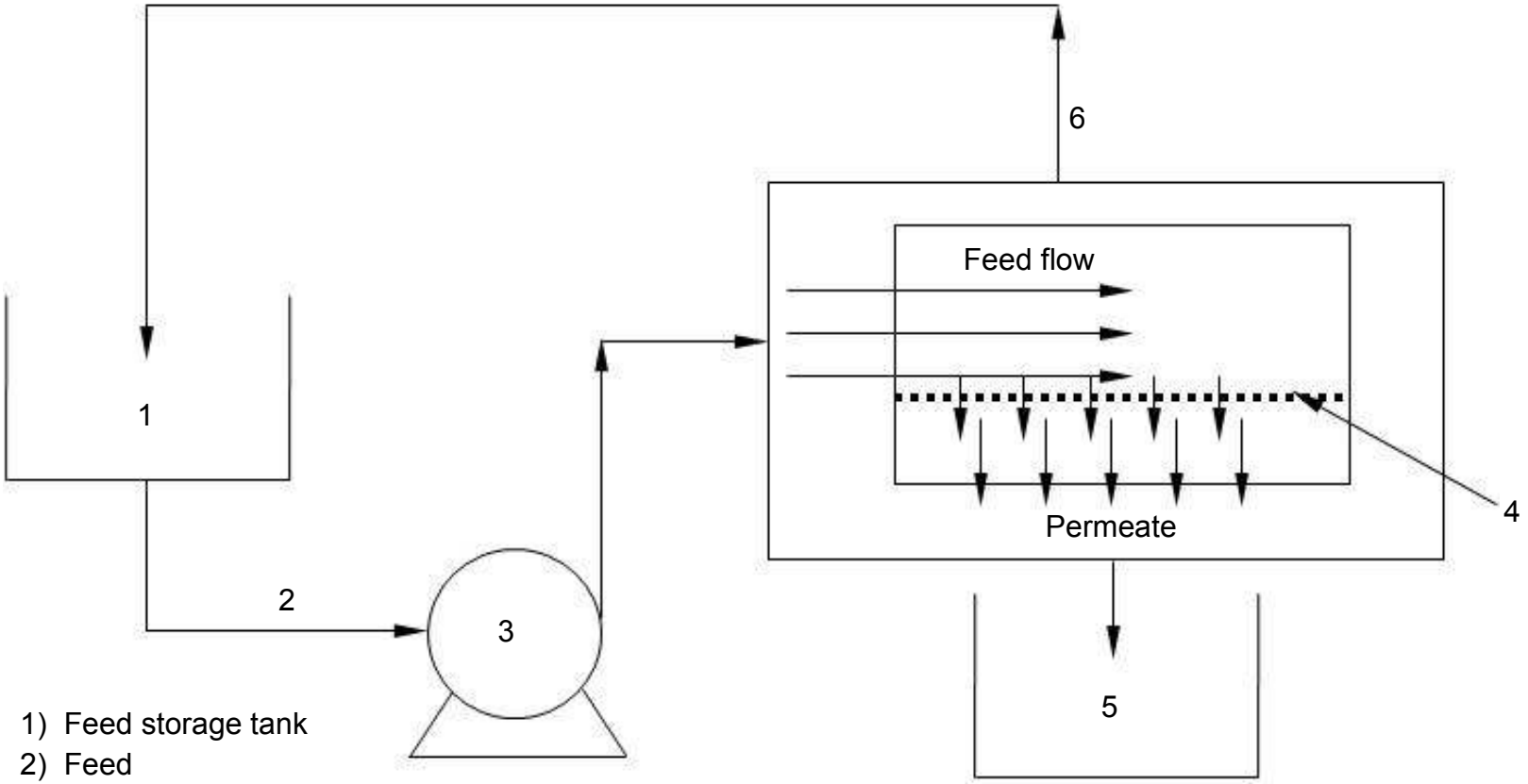
Fig. 2.1 Self-stirred flat membrane test cell

adsorption to membrane surface. The effect of stirring on loss of PG activity and protein was studied at various speeds (0, 200, 400, 600 and 800 rpm) up to 3 h of operation at room temperature (28±2°C). Fifteen millilitre culture broth containing PG was subjected to the chosen stirring speed and assays were carried out for every 1 h. Experimental runs were also conducted with BSA solution (model) at a concentration of 0.02% equal to the protein content of the SmF culture broth.

2.2.2.2 Laboratory cross-flow filtration system

Experiments were conducted in a cross-flow flat membrane cell (Model: C10-T, M/s Nitto Denko Corporation, Kusatsu, Japan) fitted with a peristaltic pump (M/s Bredel Hose Pumps B.V., Delden, Netherlands), pressure gauge and pressure/flow control valve as illustrated in Fig. 2.2. Pressure applied depended on the type of membrane process (MF: 0.1 MPa; UF: 0.5 MPa) and was adjusted using the pressure regulating valve (needle valve). The hold-up volume in the system was ~200 ml. The unit was operated in batch mode for process studies and the experimental run was stopped upon achieving the desired VCR. It was also operated under recirculation mode for assessing flux and solute permeability. Flux measurement and membrane cleaning were carried out as described earlier (section 2.2.2.1).

The pumping effect or shear stress on enzyme activity was studied in the cross-flow filtration system (Model: C10-T, M/s Nitto Denko Corporation) fitted either with a gear pump (M/s Tuthill Corporation, California, USA) or peristaltic pump (M/s Bredel Hose Pumps B.V.), pressure gauge and pressure/flow control valve but without fitting the membrane module.



1) Feed storage tank
2) Feed
3) Feed pump
4) Membrane
5) Permeate
6) Retentate

Fig. 2.2 Cross-flow filtration system

Accordingly, 400 ml culture broth was subjected to pumping up to 3 h of operation at room temperature. Enzyme activity and protein were assayed for every 30 min. The unit was operated in recirculation mode at a flow rate of 0.97 l/min. Experimental run with BSA model solution was conducted only with the gear pump.

2.2.2.3 Pilot scale filtration systems

Scale up experiments on MF (M/s Techniques Industrielles Appliquees, Bollene, France) and UF (M/s PCI Membrane Systems Ltd.) process steps were conducted in cross-flow pilot membrane systems. MF system was fitted with a rotary lobe pump (M/s Hilge Pumps Ltd., West Sussex, England) while the UF system with a triplex plunger pump (M/s CAT pumps Ltd., Minneapolis, USA), for developing the required pressure for the process. These systems were also fitted with a heat exchanger to maintain the temperature. Pressure applied for MF (0.1 MPa) and UF (0.5 MPa) system was adjusted using the pressure control valve (diaphragm valve). The hold-up volume in both MF and UF system was ~6.5 l. The experimental runs were conducted in batch mode and stopped upon achieving the desired VCR. Flux measurement and membrane cleaning were carried out as described earlier (section 2.2.2.1).

2.2.3 Integrated membrane processing

Crude culture broth containing PG was filtered through ordinary filter paper. The filtrate obtained was subjected to MF followed by UF in diafiltration mode (Fig. 2.3). The discontinuous diafiltration was performed with 100% dilution in two steps after the primary run. Initially, the unit was charged with 50 ml, 1 l

and 50 l of culture broth in self-stirred filtration, laboratory cross-flow filtration and pilot scale filtration systems, respectively and processed until the desired VCR was reached. Then the retentate fraction was diluted with 0.85% NaCl solution (50% of initial feed volume) and the experimental run was continued as before and stopped upon permeation of same quantity as that of addition. In a similar manner the second step of diafiltration was conducted.

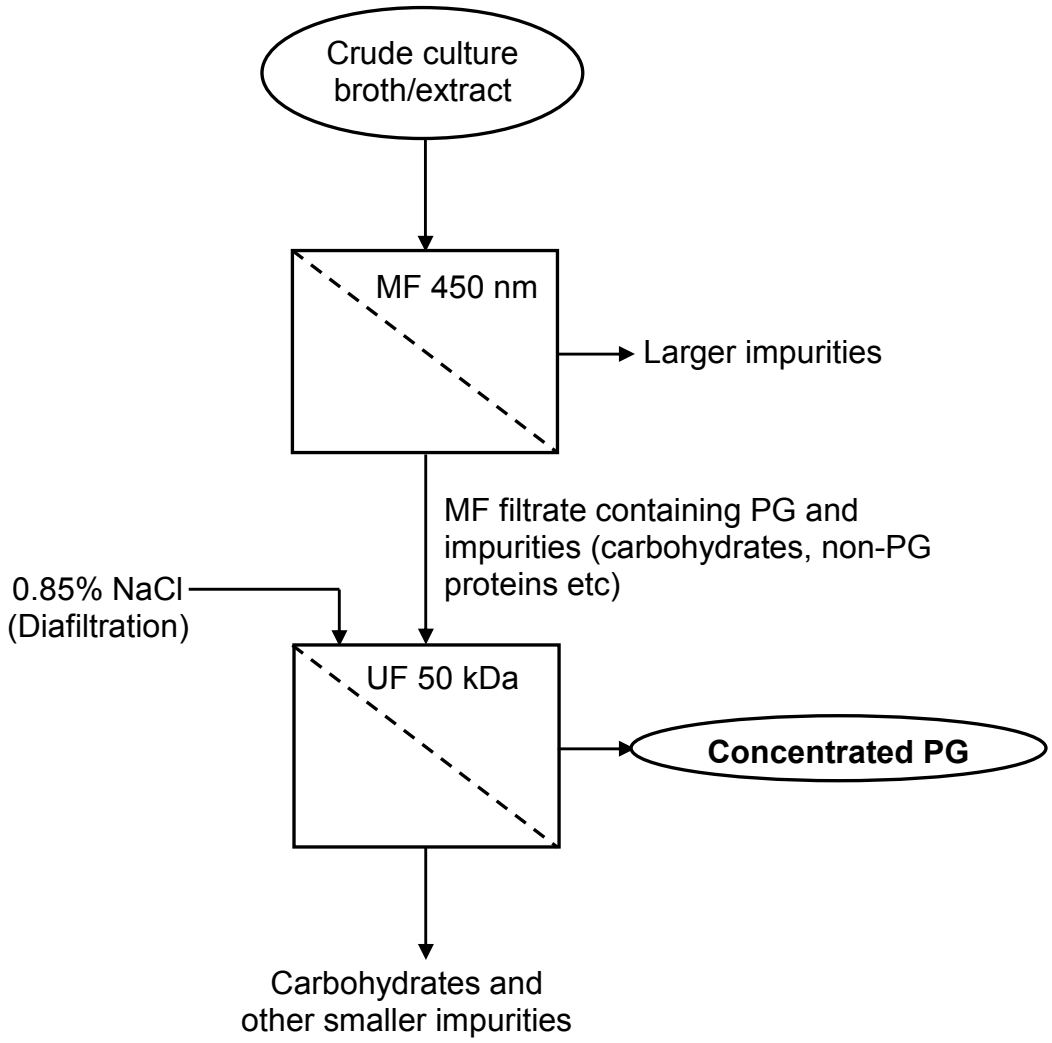


Fig. 2.3 Scheme for integrated membrane processing

2.2.4 Alginate affinity purification

Crude culture extract or culture broth (10 ml) was mixed with 20 ml of 0.5% sodium alginate prepared in distilled water (pH 3.8). The mixture was stirred

for 5 min, allowed to stand for 30 min at 30°C and the alginate enzyme complex was precipitated by adding 20 ml of 0.02 M $CaCl_2$ (pH 3.8). The mixture was allowed to stand undisturbed for 15 min at 30°C for uniform precipitate formation. The precipitate was separated by centrifugation at 3000 x g for 15 min and washed with 20 ml of 0.02 M $CaCl_2$ (pH 3.8) for three times (Gupta *et al.*, 1993). The scheme for AAP is shown in Fig. 2.4.

The enzyme from the alginate precipitate was eluted using three different eluting conditions. Elution-A was carried out using 10 ml of pH adjusted distilled water (pH 5.9) containing 1 M NaCl and 0.2 M $CaCl_2$ for four times (Gupta *et al.*, 1993). In Elution-B, the enzyme alginate precipitate was dissolved in 15 ml of 1 M NaCl (pH 4.3) and stirred for 5 min, allowed to stand for 18 h at 4°C and the alginate was precipitated by the addition of 15 ml of 0.2 M $CaCl_2$ of pH 4.3 (Mondal *et al.*, 2004). Elution-C followed was similar to Elution-B while the incubation time (2 h) and temperature (30°C) were modified. Concentration of Ca^{2+} greater than 0.01 M reduced the binding of the enzyme to alginate beads (Smidsrod and Sjak-Braek, 1990). Therefore, the eluted aliquots were dialyzed against pH adjusted distilled water (pH 4.3) and equilibrated by dialysis against 0.1 M sodium acetate buffer (pH 4.3) at 4°C. The enzymes in the dialyzed aliquots were concentrated by osmotic effect by immersing the dialysis tube in PEG-20000. PEG was reused by recovering from its aqueous solution by preferential dissolution into chloroform followed by removal of chloroform in a flash evaporator.

2.2.4.1 Pretreatment using charcoal

The culture extract was treated with activated charcoal at various

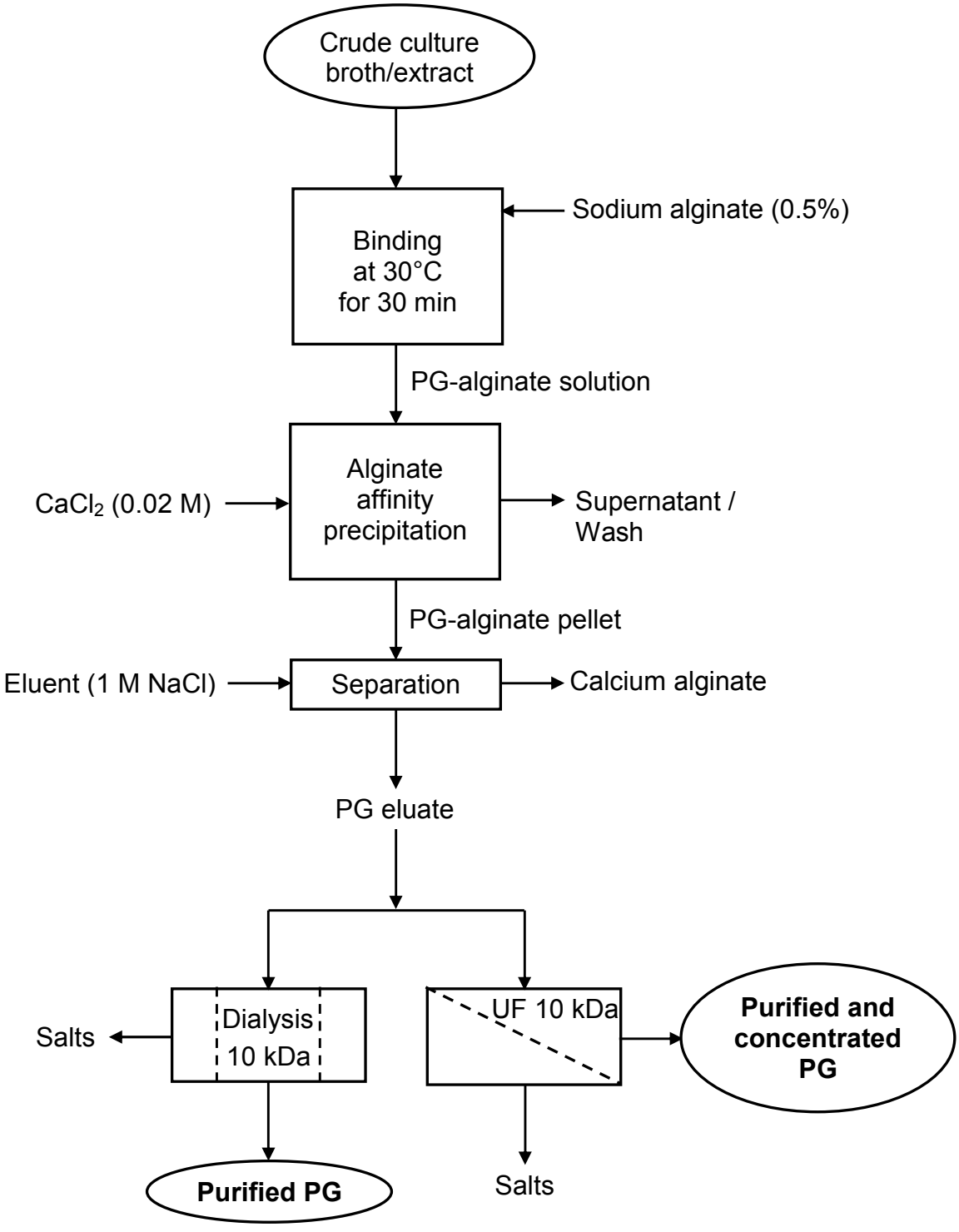


Fig. 2.4 Scheme for alginate affinity purification

concentrations (1, 5, 10, 20, 30, 40 and 50 mg/ml). Accordingly, a predecided quantity of activated charcoal was added to 50 ml culture extract and mixed

for 5 min at 200 rpm. The treated culture extract was filtered through Whatman No. 1 filter paper to remove the colour compounds adsorbed to the activated charcoal. The pretreated extract thus obtained was subjected to AAP as described above.

2.2.4.2 Desalting using UF

The cell was initially charged with 50 ml eluate containing PG and processed using a 10 kDa membrane until a desired VCR of 10 was reached. Then the retentate fraction was subjected to discontinuous diafiltration as described earlier (section 2.2.3).

2.2.5. Determination of shear stress in a stirred cell

The shear stress in a simulated stirred cell was determined using the following equations as proposed by Kosvintsev *et al.* (2005).

$$\tau = 0.825\ \mu\ \omega\ r\ \frac{1}{\delta} \qquad \text{when } r < r_c \qquad (1)$$

$$\tau = 0.825\ \mu\ \omega\ r_c \left(\frac{r_c}{r}\right)^{0.6} \frac{1}{\delta} \qquad \text{when } r > r_c \qquad (2)$$

Where τ is the shear stress (Pa), μ is the dynamic viscosity of culture broth (1.333×10^{-3} Pa·s), ω is the angular velocity (rad/s), r is the radius of stirrer (half length of magnetic bead, 0.015 m), r_c is the critical radius of the stirrer (m) and δ is the momentum boundary layer thickness (m).

Critical radius and momentum boundary layer thickness were calculated using the equations (3) and (4), respectively (Kosvintsev *et al.*, 2005).

$$r_c = \frac{D_i}{2} 1.23 \left(0.57 + 0.35 \frac{D_i}{D_t} \right) \left(\frac{h}{D_t} \right)^{0.036} n_b^{0.116} \frac{Re_i}{1000 + 1.43 Re_i} \quad (3)$$

$$\delta = \sqrt{\frac{\mu}{\rho \omega}} \quad (4)$$

Where D_i is the stirrer diameter (length of magnetic bead, 0.03 m), D_t is the stirred cell diameter (0.036 m), h is the stirrer height (diameter of magnetic bead, 0.008 m), n_b is the number of stirrer blades (2), Re_i is the impeller Reynolds number and ρ is the density of culture broth (1046 kg/m^3).

2.2.6 Resistance-in-series model

The permeate flux decline with time was analyzed by a resistance-in-series model generally as described by Rai *et al.* (2007). At any time, the permeate flux could be expressed in terms of total resistance,

$$J = \frac{\Delta P}{\eta R_t} \quad (1)$$

$$\Delta P_{Dead\ end} = P_{inlet} - P_{permeate} \quad (2)$$

$$\Delta P_{Cross\text{ - }flow} = \frac{P_{inlet} + P_{outlet}}{2} - P_{permeate} \quad (3)$$

Where, P is pressure, ΔP is transmembrane pressure, η is permeate viscosity, R_t is the total resistance. Total resistance, R_t is composed of three resistances as,

$$R_t = R_m + R_c + R_f \quad (4)$$

Where, R_m is the intrinsic membrane resistance, R_c is the cake layer resistance and R_f is the fouling resistance. The intrinsic membrane resistance (R_m) was calculated by measuring the pure water flux as,

$$R_m = \frac{\Delta P}{\eta_w J_w} \quad (5)$$

Where, η_w was the viscosity of water and J_w was the water flux. The membrane was rinsed thoroughly with distilled water after each run and the permeate flux using distilled water was measured at the same pressure and stirring speed/flow rate employed in the process. The resistance thus estimated accounts for irreversible fouling due to the adsorption of the particles present in the crude culture broth on to the membrane. The absolute R_f was then calculated using the following equation:

$$R_f = \frac{\Delta P}{\eta_w J_f} - R_m \quad (6)$$

The resistance caused by the cake layer was estimated using the following equation:

$$R_c = R_t - R_m - R_f \quad (7)$$

2.2.7 Enzymatic extraction and clarification of apple juice

Apples purchased from local market were washed, peeled and cut into small pieces. The cut apples were mashed using a food processor (Model: Slimline, M/s Chhaya Industries, Valsad, India) for 30 s with addition of ascorbic acid (0.5 g/kg). Commercial PG-1 was added at a level of 0.5 to 10 U/g of apple mash and incubated for maceration at 30, 40 and 50°C for 2 and 4 h for optimizing the process conditions. After the preliminary studies with commercial PG, the efficacy of *A. carbonarius* PG was tested at 2.5 and 5 U/g of apple mash at 30 and 50°C for 2 h. After enzymatic treatment, the mash was heated to 90°C for 5 min in a water bath to inactivate the enzymes. Juice from the mash was separated by centrifugation at 2200 x g for 10 min and

analysed for yield, clarity, alcohol insoluble solids, colour, total soluble solids, pH, viscosity and density. The scheme for enzymatic extraction and clarification of apple juice is shown in Fig. 2.5.

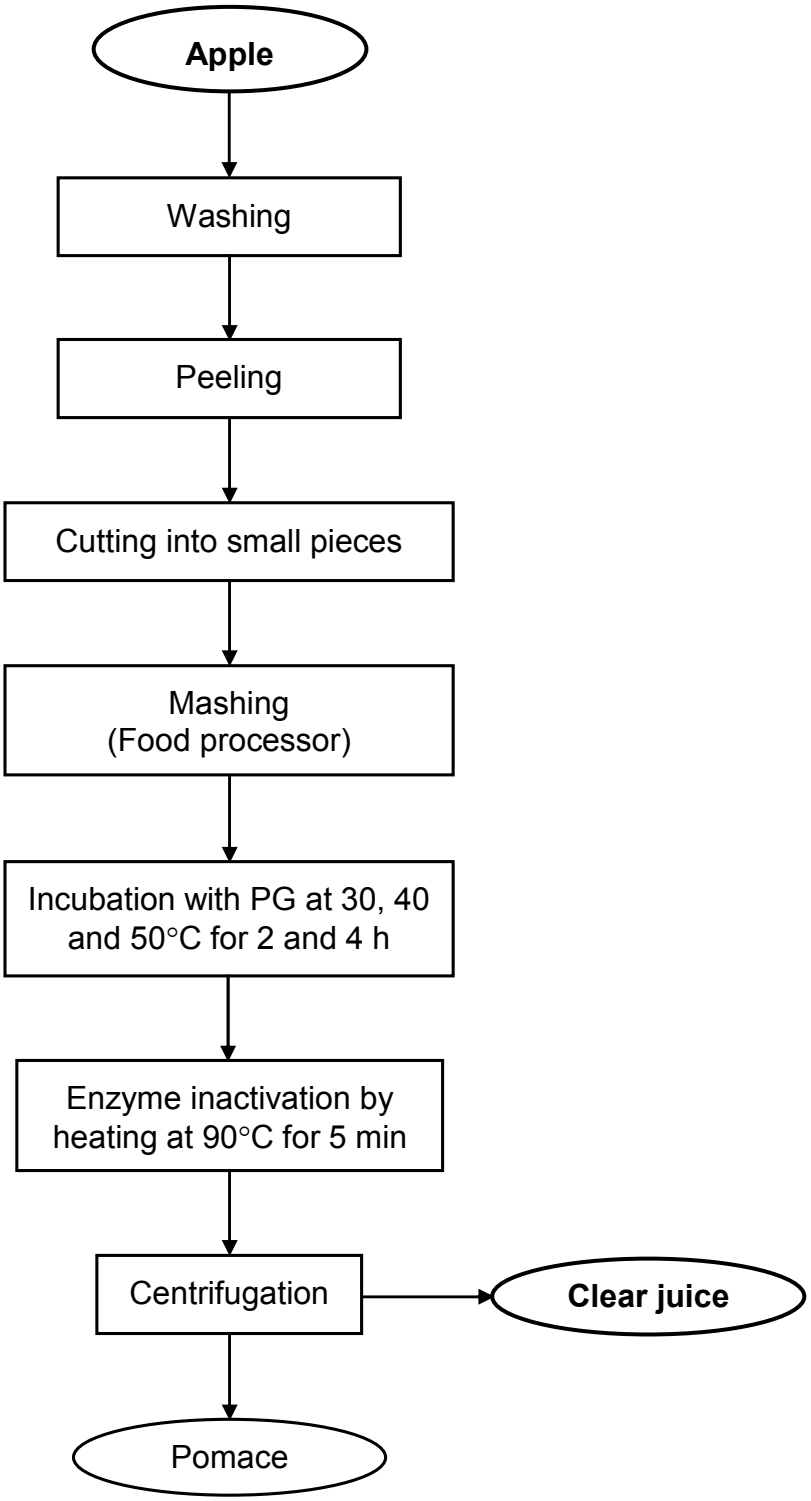


Fig. 2.5 Process flow diagram for enzymatic extraction and clarification of apple juice

2.2.8 Assays

UV-VIS spectrophotometer (UV-160A, M/s Shimadzu Scientific Instruments Inc., Kyoto, Japan) was used for all spectrophotometric analyses.

2.2.8.1 Polygalacturonase assay

PG activity was determined using 0.5% (w/v) polygalacturonic acid (sodium salt) prepared in 0.1 M sodium acetate buffer (pH 4.3) as substrate. Reducing sugars released due to enzyme activity were determined as galacturonic acid equivalents by spectrometric analysis, according to the method of Nelson (Nelson, 1944) and Somogyi (Somogyi, 1952) against galacturonic acid standards.

Polygalacturonic acid substrate (2 ml) equilibrated to 50^{o}C in a water bath was treated with 50 µl of suitably diluted enzyme and the reaction was allowed to proceed for 10 min. The reaction was stopped by drawing 100 µl and mixing with 1 ml freshly made alkaline copper sulphate solution. To this mixture, 900 µl of distilled water was added and kept in boiling water bath for 20 min. After cooling, the colour was developed with 1 ml arsenol molybdate reagent and the volume was made to 10 ml with distilled water. Galacturonic acid formed was estimated by determining absorbance at 540 nm read against enzyme blanks. Activity corresponded to µmole galacturonic acid released per min per ml of culture broth or one gram moldy bran.

Alkaline copper sulphate reagent

Solution A

Sodium carbonate (anhydrous) : 2.5 g

Sodium bicarbonate : 2.5 g

Sodium potassium tartarate : 2.5 g

Solution B

Copper sulphate pentahydrate : 15 g

Concentrated sulphuric acid : 1-2 drops

These constituents were dissolved in distilled water separately for solutions A and B, volumes made to 100 ml and stored at room temperature in stoppered brown bottles. Alkaline copper sulphate reagent was prepared fresh for assay by mixing solutions A and B in the ratio of 25:1.

Arsenol molybdate reagent

Ammonium molybdate : 5 g

Concentrated sulphuric acid : 4.2 ml

Sodium arsenate : 0.6 g

The above constituents were dissolved in distilled water and the volume made to 100 ml. The solution was incubated at 37^{o}C on an orbital shaker overnight. The reagent was stored at 4^{o}C in a stoppered brown bottle.

2.2.8.2 Amylase assay

Amylase activity was determined using 2% starch (w/v) prepared in 0.1 M sodium acetate buffer (pH 4.3) as substrate. Reducing sugars released due to enzyme activity were estimated by dinitrosalicylic acid method (Bernfeld, 1955) against maltose standards.

Starch substrate (5 ml) equilibrated to 40°C in a water bath was treated with 1 ml of suitably diluted enzyme and the reaction was allowed to proceed

for 30 min. The reaction was stopped by drawing 1 ml and mixing with 1 ml of 4 M NaOH. To 1 ml of this mixture, 1 ml of dinitrosalicylic acid reagent was added and kept in boiling water bath for 5 min. After cooling, the volume was made to 7 ml with distilled water. Maltose equivalents formed was estimated by determining absorbance at 540 nm read against enzyme blanks. Activity corresponded to µmole maltose released per min per ml of culture broth or one gram moldy bran.

Dinitrosalicylic acid reagent

3, 5 - dinitrosalicylic acid	:	10 g
Sodium hydroxide (anhydrous)	:	16 g
Sodium potassium tartarate	:	300 g

The above constituents were dissolved in distilled water and the volume made to 1 l. The reagent was stored at room temperature in a stoppered brown bottle.

2.2.8.3 Acid protease assay

Protease activity was determined using 2% (w/v) casein prepared in distilled water (pH 2.7) as substrate. Reducing sugars released due to enzyme activity were estimated as tyrosine equivalents using Folin-Ciocalteau Phenol reagent (Ichishima, 1970) against tyrosine standards.

Casein substrate (1 ml) equilibrated to 30°C in a water bath was treated with 0.2 ml of suitably diluted enzyme and the reaction was allowed to proceed for 30 min. The reaction was stopped by adding 1.2 ml of 0.4 M trichloroacetic acid and the reaction mixture was filtered through Whatman

No. 1 filter paper. To 0.5 ml of filtrate, 2.5 ml of 0.4 M sodium carbonate solution and 0.5 ml Folin-Ciocalteau Phenol reagent (1:5 diluted with water) were added and incubated at 30°C for 30 min. The tyrosine equivalents released was estimated by determining absorbance at 660 nm read against enzyme blanks. Activity corresponded to µmole tyrosine released per min per ml of culture broth or one gram moldy bran.

Casein (2%)

Casein (2 g) was suspended in 10 ml of distilled water for 15 min at 30°C. To this, 60 ml water and 1.5 ml of 1 N HCl were added and the casein was dissolved by stirring the suspension on boiling water bath for 15 min. After cooling to room temperature, the volume was made to 100 ml with water and the pH was adjusted to 2.7 using 0.1 N HCl.

2.2.8.4 Estimation of protein

Protein concentrations were determined by the dye binding method (Spector, 1978) using Coomassie Brilliant Blue G 250. The detection reagent for this assay consisted of Coomassie Brilliant Blue G 250 prepared in 3% perchloric acid (optical density of the prepared reagent at 465 nm was adjusted between 1.3–1.5). Protein was quantitated by adding appropriately diluted sample to 1.5 ml of the reagent and making up the volume to 3 ml with distilled water. Optical density was measured at 595 nm against blank. Bovine serum albumin was used as standard. Specific activity of enzymes in liquid cultures corresponded to activity estimated for one milligram protein.

2.2.8.5 Electrophoresis

SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis) was performed in 10% gels (Laemmli, 1970). The protein samples for loading the gel were prepared in sample buffer containing dithiothreitol. The proteins were visualized by silver staining (Morrisey, 1981). Molecular mass of proteins was determined by running the following standards alongside the protein samples in SDS-PAGE.

Molecular mass standards	kDa
Phosphorylase b	97.4
Bovine serum albumin	66.0
Ovalbumin	43.0
Carbonic anhydrase	29.0
Soyabean trypsin inhibitor	20.1
Lysozyme	14.3

2.2.8.6 Estimation of total carbohydrates

Total carbohydrate was estimated by the phenol-sulphuric acid method using dextrose as standard. 0.3 ml of 5% phenol and 1.8 ml of concentrated sulphuric acid was added to the sample and made up the volume to 2.6 ml using distilled water and kept for 20 min at room temperature. Optical density was measured at 480 nm against blank (Rao and Pattabiraman, 1989).

2.2.8.7 Estimation of inorganic phosphate

The sample mixture (0.3 ml) containing inorganic phosphate was added to 0.7 ml of solution (1 part of ascorbic acid and 6 parts of ammonium molybdate),

and kept at 45°C for 20 min. It was cooled to room temperature and absorbance was read at 820 nm against blank. Potassium dihydrogen phosphate was used as standard (Ames, 1966).

Ascorbic acid

Dissolve 10 g of ascorbic acid in 100 ml of distilled water and store at 4°C.

Ammonium molybdate

Ammonium molybdate tetrahydrate : 0.42 g

Concentrated sulphuric acid : 2.86 ml

The above constituents were dissolved in distilled water, volume made to 100 ml and stored at room temperature in a stoppered brown bottle.

2.2.8.8 Estimation of total acids

Sample was titrated with 0.1 N sodium hydroxide using 1-2 drops of phenolphthalein indicator. The end point is the colour change from colorless to light pink. These results are calculated as percent anhydrous citric acid (Ranganna, 2003).

2.2.8.9 Estimation of juice yield

The juice yield was determined using the following equation.

$$\text{Juice yield (\%)} = \frac{\text{Final juice weight}}{\text{Initial pulp weight}} \text{ x } 100$$

2.2.8.10 Clarity and turbidity measurements

Clarity of juice was measured as percent transmittance according to the method of Krop and Pilnik (1974). Percent transmittance was determined at 660 nm in the spectrophotometer.

Turbidity of juices was measured using a benchtop turbidity meter (Model: Cyberscan Turbidimeter TB1000, M/s Eutech Instruments Pte Ltd., Singapore) and expressed in nephelometric turbidity unit (NTU). It is the measurement of optical clarity based on light scattering technique. The greater the intensity of the scattered light, the higher is the turbidity and lower is the clarity.

2.2.8.11 Estimation of alcohol-insoluble-solids

The content of pectinnious materials present in the apple juice was measured in terms of alcohol-insoluble-solids (AIS). AIS were determined by boiling 5 g juice with 75 ml of 80% ethanol, simmering for 30 min and filtering through Whatman No. 1 filter paper. The filtered residue was washed again with 80% ethanol. The residue was dried at 100°C for 2 h and expressed in percentage by weight (Hart and Fisher, 1971).

2.2.8.12 Hunter colour values

Colour of juice was measured using a Hunter Lab Colour Measuring System (Model: Labscan XE, M/s Hunter Associates Laboratory Inc., Reston, USA) at 2°/C angle of illumination (Garg *et al.*, 2009). Values were measured in terms of lightness (L) and colour (+a: red, −a: green, +b: yellow, −b: blue).

2.2.8.13 Determination of viscosity, density and total soluble solids

Viscosity was measured using a glass Oswald capillary viscometer (M/s Borosil Glass Work Ltd., Mumbai, India) at a constant water bath temperature of 30±2°C. Density was determined using a specific gravity bottle (M/s Borosil Glass Work Ltd.). Total soluble solids (TSS) in juice were measured using a hand held refractometer (Model: RHB-32(ATC), M/s HTA Instrumentation Pvt. Ltd., Bangalore, India) and expressed as °Brix.

2.2.8.14 pH measurement

pH was measured using a digital glass electrode pH meter (M/s Control Dynamics Instrumentation Pvt. Ltd., Bangalore, India). It was calibrated using buffer solutions of pH 4.0 and 7.0 at room temperature (28±2°C).

2.2.9 Statistical analysis

The data obtained on apple juice extraction and clarification were analyzed using one-way analysis of variance (ANOVA) and means for each pair were compared at 5% significance level by using t-test, in Microsoft Excel Data Analysis Tool Pack. All other experiments were carried out in triplicate and samples were analyzed in triplicate, and the mean values are reported.

2.2.10 Performance parameters

The performance of the membrane process was expressed as percentage observed rejection (R_o) of each component. R_o was determined, assuming that it was constant for each batch experiment (Cheryan, 1998). Other performance parameters of MF and UF were determined as follows:

$$R_o = \frac{\ln (C_R / C_F)}{\ln (V_F / V_R)} \times 100 \quad (1)$$

$$\text{Volume concentration ratio (VCR)} = \frac{V_F}{V_R} \quad (2)$$

Microfiltration

$$\text{Recovery (\%)} = \frac{C_P V_P}{C_F V_F} \times 100 \quad (3)$$

$$\text{Elimination (\%)} = \frac{C_F V_F - C_P V_P}{C_F V_F} \times 100 \quad (4)$$

Ultrafiltration

$$\text{Recovery (\%)} = \frac{C_R V_R}{C_F V_F} \times 100 \quad (5)$$

$$\text{Elimination (\%)} = \frac{C_F V_F - C_R V_R}{C_F V_F} \times 100 \quad (6)$$

Where C_F, C_P and C_R are the enzyme activity (U/ml) or protein content (µg/ml) or carbohydrate content (mg/ml) or phosphate salts (mg/ml) or total acids (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, salts and acids in the multi-step process were estimated with reference to the crude culture broth or culture extract.

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

2.2.11 Economic analysis

The base-case pectinase plant was designed to produce 30 kl purified PG concentrate (3000 U/ml) in 300 working days per year. Plant design and equipment sizing were worked out based on the laboratory process performance parameters keeping a high level utilization of process equipment. Specifications were drawn for major equipment and cost estimates obtained from a plant and equipment supplier. Six-tenths rule was applied wherever necessary for estimating the price of equipment (Sinnott, 1983). The prices of raw materials were obtained from chemical catalogues or directly from suppliers. Ceramic (MF) and polymeric membranes (UF and Reverse osmosis, RO) were considered to have a life-time of 5 and 1 years respectively. Accordingly, replacement costs were built in to the cost of consumables. The activity of the concentrate product was kept close to commercial pectinase concentrates (~3000 U/ml) and the conservative selling price applied in the revenue calculations was ~20% of the price of an equivalent commercial product (INR 22000/l, net price without taxes). Total capital cost, total product cost and unitary product cost were worked out individually for upstream and downstream processing sections based on methodologies proposed by Peters and Timmerhaus (1991).

3.0 Significance and focus of the work

SmF and SSF are employed industrially for microbial production, both the processes claiming relative merits. Enzyme production by microbial cultivation generally involves use of low cost starchy substrates in growth media and the fermented broths usually contain high concentrations of target protein along with a mixture of media components, especially carbohydrates and salts. Elimination of carbohydrates from culture broth is essential that would otherwise trap the enzymes during cold storage and interfere with the purification processes leading to enzyme loss (Singh *et al*., 1999). The difference in molecular weight of PG and carbohydrates suggested the application of membrane process for the elimination of impurities. In the present work, SmF and SSF processes were assessed for the production of PG from *A. carbonarius*. Also attempts were made for the elimination of carbohydrates and other non-protein impurities from culture broth containing PG employing MF and UF membranes.

3.1 Performance assessment of upstream processes for *A. carbonarius* PG production

The performance of SmF and SSF processes for the production of *A. carbonarius* PG are presented in Table 3.1. The fermentation period by SmF was only 24 h as against 60 h by SSF while the PG activity (U/ml) was higher in SmF (230 U/ml) compared to SSF (89 U/ml). Generally, SSF provides a system with higher productivity due to larger enzyme yields and shorter fermentation periods (Solis-Pereira *et al.*, 1993). In the present study, the yield of PG (U/ml) obtained in

Table 3.1 Production of *A. carbonarius* PG by SmF and SSF[a]

Description	SmF	SSF
Main substrate	Corn flour	Wheat bran
Fermentation period (h)	24	60
Broth/extract activity (U/ml)	230	89[b]
PG specific activity (U/mg)	1190	540
PG activity (U/g of substrate)	9200	890
PG activity (U/g of starch)[c]	13100	4940
Productivity ((U/ml)/h)	9.6	1.5
Productivity ((U/g starch)/h)	548	82.4

[a]SmF-Submerged fermentation; SSF-Solid state fermentation; PG-polygalacturonase.

[b]Under recommended extraction conditions (moldy bran:acetate buffer 1:10).

[c]Starch content in corn flour and wheat bran were 70% and 18%, respectively (Ghildyal *et al.*, 1985).

SmF was 2.6 times higher than SSF while the yield was much higher to the extent of 10.3 fold when compared in terms of substrate quantity used. In absolute measure, one gram of starch yielded 13100 U by SmF as against 4940 U obtained in SSF process indicating that the utilization of starch for the production of enzyme was 2.7 times higher in SmF process. Higher yield together with shorter fermentation period resulted in 6.6 times greater productivity favoring SmF for PG production over SSF. The higher PG productivity obtained by SmF could be attributed to the use of mutant *A.*

carbonarius that was more effective in glucose regulation (Venkatesh, 2004).

3.2 Microfiltration of culture broth

The culture broths used for the study contained 103 and 113 U/ml PG. The 42 kDa high active enzyme (Devi and Rao, 1996) was the predominant protein in the culture broths (Fig. 3.1). Thus these culture broths showed a specific activity of 1140 and 1470 U/mg protein, respectively. The broth contained very little contaminating proteins and ~4.72 mg/ml total carbohydrates. The removal of these impurities by centrifugation at 10000 x g for 15 min at 4°C resulted in only 14–16% reduction in polysaccharides, suggesting the necessity for a much more effective method for their elimination. Keeping in view of the fact that feed containing proteinaceous substances such as the culture broth have a tendency to form a dynamic layer during membrane processing (Barhate *et al*., 2003), attempts were with MF membranes for the elimination of carbohydrates. In the MF experiments, membranes of four different pore sizes (70, 150, 200 and 450 nm) were screened for carbohydrate elimination from culture broth in order to obtain higher recovery and productivity (permeate flux) of PG.

3.2.1 Microfiltration of centrifuged culture broth

The experimental data (Table 3.2) was in conformity with the behavior of porous membranes in that the rejection of PG and protein were higher with 70 nm than with 150 and 450 nm membranes. Further, at the desired VCR of 5 (80% permeation), the membranes rejected 15-40% PG and 34-57% proteins while the

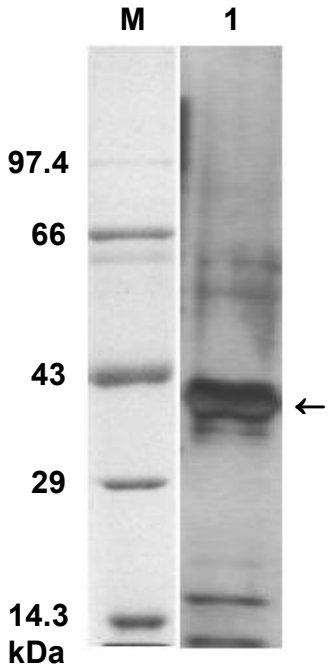


Fig. 3.1 SDS-PAGE showing crude culture broth proteins of mutant strain of *Aspergillus carbonarius.* Overproduction of 42 kDa polygalacturonase (←).

M – Molecular weight markers; 1 – Crude culture broth proteins.

carbohydrate rejection was only 10-21%. The retention performance of MF membranes is primarily controlled by the sieving effect with centrifuged culture broth. Greater recovery of 86% PG and 78% protein were obtained with 450 nm membrane owing to its large pore size. Although the smaller pore (70 and 150 nm) membranes eliminated 21-28% carbohydrates, enzyme recovery was below 69%. This suggested the use of diafiltration to improve the PG recovery during membrane processing.

Diafiltration was carried out up to 100% dilution in two steps using 0.85% NaCl solution in order to maintain the stability of proteins. The data on PG activity, protein and total carbohydrate contents during diafiltration are described

Table 3.2 Microfiltration (diafiltration) of centrifuged culture broth[a]

Membrane pore size	PG activity				Protein content				Carbohydrate content				VCR (Fold)	Avg. flux (l/m^2·h)	Specific activity (U/mg)	Activity/ dry solids (U/mg)
	R_o (%)	Total (U)		CR (%)	R_o (%)	Total (µg)		CR (%)	R_o (%)	Total (mg)		CE (%)				
		P	R			P	R			P	R					
70 nm																
MF	40	3640	2010	64	57	2010	1840	52	10	161	44	21	5	33		
DF-1		940	1070	81		640	1200	69		23	21	10	4	28		
DF-2		300	770	86		280	920	76		4	17	8	11	25	1670	2.8
150 nm																
MF	27	3920	1730	69	44	2280	1570	59	21	148	57	28	5	83		
DF-1		980	750	87		670	900	77		32	25	12	4	80		
DF-2		650	100	98		440	460	88		9	16	8	11	71	1640	3.4
450 nm																
MF	15	4840	810	86	34	3020	830	78	15	176	29	14	5	198		
DF-1		660	150	97		480	350	91		20	9	4	4	117		
DF-2		150	0	100		150	200	95		3	6	3	11	103	1550	3.2

[a]Pretreatment: Filtered through Whatman No.1 filter paper; Centrifuged at 10000 x g for 15 min at 4°C;

Feed volume: 50 ml; R_o – Percent rejection; CR – Cumulative recovery in the permeate;

CE – Cumulative elimination from the permeate; VCR – Volume concentration ratio; P – Permeate; R – Retentate; DF – Diafiltration.

Centrifugation gave ~100% recovery of PG and protein as well as 14-16% of carbohydrates elimination.

Total PG activity, protein content, and carbohydrate content in feed were 5650 U; 3850 µg and 205 mg, respectively.

Specific activity and activity/dry solids in feed were 1470 U/mg protein and 5.7 U/mg dry solids, respectively.

in Table 3.2. Although 86-100% PG could be recovered by this procedure, only 3-8% carbohydrates could be eliminated. There was only a slight improvement in the specific activity (1550-1670 U/mg protein) owing to the fact that the recovery of protein was close to that of PG. Use of salt and permeation of carbohydrates and probably other impurities along with the enzyme during MF resulted in PG activity of the dry solids (2.8-3.4 U/mg dry solids) comparable to the effective value of feed (3.7 U/mg dry solids).

3.2.2 Microfiltration of crude culture broth

The experimental data on PG and protein recovery, carbohydrate elimination and average permeate flux obtained during MF of culture broth not subjected to centrifugation are described in Table 3.3. The process rejected 41-64% PG, 68-72% proteins and 10-44% carbohydrates. These rejection values were higher than for the centrifuged culture broth (Table 3.2) owing to the presence of cell debris and suspended solids in the feed. Since the rejection of PG and carbohydrates was higher with large pore size membranes than with smaller pore size membrane, dynamic membrane or in other words secondary layer formation on the membrane surface owing to adsorption and deposition of substances contained in the feed (van Oers *et al*., 1995) appeared to aid the rejection rather than the actual pore size of the membrane (Barhate *et al*., 2003). Barhate *et al.* (2003) attributed the rejection of amylases (24 kDa) present in honey by a UF membrane (MWCO; 100 kDa) mainly to the formation of a dynamic active layer on the membrane surface. Highest recovery of PG was obtained with 70 nm

Table 3.3 Microfiltration (diafiltration) of crude culture broth[a]

Membrane pore size	PG activity				Protein content				Carbohydrate content				VCR	Avg. Flux	Specific activity	Activity/ dry solids
	R_o	Total (U)		CR	R_o	Total (µg)		CR	R_o	Total (mg)		CE				
	(%)	P	R	(%)	(%)	P	R	(%)	(%)	P	R	(%)	(Fold)	($l/m^2 \cdot h$)	(U/mg)	(U/mg)
70 nm																
MF	41	3160	1990	61	68	1800	2700	40	21	170	66	28	5	25		
DF-1		720	1270	75		500	2200	51		37	29	12	4	20		
DF-2		340	930	82		300	1900	58		15	14	6	11	19	1620	2.4
200 nm																
MF	64	2280	2870	44	72	1640	2860	36	10	181	55	23	5	133		
DF-1		720	2150	58		440	2420	46		37	18	8	4	88		
DF-2		530	1620	69		410	2010	55		14	4	2	11	85	1420	2.4
450 nm																
MF	62	2360	2790	46	69	1760	2740	39	44	140	96	41	5	152		
DF-1		830	1960	62		480	2260	50		27	69	29	4	95		
DF-2		800	1160	78		480	1780	60		10	59	25	11	91	1470	2.3

[a]Pretreatment: Filtered through Whatman No.1 filter paper; Feed volume: 50 ml; For abbreviations see Table 3.2.

Total PG activity, protein content, and carbohydrate content in feed were 5150 U; 4500 µg and 236 mg, respectively.

Specific activity and activity/dry solids in feed were 1140 U/mg protein and 4.6 U/mg dry solids, respectively.

followed by 450 and 200 nm membranes, which could probably be attributed to the nature of the membrane material. In the present study, large pore size membranes (200 and 450 nm) were hydrophilic (nylon 6,6) while 70 nm membrane was hydrophobic (PE) in nature. Besides, processing proteins and enzymes could cause pore plugging and fouling in higher pore membranes resulting in their lower transmission compared to lower pore membranes (Bailey and Meagher, 2000; Meagher *et al*., 1994). To aid higher recovery of PG in the process stream (permeate), diafiltration was attempted while processing the culture broth with a focus on 450 nm large pore size membrane. The data (Fig. 3.2) showed that diafiltration resulted in increased PG recovery (69-82%), however elimination of carbohydrates (2-25%) reduced drastically in all the three membranes.

3.3 Ultrafiltration of culture broth

The UF process with membranes of four different MWCO (10, 25, 100 and 500 kDa) using culture broth not subjected to centrifugation was in conformity with the general behavior of porous membranes in that the rejection of PG and protein was higher with 10 and 25 kDa membranes than with 100 and 500 kDa membranes (Table 3.4). At the desired VCR of 5, the 10 and 25 kDa membranes rejected 96-99% PG and 94-98% proteins while the carbohydrates rejection was only 13-22% with all the four membranes (Table 3.4). The elimination of 72-79% carbohydrates was higher with all the four membranes but greater recovery of 98% PG was obtained with 10 kDa membrane.

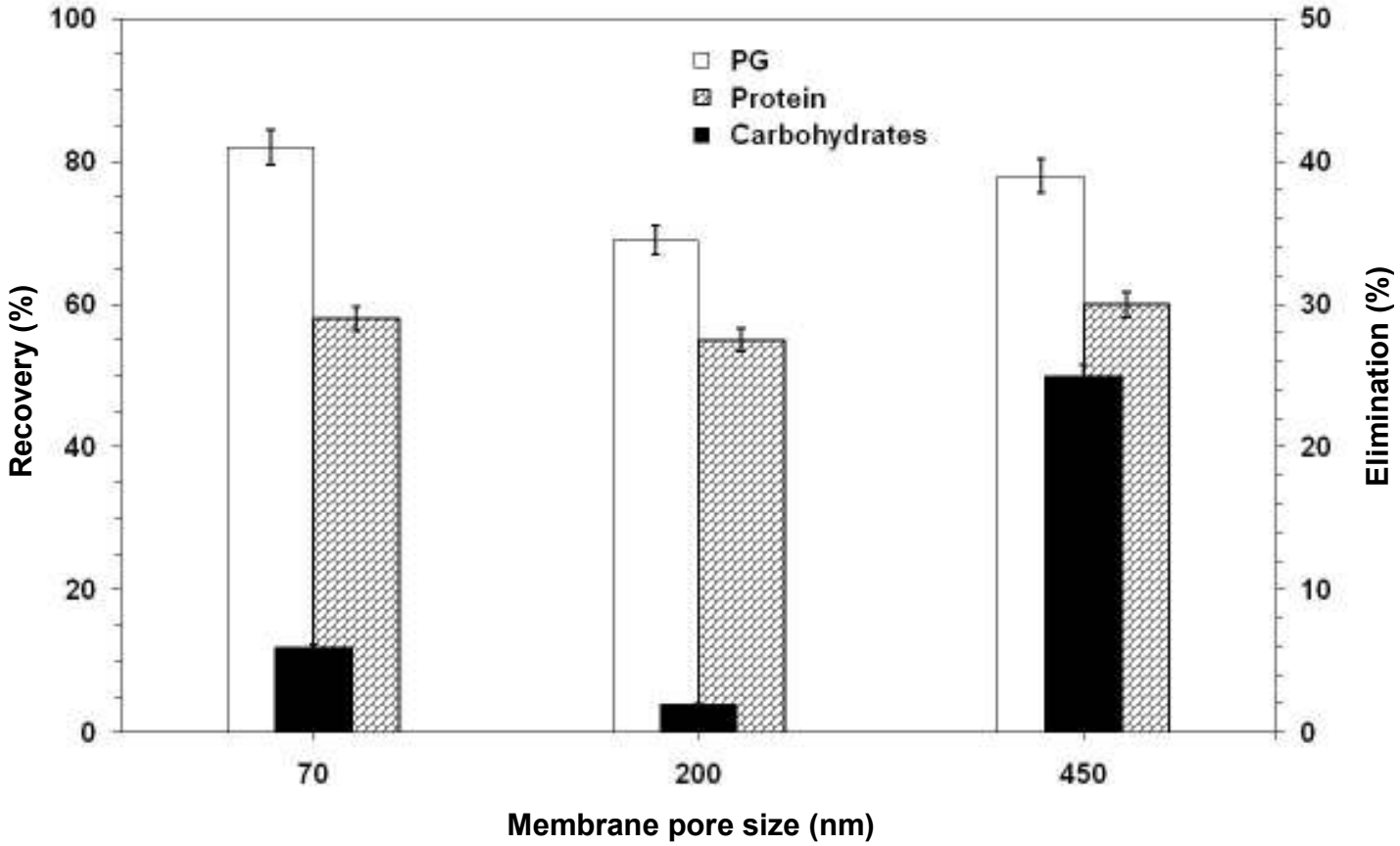


Fig. 3.2 Recovery of PG and protein and elimination of carbohydrates in the permeates during MF of culture broth in a self-stirred membrane cell (0.1 MPa, 30°C, 800 rpm and 100% diafiltration)

Table 3.4 Ultrafiltration (diafiltration) of crude culture broth[a]

	PG activity				Protein content				Carbohydrate content						Specific	Activity/
Membrane	R_o	Total (U)		CR	R_o	Total (µg)		CR	R_o	Total (mg)		CE	VCR	Avg. Flux	activity	dry solids
MWCO	(%)	P	R	(%)	(%)	P	R	(%)	(%)	P	R	(%)	(Fold)	(l/(m^2·h)	(U/mg)	(U/mg)
10 kDa																
UF	99	80	5070	98	98	120	4380	97	15	176	60	75	5	53		
DF-1		60	5010	97		80	4300	96		36	24	90	4	51		
DF-2		80	4930	96		40	4260	95		15	9	96	11	49	1160	49.4
25 kDa																
UF	96	320	4830	94	94	400	4100	91	22	169	67	72	5	60		
DF-1		280	4550	88		170	3930	87		42	25	89	4	57		
DF-2		340	4210	82		190	3740	83		16	9	96	11	55	1130	46.8
100 kDa																
UF	74	1760	3390	66	79	1280	3220	72	21	170	66	72	5	232		
DF-1		920	2470	48		440	2780	62		43	23	90	4	222		
DF-2		1090	1380	27		380	2400	53		16	7	97	11	210	580	23.0
500 kDa																
UF	73	1800	3350	65	77	1400	3100	69	13	186	50	79	5	245		
DF-1		980	2370	46		420	2680	60		33	17	93	4	227		
DF-2		1130	1240	24		380	2300	51		15	2	98	11	218	540	17.8

[a]Pretreatment: Filtered through Whatman No.1 filter paper; Feed volume: 50 ml;

CR – Cumulative recovery in the retentate; CE – Cumulative elimination from the retentate; For other abbreviations see Table 3.2.

Total PG activity, protein content, and carbohydrate content in feed were 5150 U; 4500 µg and 236 mg, respectively.

Specific activity and activity/dry solids in feed were 1140 U/mg protein and 4.6 U/mg dry solids, respectively.

Diafiltration was carried out as described earlier. Specific activity of PG did not improve with 10 and 25 kDa membranes (1160 and 1130 U/mg protein) while it reduced drastically with 100 and 500 kDa membranes (580 and 540 U/mg protein) compared with its feed (1140 U/mg protein), which revealed that some of the non-enzymatic proteins are larger in size than PG. Diafiltration with 10 kDa membrane eliminated 96% carbohydrates with 96% PG recovery (Table 3.4; Fig. 3.3). The above processing parameters also resulted in higher PG activity of 49.4 U/mg dry solids (effective feed value 3.7 U/mg dry solids). Considering the performance of 10 kDa membrane in terms of greater recovery of PG and elimination of carbohydrates with crude culture broth, the performance was further analysed.

3.4 Performance evaluation of 10 kDa UF membrane

The data on elimination of total carbohydrates, phosphate salts and total acids as well as PG recovery and average flux with crude and centrifuged culture broths using 10 kDa membrane are described in Table 3.5. The membrane eliminated 80% phosphate salts and acids besides carbohydrates. Diafiltration eliminated 96% carbohydrates, 99% phosphate salts and ~100% total acids with PG recovery of 96%. The PG activity of dry solids showed ~10.7 fold increase compared with the feed since carbohydrates and other non-protein impurities were eliminated (permeated) while the enzymes were retained (99%) by the membrane. This revealed that the majority of non-protein impurities are smaller in size than the PG and proteins. SDS-PAGE confirmed the presence of 42 and 61 kDa PG

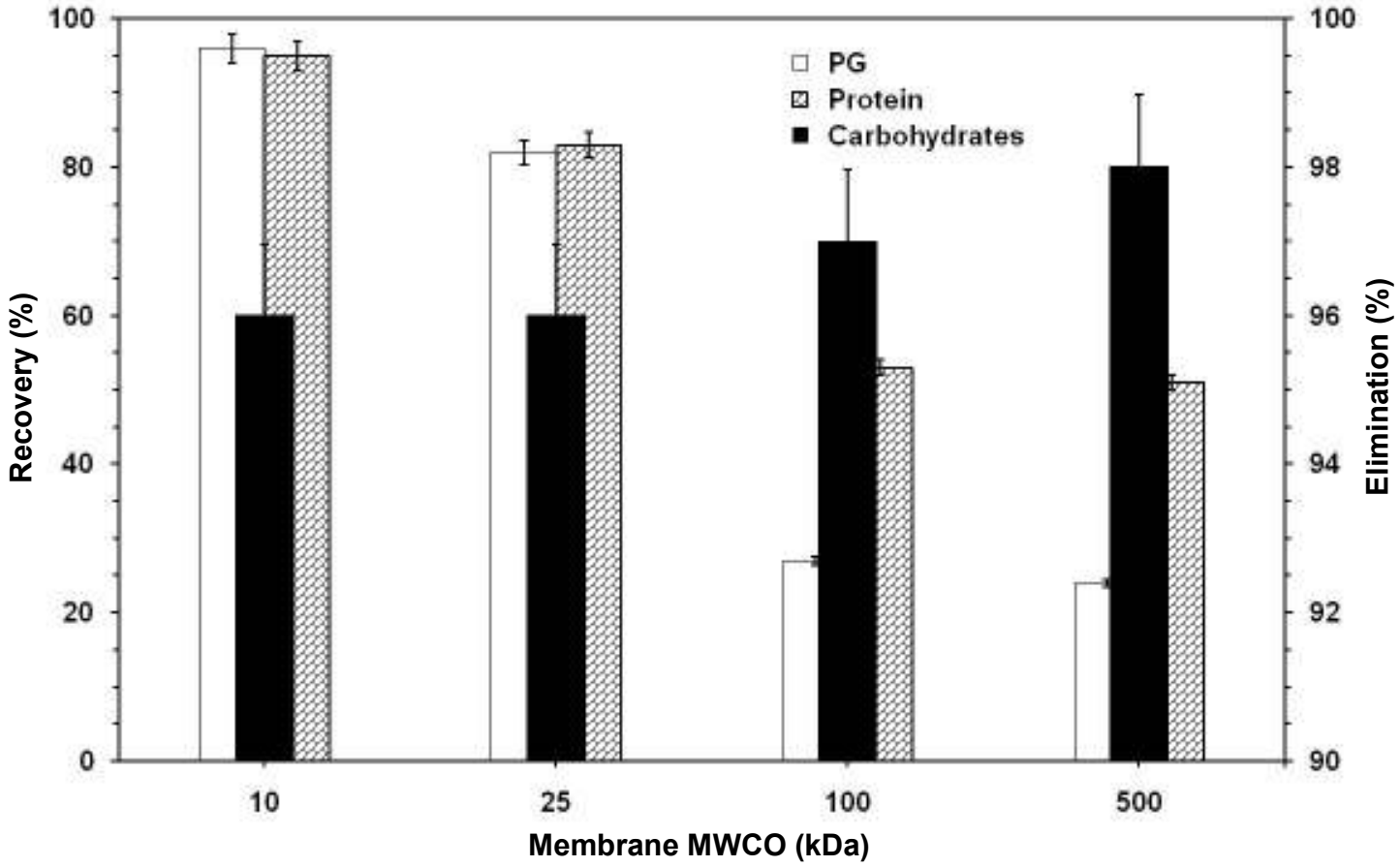


Fig. 3.3 Recovery of PG and protein and elimination of carbohydrates in the retentates during UF of culture broth in a self-stirred membrane cell (0.5 MPa, 30°C, 800 rpm and 100% diafiltration)

Table 3.5 Elimination of impurities by 10 kDa UF membrane[a]

	Carbohydrates			Phosphate salts			Total acids			PG activity	
Membrane	Total (mg)		CE	Total (mg)		CE	Total (mg)		CE	CR	Avg. flux
MWCO	P	R	(%)	P	R	(%)	P	R	(%)	(%)	($l/m^2 \cdot h$)
Crude culture broth											
UF	176	60	75	162	41	80	48.0	12.0	80	98	53
DF-1	36	24	90	31	10	95	8.0	4.0	93	97	51
DF-2	15	9	96	8	2	99	3.8	0.2	100	96	49
Centrifuged culture broth											
UF	172	30	87	162	41	80	48.0	12.0	80	98	62

[a]Feed volume: 50 ml; CE – Cumulative elimination from the retentate; CR – Cumulative recovery in the retentate;

For other abbreviations see Table 3.2.

Total carbohydrates, phosphate salts and acids in crude culture broth were 236 mg; 203 mg and 60 mg, respectively.

enzymes in feed and retentate of 10 kDa UF membrane and also confirmed the absence of PG enzymes in the permeate (Fig. 3.4).

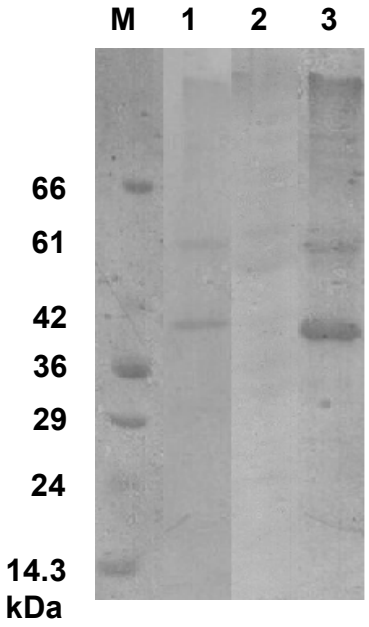


Fig. 3.4 SDS-PAGE for identifying PG enzymes in the process streams of 10 kDa UF membrane

M – Molecular weight markers; 1 – Feed; 2 – Permeate; 3 – Retentate.

UF with centrifuged culture broth did not affect either the elimination of salts and acids or the recovery of PG. However, elimination of carbohydrates (87%) was slightly higher with centrifuged culture broth because centrifugation removed suspended solids and some carbohydrates. For the same reasons, the permeate flux was greater with centrifuged culture broth (62 $l/m^2 \cdot h$) compared with crude culture broth (53 $l/m^2 \cdot h$). Although centrifugation of culture broth before processing may increase the process steps, it may be desirable to adopt

in the process as it would offer advantages such as reduced diafiltration and improved productivity in terms of increased permeate flux as well as longer processing time between cleaning cycles. The results of this study revealed that it is possible to eliminate carbohydrates and other impurities such as salts and acids completely with greater recovery of PG by employing a 10 kDa membrane in diafiltration mode.

3.5 Conclusions

Assessment of upstream processes using mutant *A. carbonarius* showed that higher yield together with shorter fermentation period resulting in greater productivity favoring SmF for PG production over SSF. Processing culture filtrate with MF membranes showed greater recovery of PG in permeates while the elimination of carbohydrates was very low owing to their permeation in the diafiltration mode. On the other hand, UF membranes exhibited greater rejection of PG and lesser rejection of impurities including carbohydrates indicating their suitability for the intended process duty. Among the UF membranes tested, 10 kDa membrane during diafiltration exhibited near complete elimination of carbohydrates, phosphate salts and acids with 96% PG recovery. The results revealed that 10 kDa UF membrane could significantly eliminate the non-protein impurities and thereby improve the enzyme activity from 4.6 to 49.4 U/mg of dry matter while retaining the proteins including enzymes in the culture broth, leading to no improvement in PG specific activity.

4.0 Significance and focus of the work

Earlier experiments using several UF membranes showed that the 10 kDa membrane in diafiltration operation removed most of the carbohydrates, phosphate salts and total acids from *A. carbonarius* culture broth with a higher PG recovery. Although the protocol was effective in eliminating non-protein impurities, there was practically no improvement in the enzyme specific activity since contaminating proteins were also recovered along with the enzyme. In the present work, a protocol for an IMP employing MF and UF membranes was developed to obtain greater enzyme-specific activity as well as recovery of PG with preprocessed as well as crude culture broth/extracts.

4.1 Improving specific activity of SmF-PG

4.1.1 Elimination of larger non-enzymatic proteins by microfiltration

MF membranes with two different pore sizes (200 and 450 nm) were tested for their ability to eliminate larger non-enzymatic proteins from culture broth centrifuged and diafiltered using 10 kDa UF membrane (Table 4.1). Lower PG rejection than contaminating proteins by both the membranes resulted in 1.38-1.40 fold improved specific activity of the enzyme in the permeate stream. This revealed that some of the larger non-enzymatic proteins were eliminated employing MF. Although the pattern of protein rejection was similar, the rejection of PG was lower with 450 nm compared to 200 nm membrane resulting in higher specific activity of PG (4440 U/mg protein). Elimination of non-enzymatic proteins, higher recovery of PG (75%) and greater productivity (37 $l/m^2 \cdot h$)

Table 4.1 Microfiltration of preprocessed culture broth[a]

Membrane pore size (nm)	PG activity		Protein content		Specific activity[b] (U/mg)	Purification[b] (Fold)	Avg. flux ($l/m^2 \cdot h$)
	R_o (%)	R^b (%)	R_o (%)	E^b (%)			
450	39	75	68	48	4440	1.40	37
200	46	71	69	49	4380	1.38	32

[a]R_o - Percent rejection; R – Recovery; E - Elimination; VCR: 10 fold; Feed volume: 50 ml; PG activity, protein content and specific activity in feed (diafiltered 10 kDa retentate after centrifugation of culture broth) were 595 U/ml, 188 μg/ml and 3170 U/mg protein, respectively.

[b]Processed permeate stream.

obtained using 450 nm membrane suggested its further evaluation for the IMP.

The performance of 450 nm membrane was examined with diluted feed and also in diafiltration mode of operation. The feed diluted to 400% resulted in 73% PG recovery compared to 65% recovery obtained with undiluted feed. However, the purity reduced from 1.48 to 1.11 fold. Increasing the dilution to 900% (close to original culture broth concentration) enabled 85% PG recovery without affecting purification (Fig. 4.1). Diafiltration resulted in higher purity (1.28 fold) with 82% PG recovery. Reduction in productivity during diafiltration was due to lower flux (21 $l/m^2 \cdot h$) compared to the run using diluted feed (220 $l/m^2 \cdot h$). The need for appropriate process conditions while purifying PG from *A. carbonarius* culture broth described above probably explains the low enzyme purity (0.69 fold) and recovery (44%) reported by Sathish-Kumar and Palanivelu (1999) processing the culture broth of *Thermomyces lanuginosus* PG using a

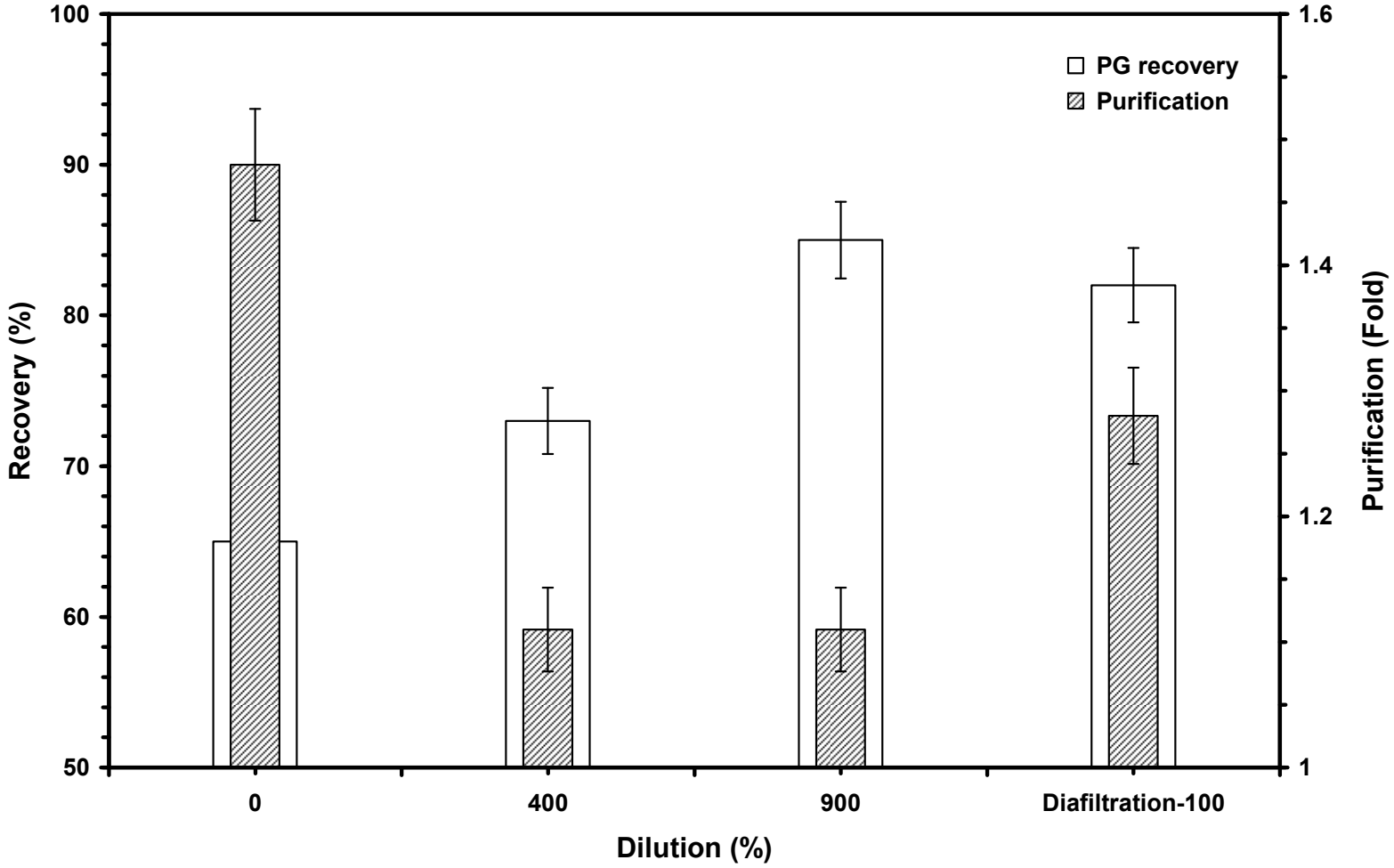


Fig 4.1 Microfiltration (450 nm) of preprocessed culture broth

similar pore size MF membrane.

4.1.2 Elimination of smaller non-enzymatic proteins by ultrafiltration

Processing with UF membranes (25, 50, 100 and 500 kDa MWCO) showed elimination of contaminating proteins from the preprocessed culture broth (Table 4.2). Least and highest retention of PG as well as proteins apparently without any selectivity was recorded with the 500 and 25 kDa membranes, respectively. The retention performance of 100 and 50 kDa UF membranes was in between the above membranes. Greater specific activity (3530 U/mg protein) and improved enzyme purity (1.11 fold) obtained in the retentate stream of 50 kDa membrane suggested its selectivity for PG. The results revealed that some of the contaminating proteins were smaller than the *A. carbonarius* PG of molecular weights 42 and 61 kDa (Davi and Rao, 1996) that could be eliminated to a limited extent with the 50 kDa UF membrane. Suitability of the membrane was also evidenced by 86% recovery and 21 $l/m^2 \cdot h$ flux. Therefore, the 50 kDa UF membrane was evaluated further for employing along with MF in an IMP for processing *A. carbonarius* PG.

Diluting the feed improved the purity as well as recovery of PG and higher the dilution greater was the improvement (Fig. 4.2). Processing 900% diluted feed improved the enzyme purity by 1.29 fold with a high recovery of 96%. The productivity (44 $l/m^2 \cdot h$) was also much higher compared to undiluted feed. During diafiltration, enzyme purity (1.21 fold), recovery (63%) and productivity (20 $l/m^2 \cdot h$) were lower compared to the above run with diluted feed.

Table 4.2 Ultrafiltration of preprocessed culture broth[a]

Membrane	PG activity		Protein content		Specific		
MWCO (kDa)	R_o (%)	R^b (%)	R_o (%)	E^b (%)	activity[b] (U/mg)	Purification[b] (Fold)	Avg. flux ($l/m^2 \cdot h$)
500	77	59	79	38	3030	0.96	27
100	92	82	91	18	3130	0.99	23
50	93	86	89	23	3530	1.11	21
25	97	94	97	8	3230	1.02	11

[a]For abbreviations see Table 4.1; VCR: 10 fold; Feed volume: 50 ml;
PG activity, protein content and specific activity in feed (diafiltered 10 kDa retentate after centrifugation of culture broth) were 595 U/ml, 188 μg/ml and 3170 U/mg protein, respectively.
[b]Processed retentate stream.

4.2 Integrated membrane processing of SmF culture broth

Based on the results described above, three different schemes were attempted for processing *A. carbonarius* culture broth employing membranes for the purification of PG.

4.2.1 Purification of PG (CF-UF-MF-UF)

In Scheme-1 PG purification involved a centrifugation (CF) step for clarifying the culture broth followed by sequential processing through 10 kDa UF, 450 nm MF and 50 kDa UF membranes (Table 4.3). The first two steps were employed for pre-processing the crude culture broth to eliminate non-protein impurities as described earlier. Processing the above culture broth after suitable dilution through 450 nm MF membrane eliminated 21% larger contaminating proteins.

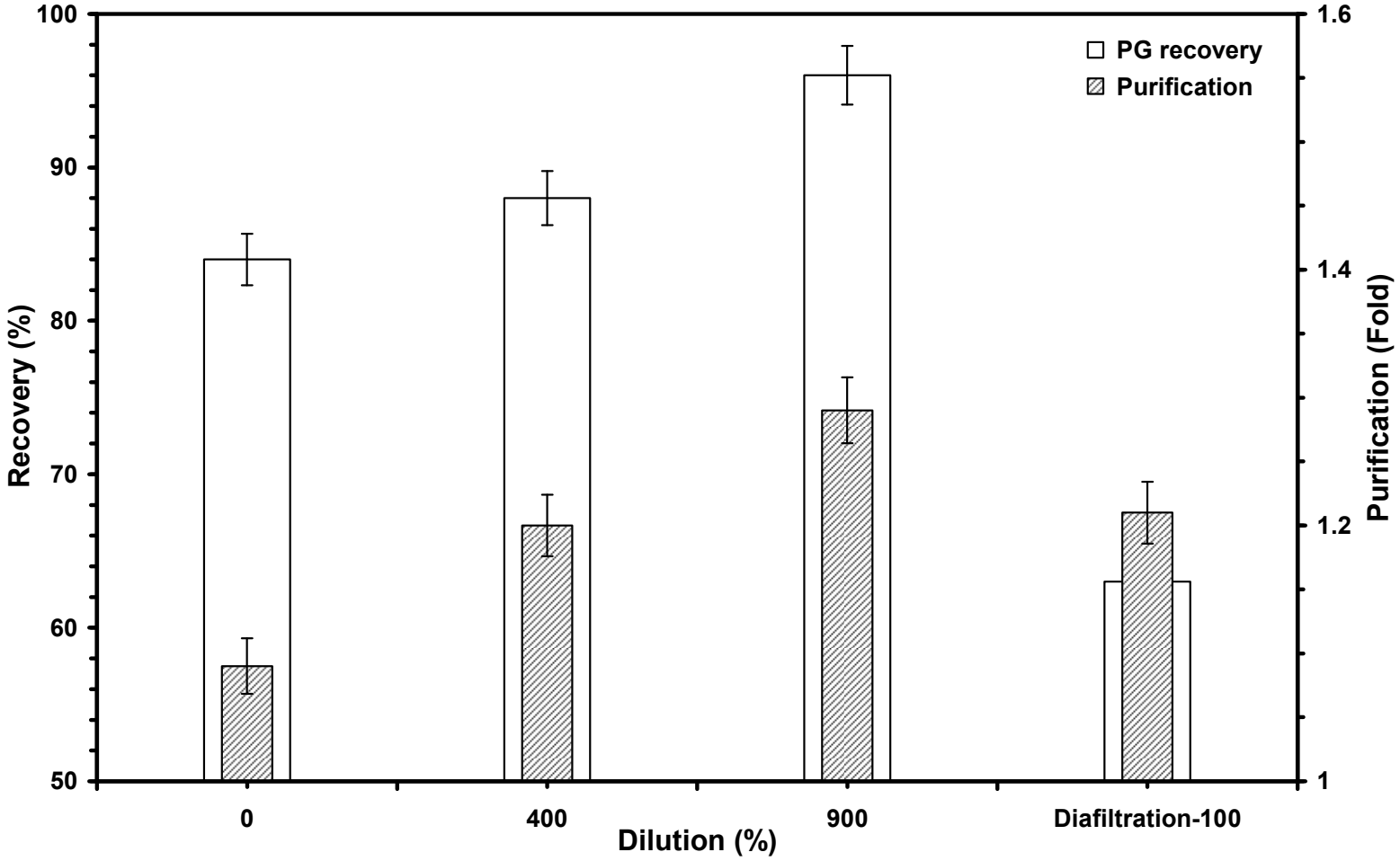


Fig. 4.2 Ultrafiltration (50 kDa) of preprocessed culture broth

Table 4.3 Summary of different schemes used for the purification of SmF-PG[a]

Process and performance description	Scheme-1	Scheme-2	Scheme-3
Process steps involved	CF + 10 kDa + 450 nm + 50 kDa	10 kDa + 450 nm + 50 kDa	450 nm + 50 kDa
Centrifugation (CF)	Suspended particles eliminated (14-16% carbohydrates) – Improvement in MF flux	-	-
UF: 10 kDa (100% diafiltration)	Near complete removal of non-protein impurities with almost complete recovery of PG enzyme	Near complete removal of non-protein impurities with almost complete recovery of PG enzyme	-
MF: 450 nm (Dilution close to crude culture broth)	Removal of larger non-enzymatic proteins 21% protein eliminated 87% PG recovery; 1.10 fold purity; 220 l/m^2·h flux	Removal of larger non-enzymatic proteins 40% protein eliminated 84% PG recovery; 1.39 fold purity; 67 l/m^2·h flux	Removal of larger non-enzymatic proteins 45% protein eliminated 85% PG recovery; 1.50 fold purity; 60 l/m^2·h flux 23% carbohydrates eliminated
UF: 50 kDa (100% diafiltration)	Removal of smaller non-enzymatic proteins	Removal of smaller non-enzymatic proteins	Removal of smaller non-enzymatic proteins

Continued…

Process and performance description	Scheme-1	Scheme-2	Scheme-3
	54% protein eliminated	68% protein eliminated	65% protein eliminated
	94% PG recovery; 24 l/m^2·h flux	95% PG recovery; 22 l/m^2·h flux	94% PG recovery; 23 l/m^2·h flux
			Near complete removal of non-protein impurities
Performance	62% total protein eliminated	82% total protein eliminated	81% total protein eliminated
	78% overall PG recovery	76% overall PG recovery	76% overall PG recovery
PG purity	1.98 fold	5.14 fold	4.69 fold
PG specific activity	2350 U/mg protein	6120 U/mg protein	5590 U/mg protein
PG activity/dry solids	248 U/mg	246 U/mg	247 U/mg
Volume processed (50 ml crude culture broth)	256 ml	256 ml	149 ml
Final product volume	3 ml	3 ml	3 ml
Membrane process time	6 h and 7 min	6 h and 16 min	3 h and 5 min

[a]Carbohydrates, phosphate salts, acids, PG specific activity and PG activity/dry solids in crude culture broth were 4.5 mg/ml, 4.0 mg/ml, 1.2 mg/ml, 1190 U/mg protein and 2.6 U/mg dry solids, respectively.

This resulted in 1.10 fold increase in enzyme purity. In the subsequent UF (50 kDa), 54% contaminating proteins (smaller) were eliminated with diafiltration resulting in improved specific activity (2350 U/mg protein). Diafiltration in this stage seemed to help achieve higher purity by eliminating more non-enzymatic proteins without sacrificing the enzyme recovery (94%). The scheme eliminated 62% total protein while retaining 78% enzymes with a specific activity of 2350 U/mg protein (Table 4.3). It also resulted in a higher PG activity of 248 U/mg dry solids as against 19 U/mg dry solids of the preprocessed culture broth. The results showed that the majority of the contaminating proteins in the culture broth, amenable for membrane separation, were predominantly <50 kDa and some were >450 nm. Since only 1.98 fold enzyme purity was obtained, modifications were attempted to improve the process efficacy.

4.2.2 Purification of PG (UF-MF-UF)

CF competes with MF many a time in process applications and at times is used as a pretreatment to MF/UF. In Scheme-2, CF was excluded since MF is also capable of removing suspended solids. The 10 kDa retentate when processed after suitable dilution using 450 nm MF membrane improved the enzyme purity to 1.39 fold owing to the elimination of 40% larger contaminating proteins (Table 4.3). The higher purity obtained could be due to secondary layer formation on the membrane surface due to adsorption and deposition of substances contained in the feed (van Oers *et al*., 1995; Barhate *et al*., 2003) that aid in the rejection of non-enzymatic proteins while allowing smaller molecular enzymes. The

exclusion of CF reduced the permeate flux during MF, probably due to concentration polarization, fouling and secondary layer formation. However, PG recovery (84%) was comparable to 87% obtained after MF in Scheme-1. In the final UF with diafiltration using 50 kDa membrane, 68% proteins (smaller) were eliminated leading to PG with a specific activity of 6120 U/mg protein (Table 4.3). The higher PG recovered (95%) compensated the lower recovery obtained during MF. The scheme also resulted in 246 U/mg dry solid comparable to that obtained in Scheme-1 despite starting with a lower activity of 11.4 U/mg dry solids.

Though the overall PG recovery (76%) was similar to that obtained with Scheme-1, elimination of 82% total proteins improved specific activity by 5.14 fold (1.98 fold obtained with Scheme-1). Based on the above results, another scheme was attempted in order to further reduce the process steps.

4.2.3 Purification of PG (MF-UF)

The 10 kDa UF membrane processing of the crude culture broth, which removed non-protein impurities, was deleted in Scheme-3 on the assumption that diafiltration using 50 kDa membrane can also aid their removal along with non-enzymatic proteins. To test the hypothesis, crude culture broth containing PG was filtered through Whatman No. 1 filter paper and directly processed in a two step membrane process with 450 nm MF and 50 kDa UF membranes (Table 4.3). During MF, 45% larger contaminating proteins were eliminated leading to 1.50 fold increase in enzyme purity and 85% recovery. The performance was

comparable with MF step in Scheme-2 except for a small decline in the permeate flux. Diafiltration with 50 kDa UF membrane eliminated 65% contaminating proteins (smaller). It was also effective in eliminating non-protein impurities, carbohydrates (96%), and almost total phosphate salts and acids. The final specific activity and recovery of the enzyme achieved in this scheme were also comparable with the values obtained in Scheme-2. It also resulted in a higher PG activity of 247 U/mg dry solids as that of the other two schemes even though the starting value was much lower (2.6 U/mg dry solids in crude culture broth). The PG purity was also confirmed by SDS-PAGE (Fig. 4.3). The overall performance achieved with Scheme-3 was significant since only two steps were involved that offered ~50% reduction in process time and lesser handling volume (Table 4.3).

In this study, membranes could be reused for several experimental runs (~200 h of operation) after cleaning. Although durability of the membrane was not assessed in terms of its replacement time, such membranes are expected to give a long life in this kind of process environment.

4.3 Integrated membrane processing of SSF culture extract

The IMP, earlier described for the purification of *A. carbonarius* PG from culture broth after growth in SmF when attempted for the purification of PG produced by the fungus in SSF eliminated 98% carbohydrates with 72% enzyme recovery. However, the improvement in enzyme specific activity was only 1.2 fold which contrasted the increased specific activity of 4.69 fold (5590 U/mg protein)

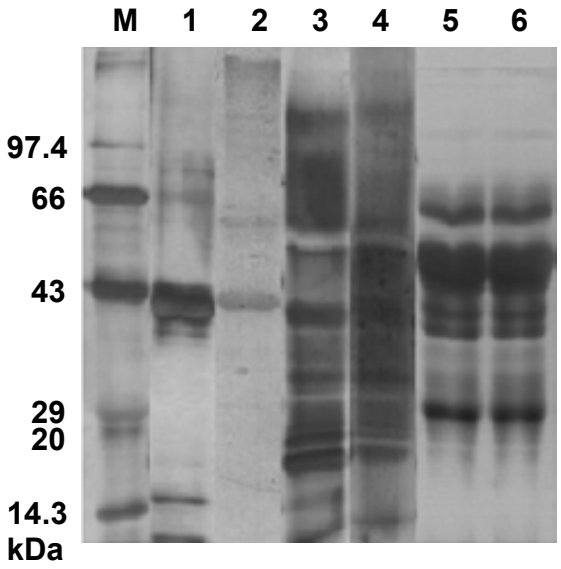


Fig. 4.3 SDS-PAGE pattern of PG purified by integrated membrane process

M - Molecular weight markers; 1 – SmF feed; 2 – SmF retentate; 3 – SSF feed; 4 – SSF retentate; 5 – Commercial feed; 6 – Commercial retentate.

obtained by IMP of SmF-PG. This drastic difference in process performance could be solely attributed to the presence of other contaminant proteins; significantly greater secretion in SSF cultures (Fig. 4.3) while it was much lower in SmF culture broth as evidenced by SDS-PAGE. The IMP also recovered other contaminant proteins along with PG, including protease and amylase present in the SSF culture extract. These enzymes were recovered to the extent of 49% and 41% while their specific activities were reduced to 0.24 and 3.16 U/mg protein, respectively (Table 4.4). Although protease was identified in the culture extract, it did not affect the stability of SSF-PG as it was in the completely glycosylated form. The presence of other similar or higher molecular mass proteins in the crude culture extract was also confirmed by SDS-PAGE (Fig. 4.3)

Table 4.4 Integrated membrane processing of SSF crude culture extract for the purification of PG[a]

Scheme description	VCR (Fold)	PG		Protease		Amylase		Protein		CHO		Specific activity			Avg. flux ($l/m^2 \cdot h$)
		R_o (%)	CR (%)	R_o (%)	CR (%)	R_o (%)	CR (%)	R_o (%)	CR (%)	R_o (%)	CE (%)	PG (U/mg)	Protease (U/mg)	Amylase (U/mg)	
Crude culture extract[b]															
MF (450 nm)	10.0	5	89	9	88	1	90	19	85	2	10	550	0.28	4.60	6000
UF (50 kDa)	10.0	94	74	88	63	75	48	93	68	36	80	570	0.25	3.03	43
DF-1	9.3	-	72	-	53	-	42	-	66	-	96	610	0.24	2.93	40
DF-2	9.3	-	72	-	49	-	41	-	65	-	98	640	0.24	3.16	36
Diluted culture extract[c]															
MF+UF+DF	9.3	98	80	-	-	-	-	95	72	26	99	550	-	-	-

[a]Pretreatment: Filtered through ordinary filter paper followed by Whatman No. 1 filter paper; Feed volume: 50 ml;
VCR: Volume concentration ratio; CHO: Carbohydrates; R_o: Percentage rejection;
CR: Cumulative recovery (MF – permeate; UF – retentate); CE: Cumulative elimination (MF – permeate; UF – retentate);
DF-1: Diafiltration - 1 (50%; 25 ml of 0.85% NaCl); DF-2: Diafiltration – 2 (50%; 25 ml of 0.85% NaCl); DF: Diafiltration (DF-1+DF-2).
[b]PG, protease and amylase activities and their specific activities in crude culture extract were 80 U/ml (1410 U/g of dry bran), 0.041 U/ml, 0.660 U/ml and 530 U/mg protein, 0.27 U/mg protein, 4.34 U/mg protein, respectively.
Protein and CHO contents in crude culture extract were 152 μg/ml and 4.8 mg/ml, respectively.
[c]PG activity, protein content, CHO content and PG specific activity in diluted culture extract were 26 U/ml, 56 μg/ml, 1.8 mg/ml and 460 U/mg protein, respectively.

which was responsible for low membrane selectivity restricting the improvement in PG purity. In order to examine the role of protein concentration in selective partition of PG during IMP, the crude SSF culture extract was diluted to similar levels of protein concentrations used for IMP of SmF culture broth. Though, in this process, PG recovery increased to 80% while retaining the level of carbohydrates elimination, there was no improvement in the specific activity of PG (Table 4.4). Since an increased permeate flux of 44 $l/m^2 \cdot h$ was obtained with the diluted culture extract, the performance appeared to give greater productivity but not so in real terms considering the enzyme concentrations. The contaminant proteins affected the membrane selectivity even under diluted conditions.

4.4 Integrated membrane processing of commercial enzyme preparation

The commercial enzyme preparation (commercial PG-1) analyzed by SDS-PAGE (Fig. 4.3) revealed the presence of other contaminant proteins including enzymes such as protease and amylase (Table 4.5). This enzyme preparation was used as a test sample, after diluting close to SSF protein concentrations, in order to study the interference of other contaminant proteins during IMP. The PG specific activity improved only to the extent of 1.05 fold during MF which improved marginally to 1.28 fold after UF with diafiltration (Table 4.5). On the whole, the overall performance obtained with commercial enzyme preparation (97% carbohydrates elimination; 72% PG recovery; 1.28 fold increase in specific activity) was similar to that of SSF culture extract (Table 4.4).

Table 4.5 Membrane processing of commercial enzyme preparation for PG purification[a]

Scheme description	VCR (Fold)	PG		Protease		Amylase		Protein		CHO		Specific activity			Avg. flux ($l/m^2 \cdot h$)
		R_o (%)	CR (%)	R_o (%)	CR (%)	R_o (%)	CR (%)	R_o (%)	CR (%)	R_o (%)	CE (%)	PG (U/mg)	Protease (U/mg)	Amylase (U/mg)	
MF (450 nm)	10.0	9	88	1	90	7	88	20	84	8	12	220	0.16	16.3	3830
UF (50 kDa)	10.0	96	76	92	72	94	73	96	73	34	82	250	0.16	17.9	31
DF-1	9.3	-	74	-	66	-	67	-	71	-	93	270	0.15	13.6	23
DF-2	9.3	-	72	-	61	-	64	-	71	-	97	270	0.12	14.5	19

[a]Commercial PG-1; Feed volume: 50 ml; For other abbreviations see Table 4.4;

PG, protease and amylase activities and their specific activities in commercial enzyme preparation were 39 U/ml, 0.027 U/ml, 2.89 U/ml and 210 U/mg protein, 0.15 U/mg protein, 15.6 U/mg protein, respectively.

Protein and CHO contents in commercial enzyme preparation were 186 μg/ml and 0.43 mg/ml, respectively.

The above results showed that there was no appreciable improvement in PG specific activity and Fig. 4.3 showed that there were other contaminant proteins present in the retentate which would not be eliminated by IMP due to their molecular mass similarity with PG.

4.5 Conclusions

IMP employing 450 nm MF and 50 kDa UF membranes revealed its capability for improving PG specific activity by selectively eliminating the contaminant proteins from SmF culture broth of *A. carbonarius* containing PG taking advantage of the differences in their molecular sizes. Besides, this approach appears to be easily adaptable in downstream processing of any microbial culture broth and would bring down the size and cost of the subsequent purifications steps. On the other hand, IMP is not suitable for the purification of PG from SSF culture extract since proteins including enzymes such as amylase and protease of molecular mass similar to PG interfered in downstream processing.

5.0 Significance and focus of the work

IMP improved the specific activity of PG from SmF culture broth but did not improve the specific activity of SSF-PG due to the presence of contaminating proteins of molecular mass similar to PG and suggested the need for alternate purification process. AAP 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. AAP has been employed predominantly for the purification of commercial enzyme preparations (Jain *et al.*, 2006). In the present work, affinity purification was attempted for the purification of PG to obtain greater PG specific activity and recovery by eliminating similar molecular mass contaminant proteins from the crude culture extract/broth of *A. carbonarius*.

5.1 Alginate affinity purification of PG

For PG purification, eluent (sodium chloride) concentration for the separation of bound PG from the alginate precipitate and charcoal concentration for the removal of colour compounds from the crude culture extract that might interfere during affinity precipitation were optimized.

5.1.1 Optimization of eluent concentration

Eluent (sodium chloride) concentration was optimized against PG recovery and its purity. PG recovery increased sharply when the eluent concentration was increased from 0.5 to 1 M and thereafter increased gradually with increase in

eluent concentration. While the PG purity decreased from 3.6 to 2.5 fold as the eluent concentration was increased from 0.5 to 2.5 M. The decrease in PG purity with increase in eluent concentration was due to desorption of other bound proteins. At 1 M NaCl concentration, PG recovery was reasonably higher (54%) with only a small loss in purity, hence construed as optimum eluting condition (Fig. 5.1) and employed in subsequent experiments.

5.1.2 Optimization of charcoal concentration

Although increase in charcoal concentration increased the colour reduction, it affected the PG recovery and purity. Colour reduction increased sharply with increase in charcoal concentration from 1 to 5 mg/ml and gradually increased thereafter up to a concentration of 40 mg/ml before attaining a plateau. Charcoal concentration had a great influence on PG recovery and purity; increase in concentration from 5 to 50 mg/ml, affected the PG recovery from 65% to 1% and purity from 0.87 to 0.34 fold. A concentration of 5 mg/ml was considered optimum owing to a substantially high colour reduction (83%) without much loss of PG and its purity (Fig. 5.2).

5.2 Purification of PG from SSF crude culture extract

The alginate precipitate containing bound PG was separated from enzyme alginate solution by centrifugation. The supernatant contained 81% of carbohydrates and 61% colour compounds along with 17% PG. The precipitate containing bound PG was washed with 0.02 M $CaCl_2$ (pH 3.8) for three times to

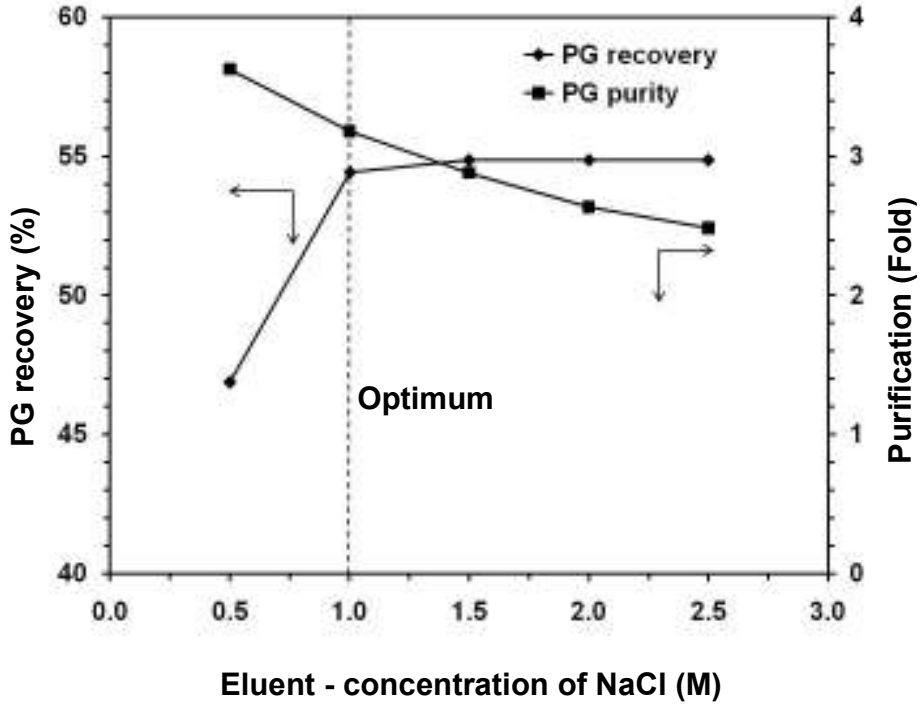


Fig. 5.1 Recovery and purity of PG with various eluent concentrations

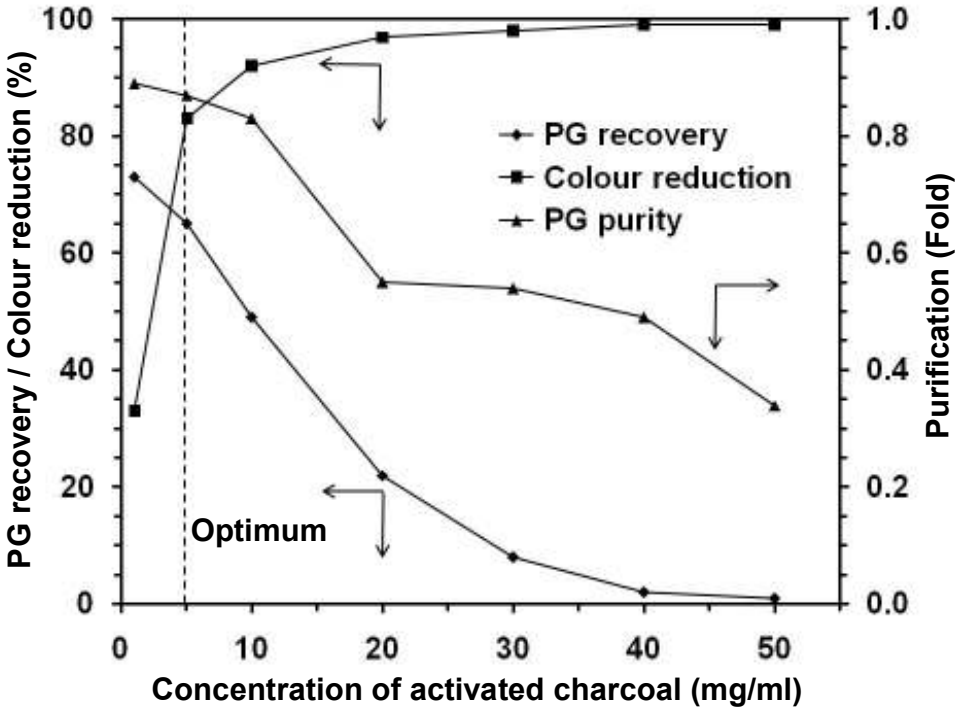


Fig. 5.2 Pretreatment of crude culture extracts using activated charcoal

remove the unbound proteins. This step resulted in the removal of additional 16% carbohydrates and 35% colour compounds; however, 2% of PG was also lost in the wash (Table 5.1).

The PG elution was attempted with three different eluting conditions as described earlier (section 2.2.4). The PG bound to the cross-linked alginate beads was eluted using a 0.1 M acetate buffer containing 1 M NaCl. Dissolution and reprecipitation of enzyme-alginate complex improved the enzyme yield to 60% with a marginal increase in specific activity (2450 U/mg protein; 4 fold) compared to four consecutive steps of elution (Table 5.1). The performance remained unaffected when the incubation period was decreased to 2 h even under ambient temperature that avoided the long incubation periods and application of cold conditions. The elimination of carbohydrates and colour compounds was nearly complete in all the cases (Table 5.1). The results exhibited the binding of PG to alginate significantly improving its purity that was confirmed by SDS-PAGE (Fig. 5.3). The purity of *A. carbonarius* PG, showing a single band on SDS-PAGE, was comparable with the purification of commercial enzyme preparation reported by Gupta *et al.* (1993). In the present case, the improvement in PG purity was achieved in a shorter incubation period without resorting to refrigerated conditions.

5.3 Purification of PG from pretreated SSF culture extract

Crude culture extract was pretreated using charcoal (5 mg/ml) for the removal of colour compounds. This pretreatment removed 83% of colour compounds and

Table 5.1 Purification of PG from SSF crude culture extract using alginate affinity precipitation[a]

Sample description	PG activity (U)	Protein (mg)	Specific activity (U/mg)	CHO elimination (%)	Colour reduction (%)	PG recovery (%)	Purification (Fold)
Crude culture extract	890	1.440	620	-	-	100	1.0
Supernatant	155	0.437	350	81	61	17	0.6
Wash	17	0.168	100	16	35	2	0.2
Eluate-A (1 M NaCl containing 0.2 M $CaCl_2$ (pH 5.9) at 30°C for 0.5 h) After four elutions	497	0.205	2430	97	96	56	3.9
Eluate-B (1 M NaCl in 0.1 M acetate buffer (pH 4.3) at 4°C for 18 h)	534	0.218	2450	97	96	60	4.0
Eluate-C (1 M NaCl in 0.1 M acetate buffer (pH 4.3) at 30°C for 2 h)	543	0.224	2450	97	96	61	4.0

[a]Pretreatment: Filtered through ordinary filter paper followed by Whatman No.1 filter paper; Feed volume: 10 ml.

All estimations of process stream samples were carried out after dialysis.

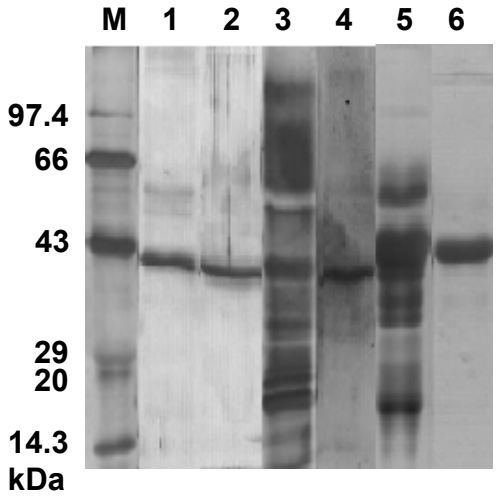


Fig. 5.3 SDS-PAGE pattern of PG purified by alginate affinity purification

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

subsequent affinity precipitation increased the PG specific activity to 2230 U/mg protein (3.6 fold) with a recovery of 52% PG (Table 5.2). A second elution step did not practically improve the PG recovery. The lesser PG purity and recovery was due to the considerable loss of PG (35%) during the pretreatment step (Table 5.2). Further, the overall colour reduction in the purified PG was nearly total (~97%) even without employing a pretreatment step (Table 5.1). The above results suggested that color compounds were not interfering and that additional pretreatment step is not needed in the affinity precipitation process.

5.4 Purification of PG from SmF culture broth

The process efficacy of AAP was tested for the purification of PG from SmF culture broth. The specific activity of SmF-PG enhanced to 9770 U/mg protein

Table 5.2 Purification of PG from pretreated culture extract (after colour removal) using alginate affinity precipitation[a]

Sample description	PG activity (U)	Protein (mg)	Specific activity (U/mg)	PG recovery (%)	Purification (Fold)
Crude culture extract	890	1.440	620	100	1.0
After activated charcoal (5 mg/ml) treatment	576	1.072	540	65	0.9
Supernatant	110	0.403	270	12	0.4
Wash	8	0.146	60	1	0.1
First eluate	464	0.209	2230	52	3.6
Second eluate	8	0.041	200	1	0.3

[a]Pretreatment: Filtered through ordinary filter paper followed by Whatman No. 1 filter paper; Feed volume: 10 ml. Elution condition: 1 M NaCl in 0.1 M acetate buffer (pH 4.3) at 30°C for 2 h.

Estimations were carried out as described in Table 5.1.

(8.7 fold) with a PG recovery of 74% (Table 5.3) while the corresponding performance obtained with SSF crude culture extract were 4 fold increase in purity (2450 U/mg protein) and 61% recovery (Table 5.1). The above difference in performance revealed that SmF culture broth contains lesser contaminant proteins and other impurities compared to SSF culture extract. Besides, AAP exhibited complete elimination of carbohydrates (Table 5.3). The purity of SmF-PG was also confirmed by SDS-PAGE (Fig. 5.3).

Table 5.3 Purification of PG from SmF crude culture broth using alginate affinity precipitation[a]

Sample description	PG activity (U)	Protein (mg)	Specific activity (U/mg)	CHO elimination (%)	PG recovery (%)	Purification (Fold)
Crude culture broth	2300	2.040	1130	-	100	1.0
Supernatant	64	0.471	140	95	3	0.1
Wash	142	0.142	1000	4	6	0.9
Eluate	1700	0.174	9790	99	74	8.7

[a]Pretreatment: Filtered through Whatman No. 1 filter paper; Feed volume: 10 ml.
For estimations and elution conditions see Table 5.2.

5.5 Purification of PG from commercial enzyme preparation

Commercial enzyme preparation (commercial PG-1) was diluted close to the PG activity of solid-state cultures and process efficacy of AAP was also tested for the purification of PG. The specific activity of PG enhanced to 1480 U/mg protein (4.4 fold) with a recovery of 64% (Table 5.4) while the corresponding

Table 5.4 Purification of PG from commercial enzyme preparation using alginate affinity precipitation[a]

Sample description	PG activity (U)	Protein (mg)	Specific activity (U/mg)	PG recovery (%)	Purification (Fold)
Commercial preparation	1040	3.120	330	100	1.0
Supernatant	140	0.912	150	14	0.5
Wash	36	0.358	100	3	0.3
First eluate	666	0.450	1480	64	4.4
Subsequent eluate	30	0.077	390	3	1.2

[a]Commercial PG-1; Feed volume: 10 ml.
For estimations and elution conditions see Table 5.2.

performance obtained with SmF culture broth were 8.7 fold increase in purity (9770 U/mg protein) and 74% recovery (Table 5.3). Gupta *et al.* (1993) reported 10 fold increase in purity (2202 U/mg protein) and much higher recovery (91%) while processing a commercial enzyme preparation. The above differences in performance could be due mainly attributed to the differences in the feed stock employed in the process. However, it may be worth noting that the purity of commercial PG obtained in this study, showing a single band on SDS-PAGE (Fig. 5.3) was comparable with the purities reported for affinity purification of commercial enzymes (Gupta *et al.,* 1993).

Although affinity purification enhanced the PG specific activity, it is necessary to eliminate calcium salts present in the eluate owing to its inhibitory effects on the enzyme activity (Cabanne and Doneche, 2002). Hence, UF was attempted as a single step process for desalting and

concentration of eluted PG after AAP.

5.6 Ultrafiltration for desalting and concentration of eluted PG

Desalting of eluted enzymes is generally done by dialysis which requires large quantities of buffer with longer processing time under refrigerated temperatures. Besides, the enzyme gets diluted during dialysis necessitating a concentration step. Osmotic concentration using PEG practiced in the laboratory may not be suitable for industrial applications. The process requires a concentration step adaptable for large scale operation. As an alternative to dialysis, UF under diafiltration mode was attempted for desalting and concentration of eluted SSF-PG using a 10 kDa membrane. The PG purity obtained by UF was low (1.6 fold) and so also its recovery (21%) with self-stirred membrane cell at 4°C operating temperature (Fig. 5.4). The reduced PG purity and recovery compared to dialysis could be due to the shear forces induced by stirring during UF. Under otherwise similar operating conditions but by not employing stirring, the PG purity improved to 4.1 fold (2520 U/mg protein) which supported the role of shear forces. Ambient temperature operation gave similar performance as that of refrigerated conditions. It was noticed that the PG recovery was generally low (maximum 36%) during UF which got affected further when stirring was employed (Fig. 5.4). This could be mainly due to difficulties in completely recovering the PG adhering to the membrane and the system. Stirring is generally employed to minimize the concentration polarization effect in the membrane cell; however, the results showed that the enzymes are shear sensitive suggesting additional care while processing such delicate materials. UF offers the advantage of

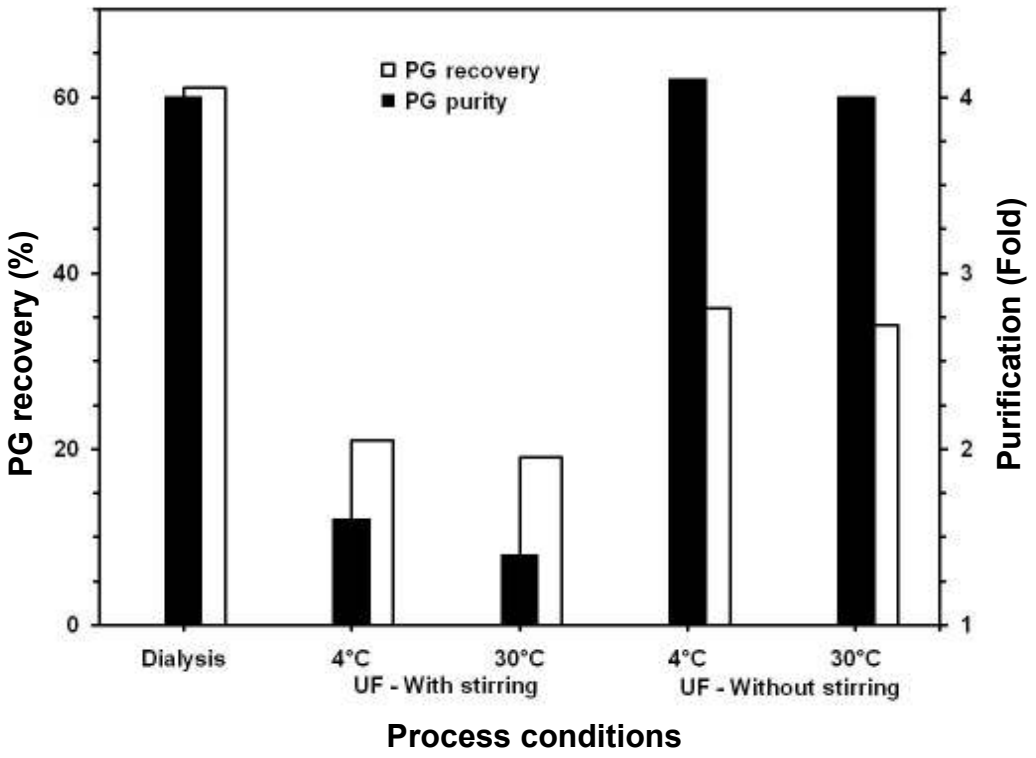


Fig. 5.4 Concentration and desalting of eluted PG by 10 kDa UF membrane

reduced handling volume and processing time besides being a single step process for desalting and concentration of eluted PG.

5.7 Conclusions

AAP exhibited excellent separation ability between PG and other impurities including similar molecular mass contaminant proteins owing to the selective affinity of alginate towards any particular protein (enzyme) under appropriate process conditions. Besides, AAP revealed its suitability for the purification of SmF- and SSF-PG. IMP offers several advantages over other downstream processes including AAP. 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.

6.0 Significance and focus of the work

Studies on upstream processes using mutant *A. carbonarius* showed that higher yield together with shorter fermentation period resulting in greater productivity favoring SmF for PG production over SSF. AAP enhanced the PG purity and recovery in both SmF and SSF-PGs. IMP offers several process advantages over other downstream processes including AAP but not suitable for purification of all types of PGs. This scenario suggested the need for a techno-economic analysis for configuring an appropriate process combination. In the present work, comparative techno-economic analysis was performed for both upstream and downstream processes for PG production and purification.

6.1 Laboratory process performance

6.1.1 Upstream processes

The detailed performance of SmF and SSF processes for the production of *A. carbonarius* PG were described in chapter 3. Generally, SSF provides a system with higher productivity due to larger enzyme yields and shorter fermentation periods (Solis-Pereira *et al.*, 1993). In the present study, one gram of starch yielded 2.7 times higher PG production in SmF over SSF process (Table 6.1). The results suggested that it is more appropriate to compare the enzyme yield as well as productivity in terms of the substrate used, more specifically the carbon source. Higher yield together with shorter fermentation period resulted in 6.6 times greater productivity favoring SmF for PG production over SSF.

Table 6.1 Process performance of SmF and SSF for *A. carbonarius* PG production and purification[a]

Description	SmF		SSF	
	IMP	AAP	IMP	AAP
Upstream				
Main substrate	Corn flour	Corn flour	Wheat bran	Wheat bran
PG activity (U/g of starch)[b]	13100	13100	4940	4940
Productivity ((U/g starch)/h)	548	548	82.4	82.4
PG activity (kU/INR of substrate)	239	239	61	61
Downstream				
Recovery (%)	76	74	72	61
Specific activity (U/mg)	5590	9770	640	2450
Purity (Fold)	4.7	8.7	1.2	4.0

[a]SmF-Submerged fermentation; SSF-Solid state fermentation; IMP-Integrated membrane process; AAP-Alginate affinity precipitation; PG-polygalacturonase.

[b]Starch content in corn flour and wheat bran were 70% and 18%, respectively (Ghildyal *et al.*, 1985).

6.1.2 Downstream processes

The downstream processing using IMP and AAP were optimized for the purification of PG from *A. carbonarius* SmF culture broth and SSF extracts. IMP enhanced the specific activity of SmF-PG by 4.7 fold with a recovery of 76%, but did not improve the specific activity of SSF-PG (Table 6.1) due to the presence of similar molecular mass proteins excreted by the fungus under SSF conditions (Venkatesh, 2004). Since these contaminant proteins, similar to molecular mass

of *A. carbonarius* PG were eliminated by AAP, a simple and scalable selective separation process, the method was used to compare the processes. AAP enhanced the specific activity of both SmF- and SSF-PG to a greater extent compared to IMP. The PG recovery obtained with SmF-AAP was comparable with SmF-IMP while the recovery was lower with SSF-AAP (Table 6.1). In the light of the results above, the economic analyses were carried out only for the three potential combinations, SmF-IMP, SmF-AAP and SSF-AAP processes.

6.2 Process design and total capital investment estimation

A base-case plant producing 30 kl purified PG concentrate per year was designed by first constructing process flow diagrams based on the laboratory performance parameters. These process flow diagrams (Figs. 6.1-6.3) served as the basis to identify the major equipment needed for the three upstream and downstream combinations taken up for the study. Specification and number of individual equipment required were worked out for a smooth operation and at the same time minimizing total capital investment (TCI). Upstream batch processes with long fermentation times were balanced with downstream processing equipment to avoid large idle intervals with a view to make the plant most economic by way of maximum utilization of the plant and machinery. Based on percentage equipment cost, obtained from a commercial plant and equipment supplier, capital investment was estimated as proposed by Peters and Timmerhaus (1991).

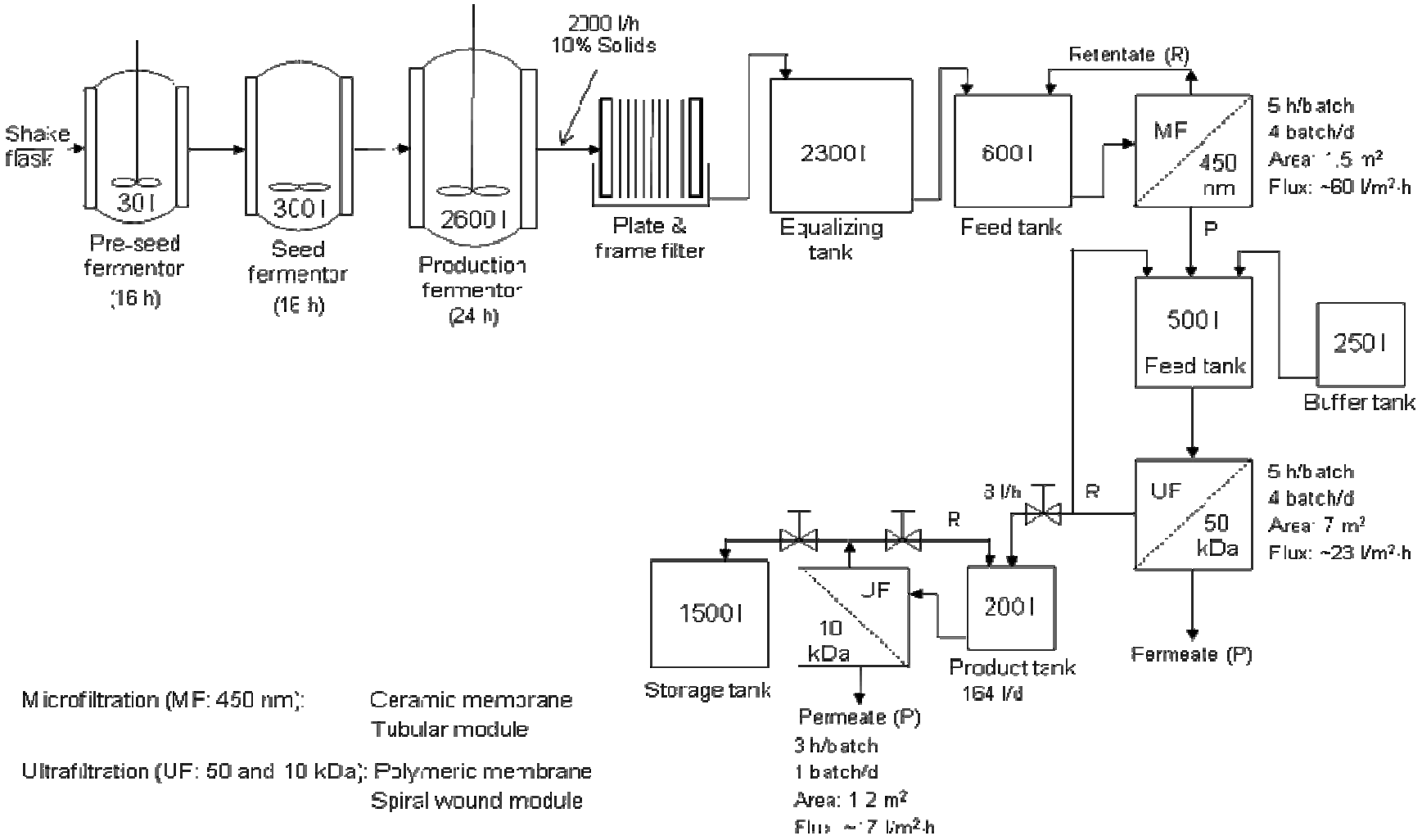


Fig. 6.1 Process flow diagram for upstream and downstream processing of *A. carbonarius* PG (SmF-IMP)

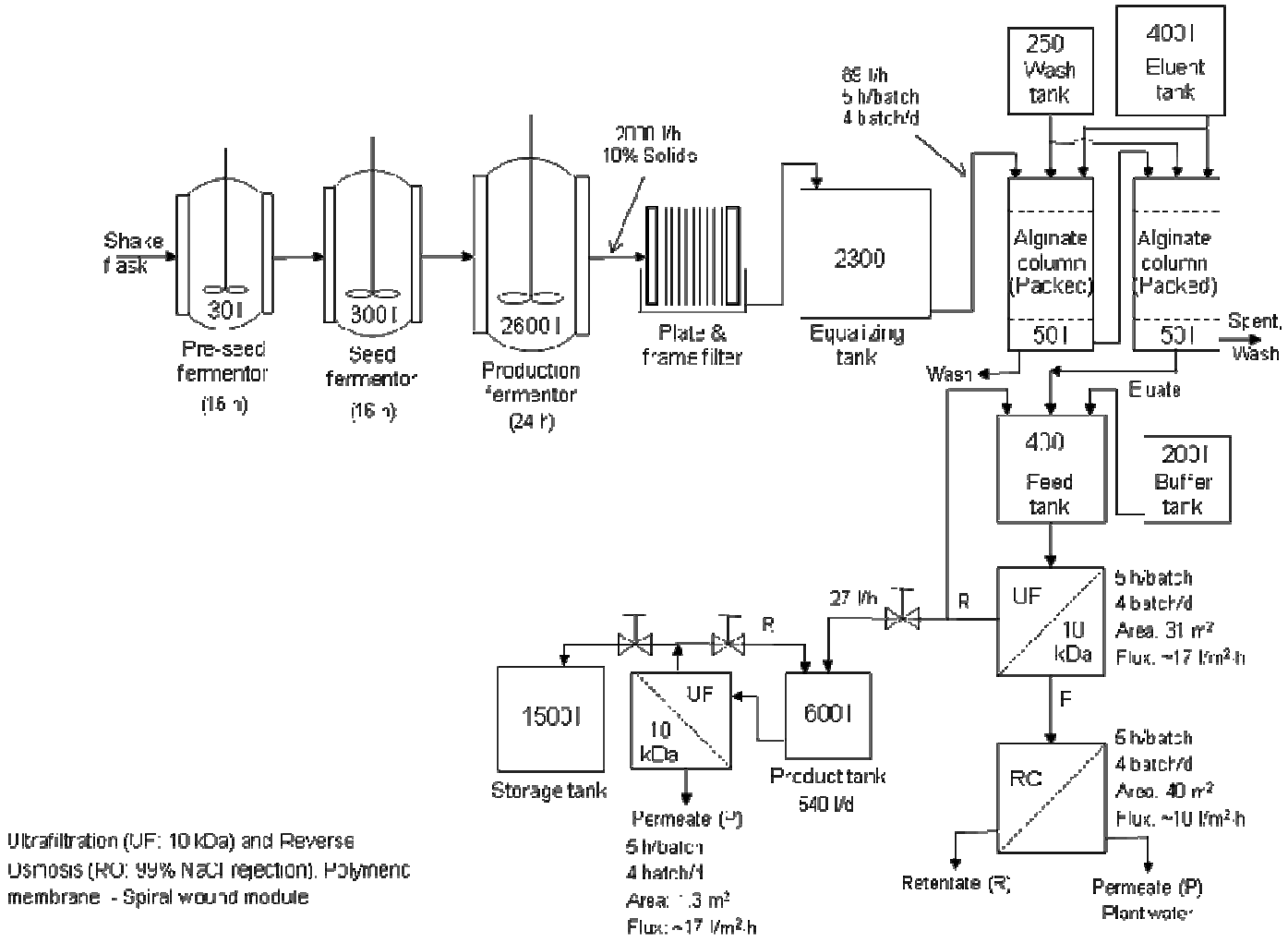


Fig. 6.2 Process flow diagram for upstream and downstream processing of *A. carbonarius* PG (SmF-AAP)

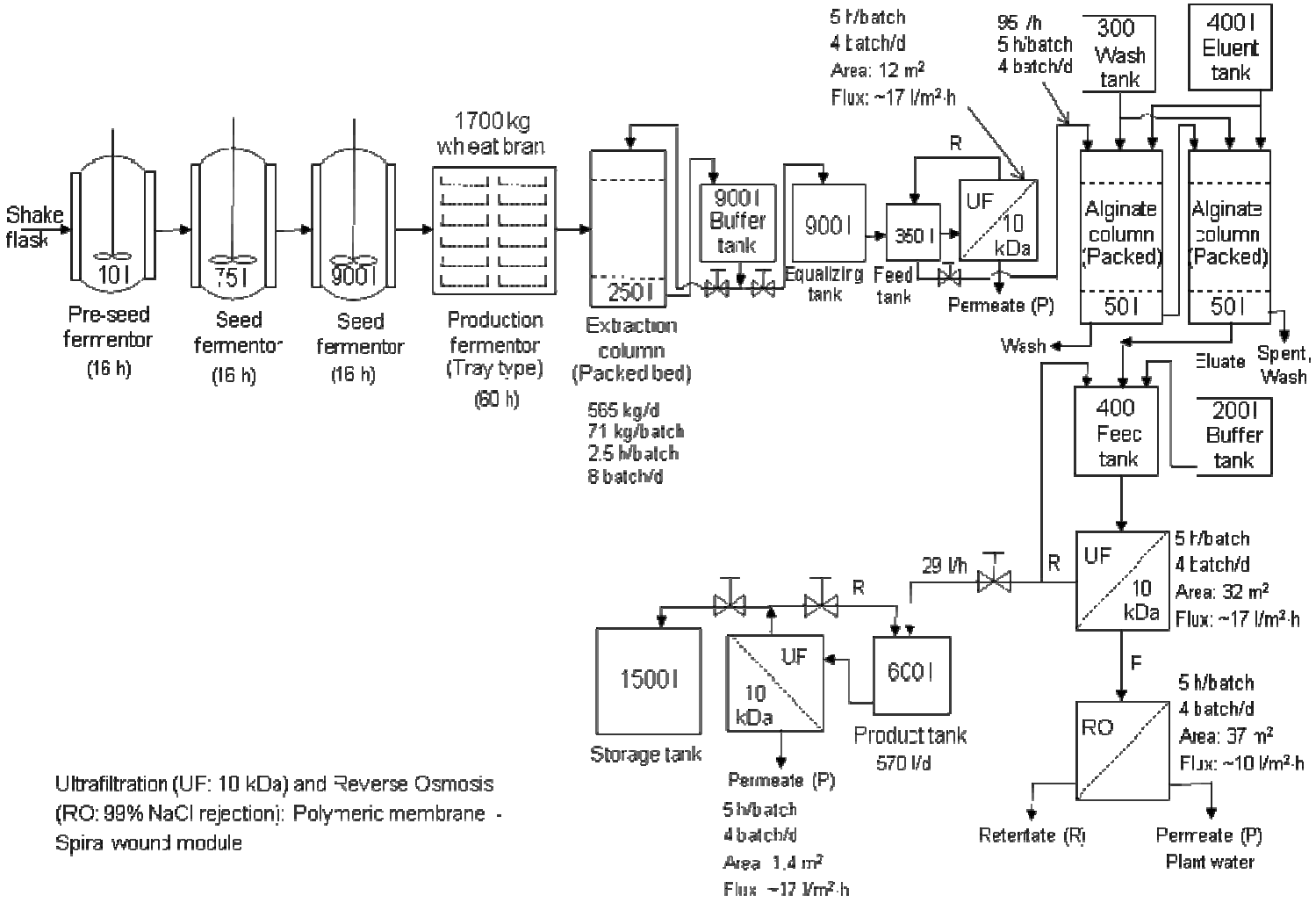


Fig. 6.3 Process flow diagram for upstream and downstream processing of *A. carbonarius* PG (SSF-AAP)

List of major equipment needed for upstream and downstream processes and their individual costs are shown in Tables 6.2a and 6.2b, respectively. There is practically no difference in upstream equipment cost for the three process combinations studied for the production of PG (Table 6.2a). In the case of downstream process, SmF-IMP resulted in lower equipment cost (INR 74 Lakhs) compared to SmF-AAP (INR 96 Lakhs), while the equipment cost was much higher with SSF-AAP (INR 116 Lakhs). The higher equipment cost obtained with SmF-AAP is due to longer processing steps and in the case of SSF-AAP it is due to lower product recovery in addition to longer processing steps.

TCI needed for three process combinations are described in Table 6.3. The SmF-IMP needed the lowest investment (INR 628 Lakhs) while SSF-AAP needed the highest investment (INR 828 Lakhs). The investment needed for SmF-IMP, SmF-AAP and SSF-AAP were in the ratio of 1: 1.17: 1.32.

There is practically no difference in the TCI requirements in the upstream processes (Table 6.4). The TCI requirements for the downstream processes were about 1.4-2.2 fold higher than upstream processes. The differences observed in the TCI of composite plants were due to the differences in the TCI of downstream processes. The differences observed in the fixed capital investment (FCI) and TCI requirements between downstream processes of SmF-AAP and SSF-AAP are mainly due to the differences in their throughputs in order to balance the differences for overall enzyme recovery (Table 6.4). Such a difference was not noticed in upstream processes of SmF-AAP and SSF-AAP, as there was no difference in the total equipment cost owing to the fact that SSF

Table 6.2a Cost of major upstream equipment required for SmF and SSF-based plants producing 30 kl purified PG concentrate per year[a]

SmF-IMP			SmF-AAP			SSF-AAP		
Equipment	Quantity	Total cost (INR Lakhs)	Equipment	Quantity	Total cost (INR Lakhs)	Equipment	Quantity	Total cost (INR Lakhs)
Pre-seed fermentor	1	5	Pre-seed fermentor	1	5	Pre-seed fermentor	1	4
Seed fermentor	1	15	Seed fermentor	1	15	Seed fermentor	1	10
Production fermentor	1	35	Production fermentor	1	35	Seed fermentor	1	20
						Production fermentor	1	20
Total		55	Total		55	Total		54

[a]For abbreviations see Table 6.1.

Table 6.2b Cost of major downstream equipment required for SmF and SSF-based plants producing 30 kl purified PG concentrate per year[a]

SmF-IMP			SmF-AAP			SSF-AAP		
Equipment	Quantity	Total cost (INR Lakhs)	Equipment	Quantity	Total cost (INR Lakhs)	Equipment	Quantity	Total cost (INR Lakhs)
Filter press	1	9	Filter press	1	9	Extraction column	1	5
Equalizing tank	1	11	Equalizing tank	1	11	Buffer tank	1	7
Feed tank	1	6	Alginate column	2	3	Equalizing tank	1	7
Microfiltration unit	1	4	Wash tank	1	2	Feed tank	2	10
Feed tank	2	12	Eluent tank	1	6	Ultrafiltration unit	1	9
Buffer tank	1	2	Feed tank	2	12	Alginate column	2	3
Ultrafiltration unit	1	6	Buffer tank	1	2	Wash tank	1	4
Product tank	1	2	Ultrafiltration unit	1	10	Eluent tank	1	6
Ultrafiltration unit	1	4	Product tank	1	6	Feed tank	2	12
Storage tank	2	18	Reverse osmosis unit	1	13	Buffer tank	1	2
			Ultrafiltration unit	1	4	Ultrafiltration unit	1	10
			Storage tank	2	18	Product tank	1	6
						Reverse osmosis unit	1	13
						Ultrafiltration unit	1	4
						Storage tank	2	18
Total		74	Total		96	Total		116

[a]For abbreviations see Table 6.1.

Table 6.3 Total capital estimate of SmF and SSF-based plants producing 30 kl purified PG concentrate per year[a]

Item	Percentage of equipment cost	Costs (INR Lakhs)		
		SmF-IMP	SmF-AAP	SSF-AAP
A. Direct costs				
Purchased equipment-delivered	100	129.00	151.00	170.00
Purchased-equipment Installation	39	50.31	58.89	66.30
Instrumentation and control (installed)	13	16.77	19.63	22.10
Piping (installed)	31	39.99	46.81	52.70
Electrical (installed)	10	12.90	15.10	17.00
Buildings (including services)	29	37.41	43.79	49.30
Yard improvements	10	12.90	15.10	17.00
Service facilities (installed)	55	70.95	83.05	93.50
Land (if purchase is required)	6	7.74	9.06	10.20
Total direct costs	293	377.97	442.43	498.10
B. Indirect costs				
Engineering and supervision	32	41.28	48.32	54.40
Construction expenses	34	43.86	51.34	57.80
Total indirect costs	66	85.14	99.66	112.20
C. Contractor's fee	18	23.22	27.18	30.60
D. Contingency	36	46.44	54.36	61.20
Fixed-capital investment (FCI: *A*+*B*+*C*+*D*)	413	532.77	623.63	702.10
E. Working capital	74	95.46	111.74	125.80
Total capital investment (TCI: FCI+*E*)	487	628.23	735.37	827.90

[a]For abbreviations see Table 6.1.

Table 6.4 Breakdown of major upstream and downstream process costs for SmF and SSF-based plants producing 30 kl purified PG concentrate per year[a]

Item	Costs (INR Lakhs)		
	SmF-IMP	SmF-AAP	SSF-AAP
Upstream			
Equipment cost	55.00	55.00	54.00
Raw material/annum	3.77	3.79	14.86
Fixed-capital investment	227.15	227.15	223.02
Total capital investment	267.85	267.85	262.98
Total manufacturing cost/annum	142.20	142.25	167.40
Total product costs/annum	194.49	194.55	225.69
Unitary product cost (INR/l)	648.29	648.50	752.29
Downstream			
Equipment cost	74.00	96.00	116.00
Raw material/annum	3.67	24.84	30.89
Fixed-capital investment	305.62	396.48	479.08
Total capital investment	360.38	467.52	564.92
Total manufacturing cost/annum	187.83	293.67	357.03
Total product costs/annum	257.31	396.31	481.60
Unitary product cost (INR/l)	857.69	1321.02	1605.32

[a]For abbreviations see Table 6.1.

fermentors are relatively cheaper than SmF fermentors.

6.3 Total product cost

Total product cost (TPC) is the estimation of costs for operating the plant and selling the product. Being the important part of an economic study, it is generally divided into the categories of manufacturing (or operating) costs and general expenses (Table 6.5). The TPC are calculated on annual basis, as a way of smoothing out any seasonal variations and of considering large but infrequently occurring expenses, such as annual change of membranes (Castilho *et al.,* 2000).

The total and unitary product costs for three process combinations are presented separately for upstream and downstream sections in Table 6.4. Raw material cost is the only independent component in the methodology proposed by Peters and Timmerhaus (1991) for the estimation of TPC. Among the raw materials used, wheat bran is the main component in the SSF while it is corn flour in the case of SmF. Generally an extremely cheap raw material is used as the main substrate in the SSF process positioning SSF with a great advantage. In the present case, the cost of substrate in SSF is significantly higher (3.9 fold) than SmF, defying the above advantage. Use of agro-waste materials, such as orange peel and apple pomace that promote pectinase synthesis in fungi have also been used in SSF process (Kashyap *et al.*, 2003; Mamma *et al.*, 2008). However, analysis revealed that raw materials accounted only for 1.9, 1.9 and 6.6% of the TPC of upstream process in SmF-IMP, SmF-AAP and SSF-AAP,

Table 6.5 Summary of total and unitary product costs for SmF and SSF-based plants producing 30 kl purified PG concentrate per year[a]

Item	Annual costs (INR Lakhs)		
	SmF-IMP	SmF-AAP	SSF-AAP
I. Manufacturing cost			
A. Direct production costs			
Raw material	7.44	28.63	45.76
Operating labour (15% of total product cost (TPC))	67.77	88.63	106.10
Direct supervisory and clerical labour (17.5% of operating labour)	11.86	15.51	18.57
Utilities (15% of TPC)	67.77	88.63	106.10
Maintenance and repairs (6% of fixed-capital investment (FCI))	31.97	37.42	42.13
Operating supplies (0.75% of FCI)	4.00	4.68	5.27
Laboratory charges (15% of operating labour)	10.17	13.29	15.91
Patents and royalties (3% of TPC)	13.55	17.73	21.22
B. Fixed charges			
Depreciation (10% of FCI)	53.28	62.36	70.21
Local taxes (2.5% of FCI)	13.32	15.59	17.55
Insurance (0.7% of FCI)	3.73	4.37	4.91
C. Plant over-head costs (10% of TPC)	45.18	59.09	70.73
Total manufacturing cost (A+B+C)	330.02	435.92	524.45
II. General expenses			
Administrative costs (4% of TPC)	18.07	23.63	28.29
Distribution and selling costs (11% of TPC)	49.70	64.99	77.80
Research and development costs (5% of TPC)	22.59	29.54	35.37
Financing (5% of total capital investment (TCI))	31.41	36.77	41.40
Total general expenses	121.77	154.94	182.86
Total product costs (TPC: *I*+*II*)	451.79	590.86	707.31
Unitary product cost (INR/l)	1505.98	1969.52	2357.71

[a]For abbreviations see Table 6.1.

respectively (Table 6.4). This indicated that employing even agro-waste materials in place of wheat bran as substrate would not have great impact on TPC.

In the downstream section, comparison of three process combinations revealed that TPC of IMP is only 53-65% of AAP (Table 6.4). The differences observed in TPC of SmF-AAP and SSF-AAP is mainly due to the difference in the overall recovery of enzyme in the downstream AAP process with SmF culture broth and SSF culture extract (Table 6.1). Taking into consideration upstream and downstream sides, the SmF-IMP combination gave the lowest TPC of INR 452 Lakhs for producing 30 kl of pectinase concentrate per year while TPC is 24% and 36% higher for SmF-AAP and SSF-AAP, respectively (Table 6.5). According to cost analysis, SmF-IMP is the cheapest for producing purified pectinase with a unitary product cost of INR 1,506/l (3000 U/ml) while the corresponding cost of crude pectinase is INR 648/l. The selling price of purified pectinase from multinational companies is in the order of INR 22,000/l (3000 U/ml) while the selling price of crude pectinase of equivalent enzyme units (3 Million Units) by the local manufacturers is at ~ INR 3750. Based on the above, a moderate selling price of INR 4000/l was considered in the present calculations, which showed great profitability for SmF-IMP process with attractive return on investment and low payback period (Table 6.6). The economic analyses performed for upstream processing of *A. carbonarius* PG nearly holds good for the production of crude enzymes considering the fact that only a filtration or extraction step is involved after fermentation. Therefore, a comparison on the production of crude and purified enzymes could be drawn by merely comparing the economic aspects

Table 6.6 Cash flow and profitability indicators for SmF and SSF-based plants producing 30 kl purified PG concentrate per year[a]

Item	Costs (INR Lakhs)		
	SmF-IMP	SmF-AAP	SSF-AAP
Total capital investment	628.23	735.37	827.90
Annual income (sales)[b]	1200.00	1200.00	1200.00
Annual production costs	451.79	590.86	707.31
Income before tax (gross profit)	748.21	609.14	492.69
Income after 40% tax	448.92	365.49	295.61
Net profit (income after tax + depreciation)	502.20	427.85	365.82
Annual cash flow	502.20	427.85	365.82
Payback period (years)	1.25	1.72	2.26
Return on investment (%)	79.94	58.18	44.19

[a]For abbreviations see Table 6.1.

[b]Selling price at INR 4000/l.

of upstream and composite (upstream and downstream) processing.

Harsa *et al.* (1993) studied a batch process for the production of PGs from *Kluyveromyces marxianus* under SmF conditions and reported a payback period of less than a year. However, the selling price (0.5 £/kU) taken for the calculations were much higher than the current international market price. Besides the purity (73 U/mg protein) achieved in the ion-exchange process employed was not significant as the activity of initial culture broth itself was extremely low (0.2 U/mg protein). Ferreira *et al.* (1995) studied a SSF process for production of 130 m^3 of pectinase concentrate per year and projected a payback period of 4 years. The present process shows

comparatively good values for the economic indicators suggesting that investing in this project is secure. Payback period was found to be 1.3 years with a return on investment of ~80%. Furthermore, the profitability remained high even while considering a 50% increase in TCI or TPC. The profitability indicators thus signify the process as very attractive from an economic point of view. A higher production scale would offer a scale gain resulting in further lowering of TPC and unitary product cost, leading to much improved profitability.

6.4 Conclusions

Techno-economic analysis revealed SmF followed by IMP as the cheapest process combination for producing purified and concentrated pectinase. The study emphasized that it is more appropriate to evaluate the enzyme yield as well as productivity in terms of carbon source. The results suggested that substrate cost need not be an influencing factor in the process selection as it formed only 2-7% of TPC of upstream process. Further downstream process costs are far more expensive than upstream process costs and adoption of appropriate methodology like IMP could make a difference.

7.0 Significance and focus of the work

Techno-economic analysis suggested that the SmF followed by IMP is a very attractive combination for producing purified and concentrated PG from *A. carbonarius*. Scale-up studies on IMP were carried out in cross-flow membrane systems to improve the process performance of IMP protocol developed earlier in the laboratory with a stirred flat membrane test cell for the purification of PG from culture broth and estimated the various resistances that are responsible for the process flux decline. Also the effect of stirring and pump performance was evaluated to assess the shear sensitive nature of PG.

7.1 Effect of stirring on PG activity

Enzymes being shear sensitive lose activity when subjected to any processing step that induce shear, such as stirring or pumping. Hence stirring speed is usually adjusted in a stirred membrane cell to a minimum level that would be sufficient to minimize concentration polarization. Loss of PG and protein from SmF culture broth was assessed against a wide range of stirring speed varying from 200 to 800 rpm under simulated conditions of a membrane cell. PG loss increased with stirring speed or to be more precise with shear stress and processing time. The loss was gradual up to 400 rpm (τ =2.1 Pa) and thereafter increased drastically with increase in stirring speed (Fig. 7.1). PG loss at higher stirring speeds, 600 (τ =4.0 Pa) and 800 (τ =6.5 Pa) rpm, was more pronounced with increase in processing time from 1 to 3 h. Thus shear stress exerted during stirring that increased with speed (Fig. 7.1) and duration of stirring reasoned the

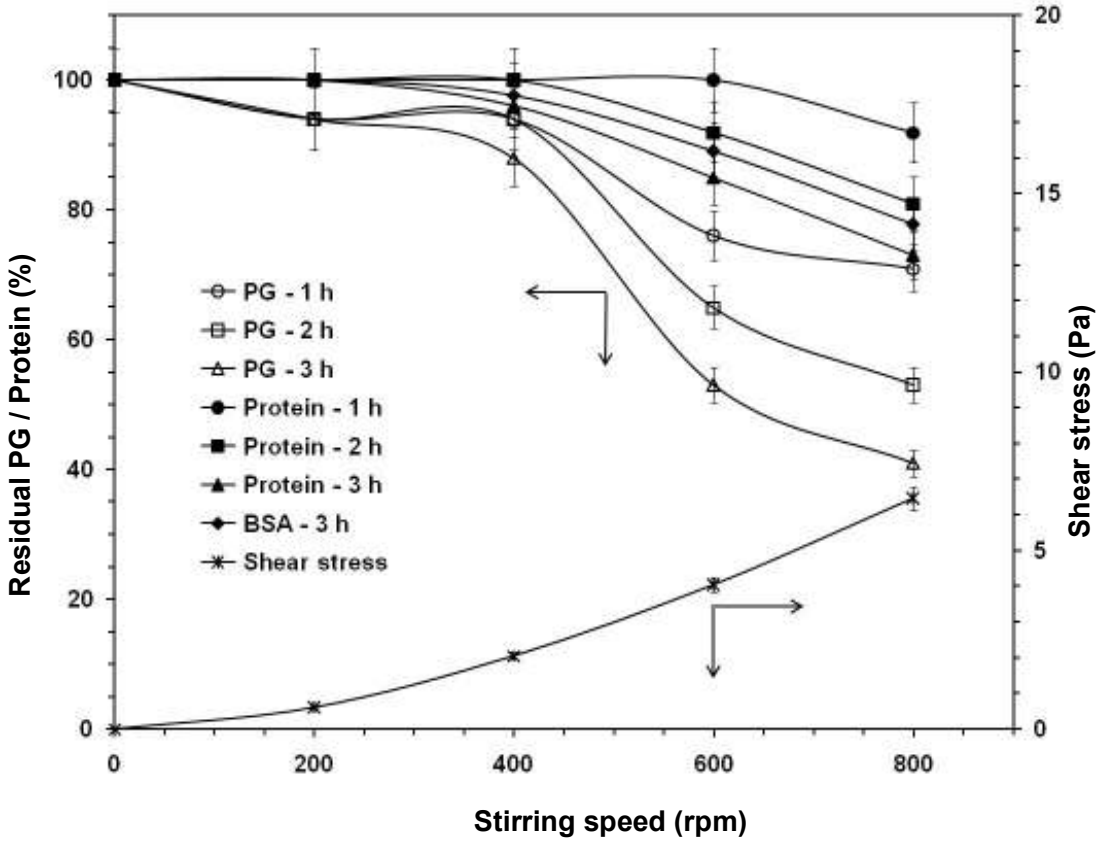


Fig. 7.1 Effect of stirring speed on PG and protein in a simulated stirred cell

loss of enzyme activity. The loss of protein also showed a similar trend with increase in stirring speed (shear stress) and duration, but to a lesser extent compared to loss of PG which may be due to the fact that non-enzymatic proteins are less shear sensitive compared to enzymes. In the case of protein, the shear stress generally affects its structure by unfolding and may cause aggregation (Cormwell *et al.*, 2006) but would not necessarily lead to lysis. Similar trend of protein loss was observed with increase in stirring speed while processing BSA solution albeit the loss was lower (Fig. 7.1). The results suggested that exceeding a stirrer speed of 400 rpm (τ =2.1 Pa) would result in greater loss of PG activity. This shear appears to be the critical shear for the

system which should not be exceeded during operation. Within this limit of operating conditions, the losses were contained and reasonably greater residual PG (88%) and protein content (96%) were obtained in the processed stream after 3 h of operation.

7.2 Pump performance during PG processing

Processing SmF culture broth using a gear pump resulted in greater loss of PG and protein to the extent of 39% and 21%, respectively in the first 30 min operation. After 2 h processing, the loss increased to 69% PG and 47% protein beyond which the losses were very gradual up to 3 h of pumping (Fig. 7.2). Similar trend was exhibited by BSA. Semi-log plots revealed that residual PG activity (Fig. 7.3) and protein content (Fig. 7.4) exhibited an exponential relationship with processing time. The residual PG and protein content in the pumping process showed similarity to classical microbial survivor curves (Bigelow, 1921) indicating that the inactivation of enzyme and protein followed a first order kinetic model. In a gear pump, the pumping action is caused by the rotation of intermeshing gears with close clearance in pump housing and the liquid flows between the toothed-gears and the casing. The loss of enzyme activity and protein in the culture broth could be due to intense mechanical shear and turbulence exerted in a gear pump leading to inactivation and denaturation. Enzymes are more sensitive to any type of stress than proteins and get inactivated rapidly.

In the case of peristaltic pump, the loss of PG and protein was negligible and the residual PG (97%) and protein (96%) remained unaffected during

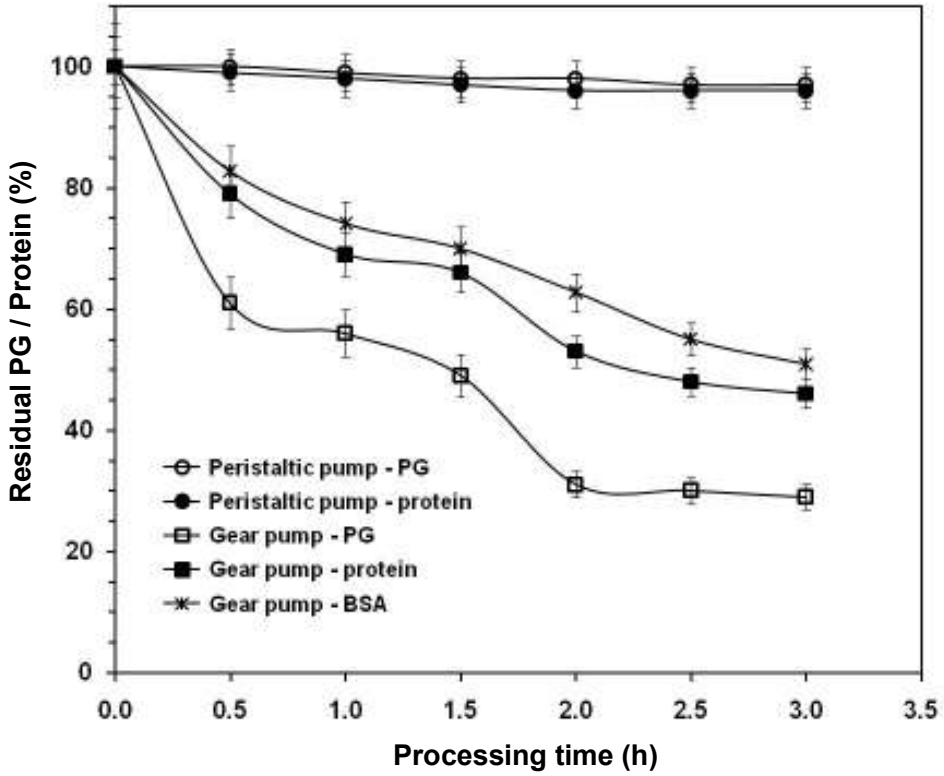


Fig. 7.2 Comparison of pump performance during processing PG

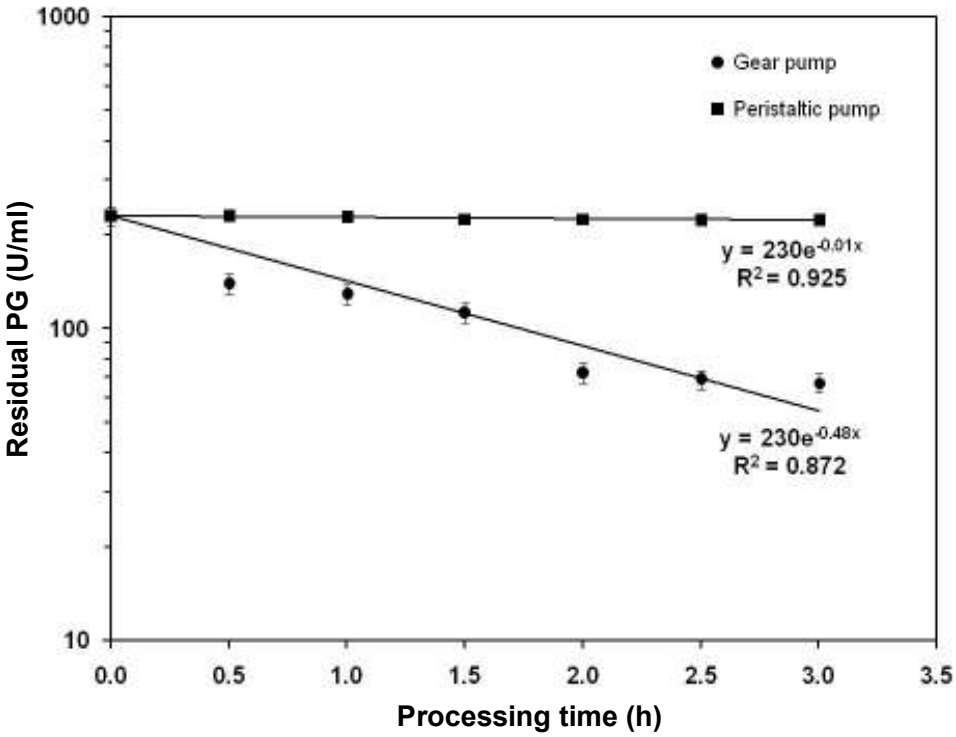


Fig. 7.3 PG loss during pumping

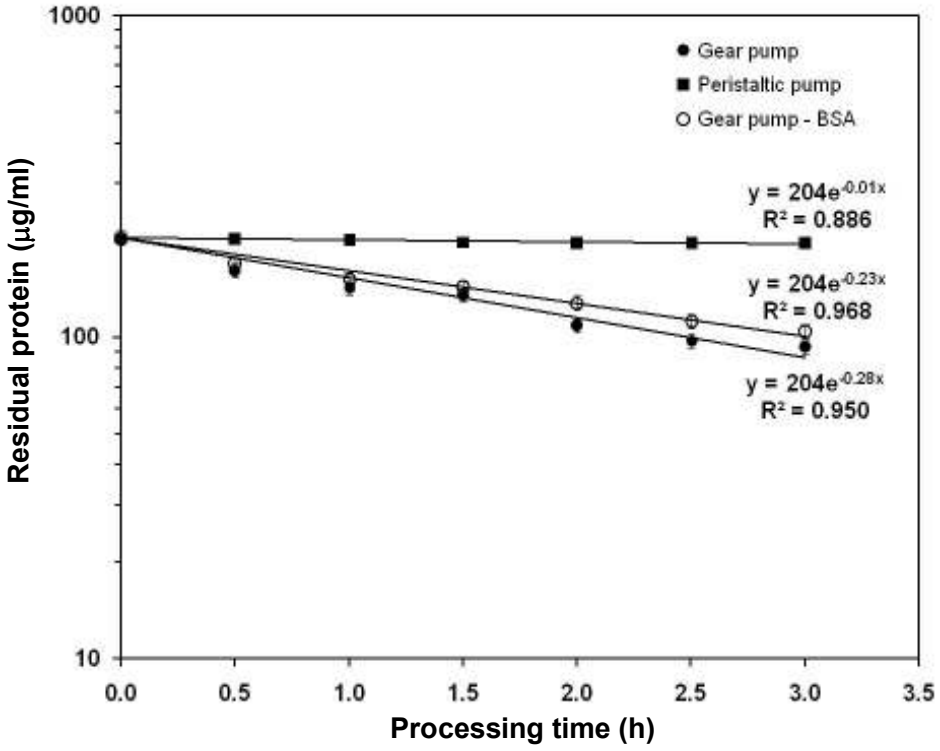


Fig. 7.4 Protein loss during pumping

processing SmF culture broth up to 3 h of pumping (Fig. 7.2). Peristaltic pump works by compressing and relaxing a hose that is positioned between a rotating device (rotor) and circular pump housing. Thus the culture broth is in contact only with hosing/tubing and not with mechanical devices. Therefore, the enzymes are not subject to intensive shear resulting in lower PG and protein losses.

Centrifugal pumps work by rotating the propeller with higher velocities. During pumping the enzyme solution comes in to contact with rotating impellers but not subjected to severe stress as in the case of a gear pump wherein the process fluid passes through the intermeshing gears. Denis and Boyaval (1991) tested the shear resistance of *E. chrysanthemi* pectate lyase in a bioreactor,

coupled with an UF system fitted with a centrifugal pump and reported that the activity was not affected up to 7.5 h of operation due to shear caused by pumping (Denis and Boyaval, 1991). Under exaggerated conditions, an activity loss of 36% was observed after 25000 passes (144 h of operation) through the pump. They concluded that pectate lyase possesses sufficient resistance to shear damage to withstand the process duty of 2500 passes (equivalent to ~14 h of operation).

There are other means of reducing the loss of shear sensitive enzymes by altering process conditions when pumps are employed in the process. The productivity in a cross flow membrane system has a strong bearing on circulation velocities. Greater the circulation velocity higher is the permeate flux which has a tendency to result in greater loss of shear sensitive enzymes. The ability of static mixer to achieve high permeate fluxes at low circulation velocities has been applied in a tubular cross flow UF system for processing pectinase solution (Krstic *et al.*, 2007). By employing a suitable static mixer in the system to maintain the productivity, the cross flow velocity reduced from 0.43 to 0.27 m/s which correspondingly reduced the pectinase loss from 44% to 20% when the system was operated in a recirculation mode, without fitting the membrane for 2 h. Pectin addition to the feed enhanced the stability of pectinase against shear deactivation (Elias and Joshi, 1997) and the loss was reduced to a mere 6% even without employing a static mixer under otherwise similar conditions (Krstic *et al.*, 2007).

7.3 Performance evaluation of IMP in self-stirred and cross-flow filtration systems

Cross-flow filtration is employed for industrial scale operation since they offer several advantages over dead end filtration systems. However, initial studies on developing IMP were carried out in a self-stirred flat membrane test cell owing to the convenience it offered for working out the process scheme. IMP (450 nm followed by 50 kDa) scheme was attempted in a laboratory cross-flow filtration system and performance compared with self-stirred flat membrane test cell (Table 7.1). Both self-stirred and cross-flow filtration systems exhibited near complete elimination of carbohydrates present in the culture broth. The PG recovery obtained with 450 nm MF membrane in cross-flow filtration system was lower compared to self-stirred filtration system while the specific activity and normalized flux were higher. In the cross-flow filtration system studied, the VCR was not exceeded above 5 fold due to process limitations of the system, requiring longer process time leading to deterioration of feed. The lower VCR in the cross flow filtration system resulted in 4.1 fold increase in PG purity (4610 U/mg protein) with 64% recovery. Higher PG purity and recovery (4.7 fold; 76% recovery) obtained in stirred membrane cell suggested similar performance could be achieved in a cross-flow filtration system by maintaining the same VCR of 10 with a larger membrane area.

The pure water flux of MF and UF membranes in cross-flow filtration system were ~1.5 fold higher than self-stirred filtration system. The average process flux obtained with MF in cross-flow filtration system was ~3 times lower

Table 7.1 Performance evaluation of IMP (450 nm-50 kDa) for PG purification in self-stirred and cross-flow filtration systems[a]

Process description	Self-stirred filtration system						Cross-flow filtration system						
	VCR (Fold)	PG-CR (%)	Protein-CR (%)	CHO-CE (%)	Specific activity (U/mg)	Average flux ($l/m^2 \cdot h$)	VCR (Fold)	PG-CR (%)	Protein-CR (%)	CHO-CE (%)	Specific activity (U/mg)	Average flux ($l/m^2 \cdot h$)	Normalized flux[b] ($l/m^2 \cdot h$)
MF (450 nm)	10.0	85	55	23	1690	60	5.0	71	35	20	2290	19	95
UF (50 kDa)	10.0	79	35	78	2460	29	4.0	67	28	79	2640	71	355
DF-1	9.3	78	23	94	3890	20	3.6	65	24	97	3340	70	350
DF-2	9.3	76	19	96	5590	19	3.6	64	20	98	4610	69	345

[a]Pretreatment: Filtered through ordinary filter paper; Feed volume: 50 ml for self-stirred filtration system and 1000 ml for cross-flow filtration system; VCR – Volume concentration ratio; PG – Polygalacturonase; CHO – Carbohydrates; CR – Cumulative recovery (MF – permeate; UF – retentate); CE – Cumulative elimination (MF – permeate; UF – retentate); DF-1 and 2: Diafiltration - 1 and 2 (50% feed volume of 0.85% NaCl).
PG activity, protein content, CHO content, and PG specific activity in feed were 230 U/ml, 204 µg/ml, 13.1 mg/ml, and 1130 U/mg protein, respectively.

[b]Normalized with respect to feed volume-to-membrane surface area of self-stirred filtration system.

compared to self-stirred filtration system and did not reflect the pure water flux measurements. When the flux was normalized with respect to feed-to-membrane area, the improvement in process flux achieved with cross flow system was apparent, and was comparable with the water flux measurements. Fifteen fold higher flux obtained with UF in cross-flow filtration system upon normalization suggested that cross flow system has the ability to reduce the resistance due to cake layer formation on the membrane surface, depending on the cross flow velocity employed.

Direction of feed flow in cross-flow filtration system result in higher flux due to reduced resistance caused by membrane, fouling and cake formation. Becht *et al.* (2008) reported the flux of dextran standard and lysozyme were ~20% higher while it was ~7% lower with BSA solution with a 50 kDa UF membrane in cross-flow system compared to self-stirred filtration system. Though cross flow system was expected to increase the permeate flux, the difference in flux obtained was not appreciable apparently due to the flow regimes of the systems. The cross-flow system was operated in laminar flow region (N_{Re} 1,560) while the self-stirred system (stirrer speed 2400 rpm) in the turbulent region (N_{Re} 57,000). The corresponding shear value 30 Pa in self-stirred filtration system was significantly higher than the cross-flow filtration system (1 Pa). The flow conditions have a direct bearing on the concentration polarization effect with a consequent effect on solute permeation or rejection. While processing protein solutions the solution environment such as ionic strength and pH also influences the rejection or transmission of proteins by changing the effective size of the

solute (Becht *et al.,* 2008).

Pilot scale studies were conducted with crude culture broth containing PG employing 200 nm MF followed by 20 kDa UF membranes since membranes suitable for the original IMP configuration (450 nm followed 50 kDa) were not available for the pilot system. The PG specific activity obtained with 200 nm MF membrane in pilot scale cross-flow filtration system was comparable with the self-stirred filtration system. The enzyme recovery of 46-53% from both the systems obtained with 200 nm MF membrane was much lower to 71-85% obtained with 450 nm MF membrane (Tables 7.1 and 7.2). This apparently reflected on the difference in the pore sizes of the membranes. The normalized process flux obtained with MF in cross-flow filtration system was 4.5 fold higher compared to self-stirred filtration system, which could be mainly attributed to the greater porosity of the ceramic membrane (Table 7.2).

The near complete elimination of carbohydrates while processing through 200 nm membrane followed by 20 kDa membrane was comparable to the IMP scheme proposed (Tables 7.1 and 7.2). The specific activity of PG improved to 2300 U/mg protein (2.1 fold) although the original IMP configuration was not employed. The specific activity of PG obtained with IMP in pilot scale filtration system was comparable with self-stirred filtration system which provided evidence that the performance could be replicated in the scale up (Table 7.2). The overall PG recovery was 10% lower in pilot scale filtration system compared to self-stirred filtration system that was reasoned to the differences in the type of MF and UF membranes used in the two systems.

Table 7.2 Performance of IMP (200 nm-20 kDa) for the purification of PG in a pilot scale filtration system[a]

Process Description	Self-stirred filtration system						Pilot scale filtration system					
	PG-CR (%)	Protein-CR (%)	CHO-CE (%)	Specific activity (U/mg)	Average flux ($l/m^2 \cdot h$)	VCR (Fold)	PG-CR (%)	Protein-CR (%)	CHO-CE (%)	Specific activity (U/mg)	Average flux ($l/m^2 \cdot h$)	Normalized flux[b] ($l/m^2 \cdot h$)
MF (200 nm)	53	31	21	1960	80	5.0	46	26	19	1910	74	370
UF (20 kDa)	50	27	74	2140	55	4.0	41	20	76	2020	50	83
DF-1	50	26	92	2550	53	3.6	40	20	91	2300	49	82
DF-2	50	26	97	2570	50	3.6	40	19	96	2300	49	82

[a]Pretreatment: Filtered through ordinary filter paper; Feed volume: 50 ml for self-stirred filtration system and 50 l for pilot scale filtration system; For feed characteristics and abbreviations see Table 7.1.

[b]Normalized with respect to feed volume-to-membrane surface area of self-stirred filtration system.

Normalized process flux obtained with UF in pilot scale cross-flow filtration system was only ~1.6 fold higher when compared to self-stirred filtration system. The improvement in flux was lower compared to the 15 fold increase obtained in the laboratory scale cross flow filtration system. Despite the fact that both the membranes were made from the same material (PS), even water flux in tubular UF membrane (89 $l/m^2 \cdot h$) was lower than that of flat sheet membrane (99 $l/m^2 \cdot h$). The age of the membranes employed seemed to affect the water flux.

The permeate flux obtained with 200 nm MF membrane in pilot scale cross-flow filtration system was ~4 fold higher compared to that obtained with larger pore size 450 nm MF membrane in laboratory scale cross-flow filtration system (Tables 7.1 and 7.2). This variation could be mainly attributed to the greater porosity of the ceramic membranes fitted in the pilot scale system. The flux obtained with UF membranes in lab scale (50 kDa; 5.39 m/min) and pilot scale (20 kDa; 9.96 m/min) cross-flow filtration systems were comparable after normalizing MWCO and cross-flow velocity. The results suggested that microfiltered feeds have less influence on the flux of the subsequent UF process.

7.4 Various resistances during IMP in self-stirred and cross-flow filtration systems

Declining permeate flux with processing time lowers the filtration efficiency. Therefore, the flux decline in the proposed IMP (450 nm followed by 50 kDa) in laboratory self-stirred and cross-flow filtration systems was analysed using a resistance-in-series model described in an earlier section (2.2.6). Using the

experimental data obtained during IMP of culture broth containing PG, the membrane, fouling, cake and total resistances were determined (Table 7.3).

Membrane resistance (R_m) is the resistance of membranes to water/solvent permeation. It is an intrinsic membrane property and expected to be fairly constant and independent of the driving force, pressure. It is characterized mainly by the pore shape and size of the membrane as well as its thickness (Choi *et al*., 2005). In the self-stirred filtration system, UF membrane exhibited a higher membrane resistance ($2.81x10^{12}$/m) compared to MF membrane ($0.04x10^{12}$/m), primarily owing to the differences in pore size/MWCO. Similar trend was observed in cross-flow filtration system with UF ($0.49x10^{12}$/m) and MF ($0.02x10^{12}$/m) membranes, however, the values were lower compared to self-stirred filtration system (Table 7.3). Choi *et al.* (2005) also reported that the intrinsic membrane resistance was much higher with 30 kDa UF membrane compared to 0.3 µm MF membrane in a cross-flow filtration system. Then the results were in general agreement because the membrane resistance increased with decreasing pore size. The resistances obtained with self-stirred and cross-flow filtration systems for the same set of membranes showed that the direction of feed flow in the system was also involved in membrane resistance.

Fouling resistance (R_f) is the resistance caused by the adsorption of feed particles on the membrane matrix and blocking of pore (Kaghazchi *et al.*, 2001). During processing culture broth, in self-stirred filtration system, MF membrane exhibited higher fouling resistance to the extent of $10.30x10^{12}$/m compared to UF membrane ($6.84x10^{12}$/m). Fouling in membrane processing of biological

Table 7.3 Various resistances during IMP (MF and UF) of *A. carbonarius* PG in self-stirred and cross-flow filtration systems[a]

Process description	R_m ($x10^{12}$) (1/m)	R_f ($x10^{12}$) (1/m)	R_c ($x10^{12}$) (1/m)	R_t ($x10^{12}$) (1/m)
Self-stirred filtration system				
MF: 450 nm	0.04	10.30	0.14	10.48
UF: 50 kDa	2.81	6.84	34.70	44.35
Cross-flow filtration system[b]				
MF: 450 nm	0.02	8.81	0.08	8.91
UF: 50 kDa	0.49	0.68	5.23	6.40

[a]Feed: Prefiltered crude culture broth for MF and microfiltered culture broth for UF;

Feed volume: 50 ml for self-stirred filtration system and 1000 ml for cross-flow filtration system; VCR – Volume concentration ratio: 5 fold;

R_m – Membrane resistance; R_f – Fouling resistance; R_c – Cake resistance; R_t – Total resistance.

[b]Normalized with respect to feed volume-to-membrane surface area of self-stirred filtration system.

suspension involves the combined effects of physical, chemical and biological factors (Chang and Lee, 1998; Nagaoka *et al.*, 1996). In cross-flow filtration system the fouling resistances reduced and more so with UF that followed MF (Table 7.3). The higher fouling resistance obtained with MF membrane was attributed to the larger membrane pore size that facilitated the adsorption of suspended solids present in crude culture broth leading to pore plugging/blocking. While processing biological solution in a cross-flow filtration system, Choi *et al.* (2005) reported that MF membrane fouled more than UF membrane (~3 to 5 times) in terms of total resistance.

Cake resistance (R_c) is the resistance due to the formation of cake layer deposited on the membrane surface (Kaghazchi *et al.*, 2001). During processing culture broth containing PG in a self-stirred filtration system, UF membrane exhibited much higher cake resistance (34.70×10^{12}/m) compared to MF membrane (0.14×10^{12}/m). The presence of carbohydrates and proteins in the microfiltered culture broth, which get adsorbed on to the membrane surface, was responsible for high value of cake layer resistance during processing culture broth with UF membrane. As in the case of fouling resistance, the cake resistance obtained in cross-flow filtration system was lower both with MF (0.08×10^{12}/m) and UF (5.23×10^{12}/m) membranes. This could be mainly attributed to the differences in direction of feed flow between the systems. In self-stirred filtration system, the feed flow is perpendicular to the membrane surface, while in cross-flow filtration system, the feed flow is parallel to the membrane surface. Earlier researchers, experimenting with polystyrol particles, baker's yeast,

Corynebacterium glutamicum and *Saccharomyces cerevisiae,* reported that the specific cake resistance should be the same with both dead-end and cross-flow filtration using MF membrane (Geissler and Werner, 1995; Tanaka *et al.*, 1994). In this study, the cake resistance obtained with MF membrane in both dead end and cross-flow filtration systems was negligible compared to their corresponding fouling resistance (Table 7.3). MF was more prone to fouling while UF offered greater cake resistance even with microfiltered culture broth.

Total resistance (R_t) comprises of membrane, fouling and cake resistances (Rai *et al.*, 2007). In self-stirred filtration system, the total resistance obtained with both MF and UF membranes was higher compared to cross-flow filtration system during processing culture broth containing PG (Table 7.3). The total resistance obtained with UF membrane in self-stirred filtration system was 4.2 fold higher than MF membrane. In cross-flow filtration system, the total resistance was greater with MF membrane compared to UF membrane (Table 7.3). The above contrasting difference observed in total resistances could be mainly attributed to greater cake resistance offered by UF in dead end filtration mode of operation in the self-stirred system.

7.5 Effect of pressure and process time on performance in cross-flow filtration system

The effect of operating pressure and process time on PG and protein permeation and flux during IMP (MF 450 nm followed UF 50 kDa) was studied at various pressures, up to 3 h, in a cross-flow filtration system under recirculation mode of

operation (Figs. 7.5-7.7). With 450 nm MF membrane processing crude culture broth, the permeate flux decline was steep during 0.5 to 1 h and then gradual up to 2.5 h and thereafter reached a plateau. The rapid initial decline in permeate flux followed by a period of relatively constant flux is common to many membrane filtration processes (Bailey and Meagher, 2000; Fane and Radovich, 1990). This could be due to the deposition or adsorption of suspended particles on to the pore walls of the membrane leading to fouling. Thus, the permeate flux decline is mainly due to the reduced pore volume. This natural phenomenon occurs during MF, primarily owing to the feed characteristics. Feed containing proteinaceous materials are more prone for fouling and cake layer formation (Choi *et al.*, 2005). The permeation of PG and protein was affected to the extent of 10 and 11%, respectively due to fouling (Fig. 7.5). With a view to improve the recovery of enzyme and productivity in the system, the applied pressure was doubled from 0.05 to 0.1 MPa. However, permeate flux and permeation of PG and protein showed only a marginal increase.

During UF of microfiltered culture broth, flux decline was not as severe as that of MF (Figs. 7.5 and 7.6). The flux declined only to the extent of 3% at 0.5 MPa after 3 h operation. Although flux decline is common even with UF membranes (Meindersma *et al.*, 1997; Akoum *et al.*, 2005; Li *et al.*, 2006), the decline was only marginal in the present study, as the feed was microfiltered and hence contained relatively lesser amount of suspended solids compared to crude culture broth. The water flux was nearly 25 fold higher than process flux at 0.5 MPa. The lower process flux obtained with UF could be mainly

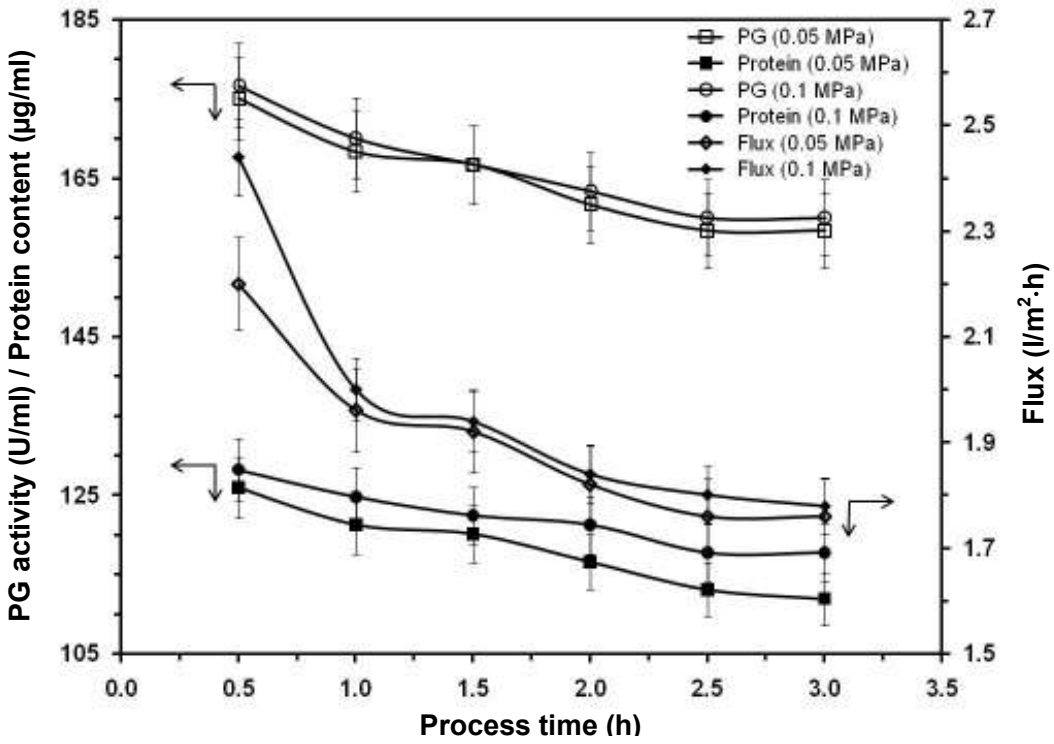


Fig. 7.5 Effect of pressure on PG and protein permeation and flux during MF (450 nm) of culture broth in a cross-flow filtration system (Recirculation mode)

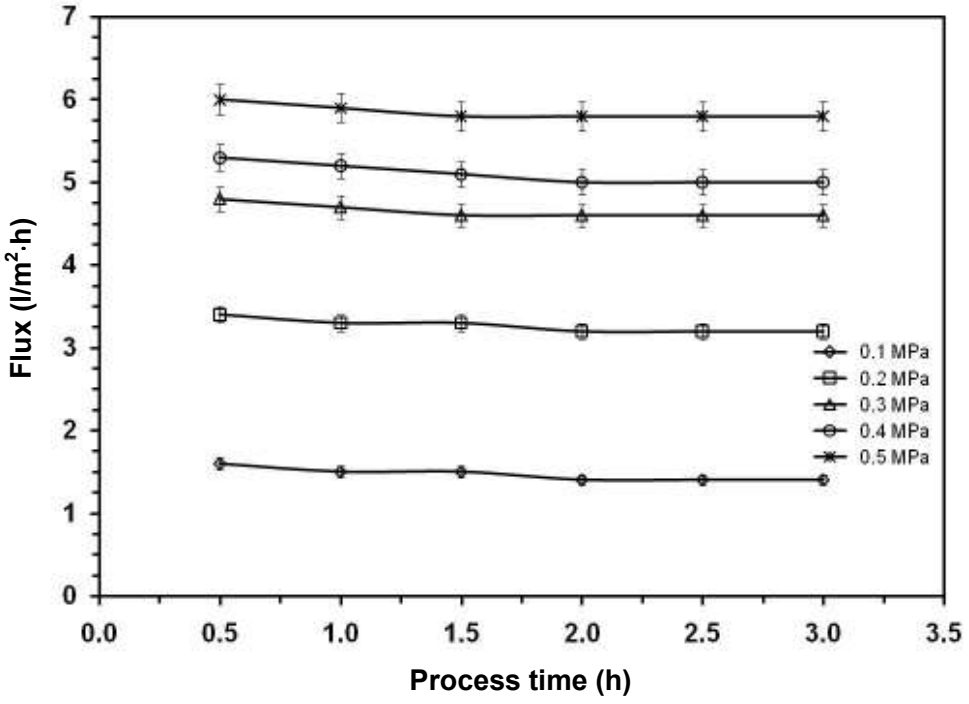


Fig. 7.6 Permeate flux during UF (50 kDa) of microfiltered culture broth in a cross-flow filtration system at various pressures (Recirculation mode)

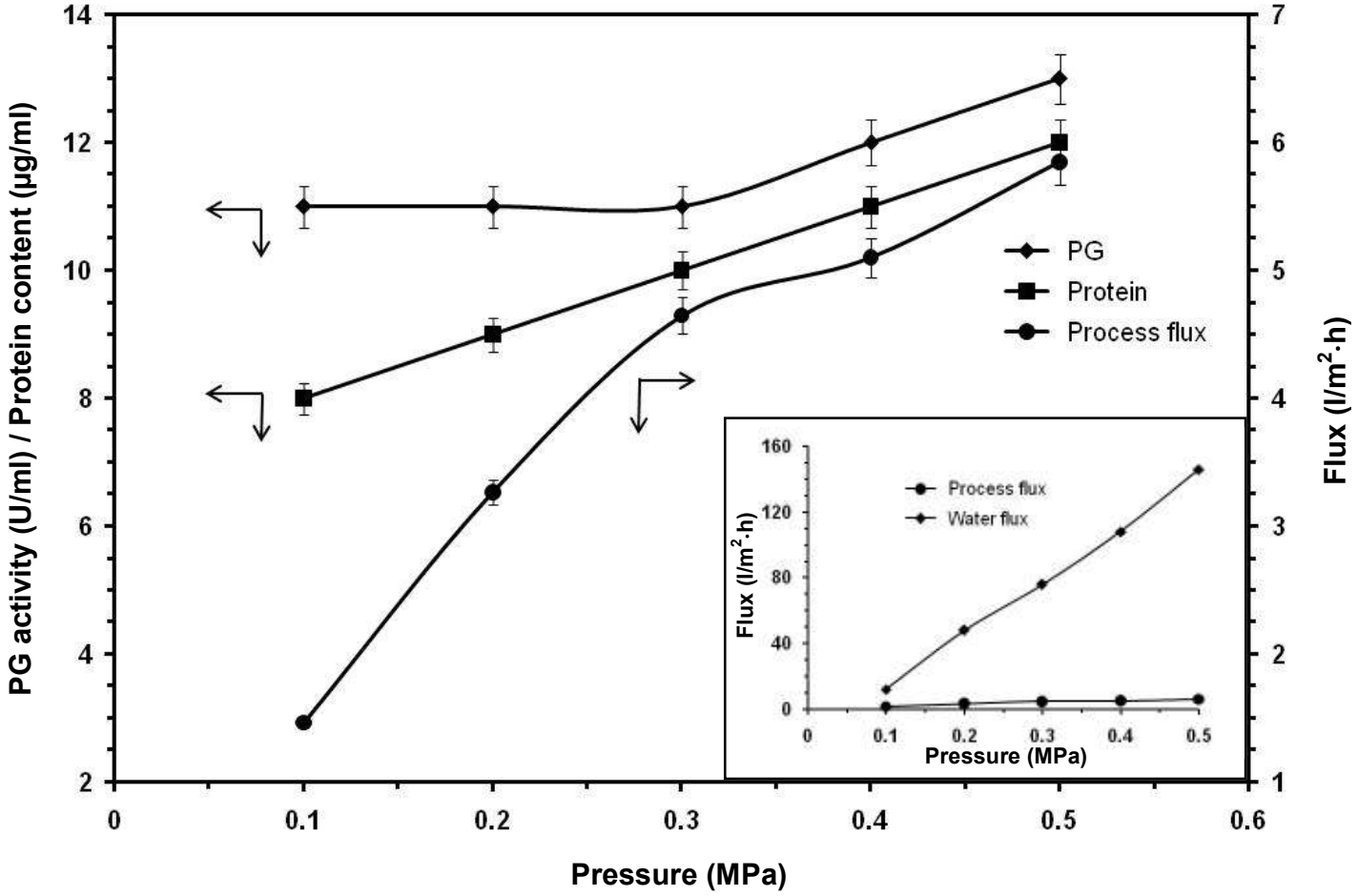


Fig. 7.7 Effect of pressure on PG and protein permeation and flux during UF (50 kDa) of microfiltered culture broth in a cross-flow filtration system (Recirculation mode after 3 h)

attributed to the cake layer resistance offered by the feed despite prior MF. The water flux increased from 12 to 146 l/m^2·h with increase in pressure from 0.1 to 0.5 MPa and the corresponding increase in process flux was from 1.5 to 5.9 l/m^2·h (Fig. 7.7). At the same time, higher operating pressure decreased the membrane rejection leading to small increase in the solute permeation; PG and protein in permeate increased from 11 to 13 U/ml and 8 to 12 µg/ml, respectively. Generally UF is operated at a higher pressure from the recommended range of 0.1-0.5 MPa (Mulder, 1998) for obtaining greater productivity. At higher pressures, the increased permeation loss of PG and protein could be considered insignificant compared to the benefits derived with increased permeate flux.

7.6 Conclusions

The study revealed that selection of stirring speed in a stirred membrane cell needs to be looked at not only from the viewpoint of concentration polarization but also from shear sensitive nature of the macromolecules present in the feed. Likewise, adequate attention is needed on the selection of pumps during processing enzymes. The results showed that the membrane, fouling and cake resistances were reduced in a cross-flow filtration system, thereby improving the productivity in the process. The study also revealed that the IMP protocol developed with a self stirred system for PG purification from *A. carbonarius* culture broth could be scaled up employing a cross-flow filtration system with improved performance.

8.0 Significance and focus of the work

PG is a major food enzyme extensively used for the extraction and clarification of fruit juices (Kashyap *et al.,* 2001). Commercial enzyme preparations generally employed contain other cell wall digesting enzymes in addition to PG to obtain greater juice yield and clarity. SSF co-produced other enzymes along with PG that have beneficial effects in terms of certain applications while SmF produced higher amount of PG with relatively lesser quantities of other enzymes. In the present work, the efficacy of *A. carbonarius* PGs (SmF-IMP-PG, SSF-IMP-PG, SmF-AAP-PG and SSF-AAP-PG) obtained in the laboratory was tested and compared with commercial PG preparations for the extraction and clarification of apple juice in terms of sugar yield and clarity.

8.1 Optimization of incubation conditions and enzyme concentration

The process conditions such as incubation temperature, period and enzyme addition were optimized using a commercial PG preparation, before testing the efficacy of *A. carbonarius* PGs. The performance of enzymatic treatment on apple mash increased with increase in levels of PG addition (0.5-2.5 U/g of mash), incubation temperature (30 and 50°C) and period (2 and 4 h) compared to control in terms of juice yield as well as TSS and clarity (Table 8.1).

Higher juice yield and clarity were observed when the apple mash was treated with PG at an elevated temperature of 50°C, compared to ambient temperature of 30°C at various levels of PG addition (0.5, 1.25 and 2.5 U/g of mash) for 2 and 4 h of incubation period (Table 8.1). Studies on extraction and

Table 8.1 Enzymatic extraction and clarification of apple juice using commercial PG-1[1]

PG addition (U/g of mash)	Temperature (°C)	Period (h)	Juice yield (%)	Clarity (%T at 660nm)	TSS (°Brix)	pH
Control	30	2	15.6 ± 1.2[a]	0.9 ± 0.2[a]	11.2	3.72
		4	20.8 ± 1.1[a]	1.5 ± 0.3[a]	11.4	3.63
	50	2	17.3 ± 1.3[a]	3.1 ± 1.0[a]	11.4	3.68
		4	22.4 ± 1.9[a]	4.3 ± 1.1[a]	11.8	3.64
0.5	30	2	43.9 ± 0.7[a]	29.2 ± 1.1[a]	11.8	3.64
		4	55.2 ± 0.5[b]	46.5 ± 0.9[b]	11.8	3.63
	50	2	57.8 ± 1.4[a]	57.0 ± 1.0[a]	11.8	3.64
		4	60.2 ± 2.0[b]	62.1 ± 1.4[a]	12.0	3.64
1.25	30	2	61.8 ± 0.8[a]	52.0 ± 1.3[a]	12.0	3.53
		4	66.7 ± 0.6[a]	62.7 ± 1.2[b]	12.0	3.49
	50	2	67.3 ± 0.5[a]	69.8 ± 0.6[a]	11.8	3.50
		4	68.2 ± 1.0[a]	74.2 ± 1.4[a]	12.2	3.48
2.5	30	2	69.0 ± 1.3[a]	70.2 ± 1.2[a]	12.2	3.40
		4	70.7 ± 1.5[a]	80.3 ± 0.9[b]	12.2	3.38
	50	2	78.5 ± 1.6[a]	83.5 ± 0.6[a]	12.2	3.36
		4	81.8 ± 1.3[a]	85.2 ± 1.4[a]	12.4	3.32

[1]PG – Polygalacturonase; TSS – Total soluble solids.

[ab]Means along a column at a particular PG addition and temperature with different duration having different superscripts are significantly different at $p<0.05$ (n=3).

clarification of apple juice using pectinolytic enzyme preparation showed that the optimum temperature could vary from 45°C to 50°C (Ishii and Yokotsuka, 1972; Kristeov and Dimitrova, 1993; Singh and Gupta, 2004). Will *et al.* (2002) reported that the highest values of juice yield, colloid concentrations and polyphenols were obtained at 50°C, which was the optimum temperature for the pectinase used in their study. In the present study, enhanced cell wall degradation at higher temperature probably led to the release of higher juice yield and greater clarity. Thus 50°C appears to be somewhat closer to the optimum temperature of the commercial pectinase used in the study. The results showed that there was practically no difference in the values of TSS and pH of the juice at two different temperatures employed (Table 8.1).

Enzymatic treatment with an incubation period of 4 h resulted in a significantly higher juice yield compared to 2 h period at a low level of PG addition (0.5 U/g of mash). However, the juice yields obtained after 2 and 4 h of incubation period were not significantly different at higher levels of PG addition (1.25 and 2.5 U/g of mash) (Table 8.1). Also, there was no significant difference in juice clarity between 2 and 4 h of incubation period at 50°C in the range of PG addition used (0.5–2.5 U/g of mash). TSS and pH of the juice did not vary with period of treatment (Table 8.1). Abdulla *et al.* (2007) reported that the incubation period was dependent on enzyme concentration. Besides, the period of treatment depends upon the nature of the enzyme, the reaction temperature and the variety of apple chosen (Kilara, 1982). In general, the time required to obtain a clear juice is inversely proportional to the concentration of enzyme used at

constant temperature over the range of 5-50°C and treatment time of 2-16 h (Rai *et al.*, 2004). Since there was no significant difference in performance between 2 and 4 h of incubation period at 50°C and higher enzyme dosages, shorter incubation period of 2 h was considered sufficient for enzymatic mash treatment in the subsequent experimental runs.

Increase in PG addition to the apple mash increased the juice yield and clarity (Table 8.1). The improvement in juice yield could be attributed to the degradation of pectins which releases cell contents. As the treatment process took place, the amount of pectin in the juices would decrease, thereby reducing the turbidity of the juice under treatment (Alvarez *et al.*, 1998). Enzymatic clarification of apple juice and carambola fruit juice using pectinolytic enzymes showed that increase in enzyme concentration increased the clarity of the juice (Singh and Gupta, 2004; Abdullah *et al.*, 2007). With increase in PG addition, there was a slight increase in TSS and decrease in pH of the juice (Table 8.1). The decrease in juice pH was due to the release of galacturonic acids during the enzymatic hydrolysis of pectin. The results revealed that the two important performance parameters, namely, juice yield and clarity increased with increase in PG addition from 0.5 to 2.5 U/g of mash. Hence, further attempts were made with much higher levels of PG addition beyond 2.5 U/g at a constant incubation period of 2 h for optimizing the level of enzyme addition for extraction and clarification of apple juice. An additional incubation temperature of 40°C was included in the experimental plan to examine whether there was a necessity to hold the mash at 50°C during the incubation period. Further, the yield

performance of enzyme treatment was evaluated more appropriately in terms of sugar yield which is a product of juice yield and TSS.

Incubation at 40°C showed only a marginal increase in sugar yield and juice clarity compared to 30°C, but the performance was much lower than the sugar yield and juice clarity obtained at 50°C at all levels of PG addition employed (Fig. 8.1). Kilara (1982) reported that increase in temperature increased the rate of enzymatic reactions which increased the rate of clarification as long as the temperature was below the denaturation temperature for the enzyme (40-60°C). In the present study, the incubation temperature of 50°C gave the best performance in the range of temperature studied (30-50°C). This range was not exceeded beyond 50°C considering the fact that higher thermal exposure could cause greater thermal damage to a delicate product like apple juice and probably would also lead to the denaturation of PG. Hence, incubation temperature of 50°C was considered as optimum for pectinase treatment of apple mash.

During enzymatic treatment, sugar yield as well as juice clarity increased sharply when the PG addition was increased from 0.5 to 2.5 U/g of mash and showed a nominal increase at 5 U/g of mash reaching a plateau, thereafter practically no difference in sugar yield and clarity was obtained (Fig. 8.1). Hence, a PG addition of 5 U/g of mash was construed as optimum. The results revealed that the maximum sugar yield of 11.9% and juice clarity ($\%T_{660}$) of 86.3% were achieved by treating apple mash with a PG addition of 5 U/g of mash and incubating for 2 h at 50°C.

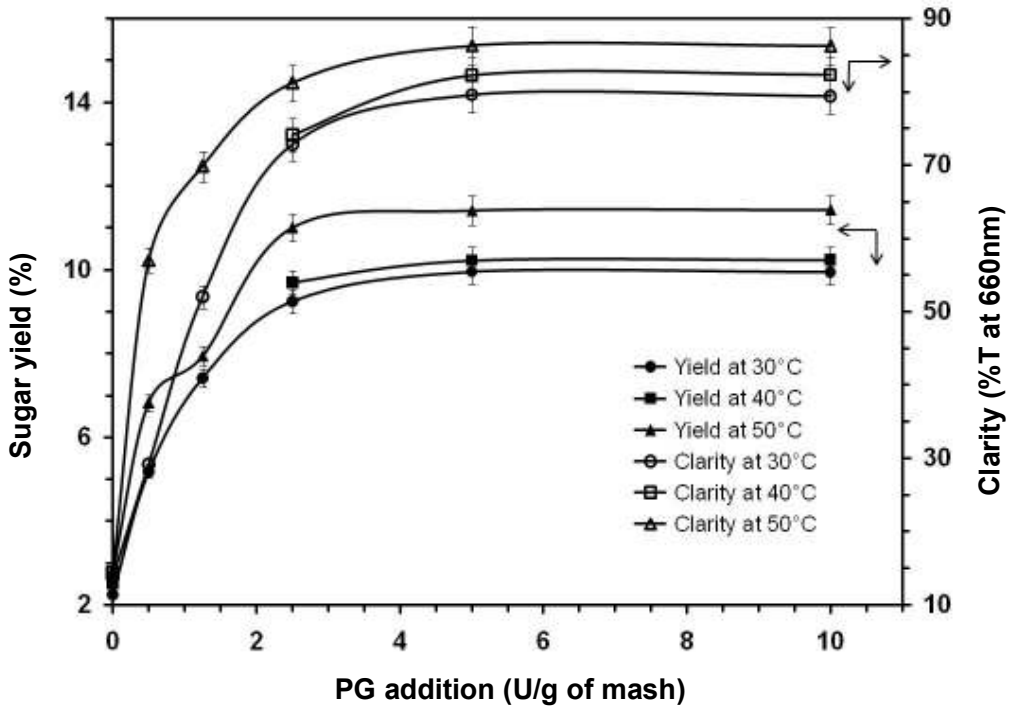


Fig. 8.1 Improvement in sugar yield and clarity of apple juice using commercial PG-1 (Incubated for 2 h)

8.2 Performance evaluation of crude and purified SmF and SSF *A. carbonarius* PG

The efficacy of crude and purified SmF- and SSF-PG of *A. carbonarius* was tested for extraction and clarification of apple juice along with two commercial PG preparations under optimum and select process conditions for comparison (2.5-5 U/g of mash at 30-50°C for 2 h). The enzymatic treatment of apple mash with crude and purified PGs obtained from the laboratory and commercial PGs resulted in much greater juice and sugar recovery as well as clarity of juice compared to the control (Table 8.2). Higher PG addition as well as higher incubation temperature increased the sugar yield with all the PGs (Fig. 8.2). Among the crude and laboratory purified PGs, the sugar yields were

Table 8.2 Enzymatic extraction and clarification of apple juice using various PG samples[1]

Type of PG	Juice yield (%)	Turbidity (NTU)	Colour			TSS (°Brix)	pH	Density ($x10^{-3}$ kg/m^3)	Viscosity (m Pa s)	AIS (wt %)	Sugar yield (%)
			L	a	B						
Control	20.5 ± 0.5^{a}	1026	35.46	8.50	32.52	14.2	4.05	1.10	3.33	1.12	2.9 ± 0.1^{a}
SmF-Crude	74.1 ± 1.2^{bfghi}	173	43.69	3.55	34.30	14.6	3.82	1.09	1.27	0.58	10.8 ± 0.2^{bfghi}
SSF-Crude	76.0 ± 1.0^{bcfghi}	99	43.53	2.87	32.41	14.6	3.81	1.09	1.20	0.56	11.1 ± 0.1^{bcfghi}
SmF-IMP	73.9 ± 1.2^{bdfghi}	102	48.45	0.44	28.11	14.6	3.82	1.09	1.27	0.54	10.8 ± 0.2^{bdfghi}
SSF-IMP	76.7 ± 1.2^{befghi}	96	48.32	1.44	31.93	14.6	3.82	1.09	1.20	0.56	11.2 ± 0.2^{befghi}
SmF-AAP	72.8 ± 0.9^{fg}	238	38.79	6.88	38.93	14.4	3.85	1.09	1.27	0.60	10.5 ± 0.1^{fg}
SSF-AAP	73.6 ± 1.3^{g}	219	42.26	4.42	36.79	14.6	3.84	1.09	1.27	0.62	10.7 ± 0.2^{g}
Commercial-1	81.6 ± 1.1^{hi}	76	45.03	2.72	34.12	14.8	3.61	1.10	1.13	0.46	12.1 ± 0.2^{hi}
Commercial-2	83.1 ± 0.8^{i}	56	49.56	-0.22	27.12	14.8	3.55	1.10	1.13	0.50	12.3 ± 0.1^{i}

[1]NTU – Nephelometric turbidity units; AIS – Alcohol insoluble solids; SmF – Submerged fermentation; SSF – Solid state fermentation; IMP – Integrated membrane process; AAP – Alginate affinity purification.

For other abbreviations see Table 8.1.

PG addition – 5 U/g of mash; Incubation at 50°C for 2 h.

[a-i]Means along a column with different superscripts are significantly different at $p<0.05$ (n=3).

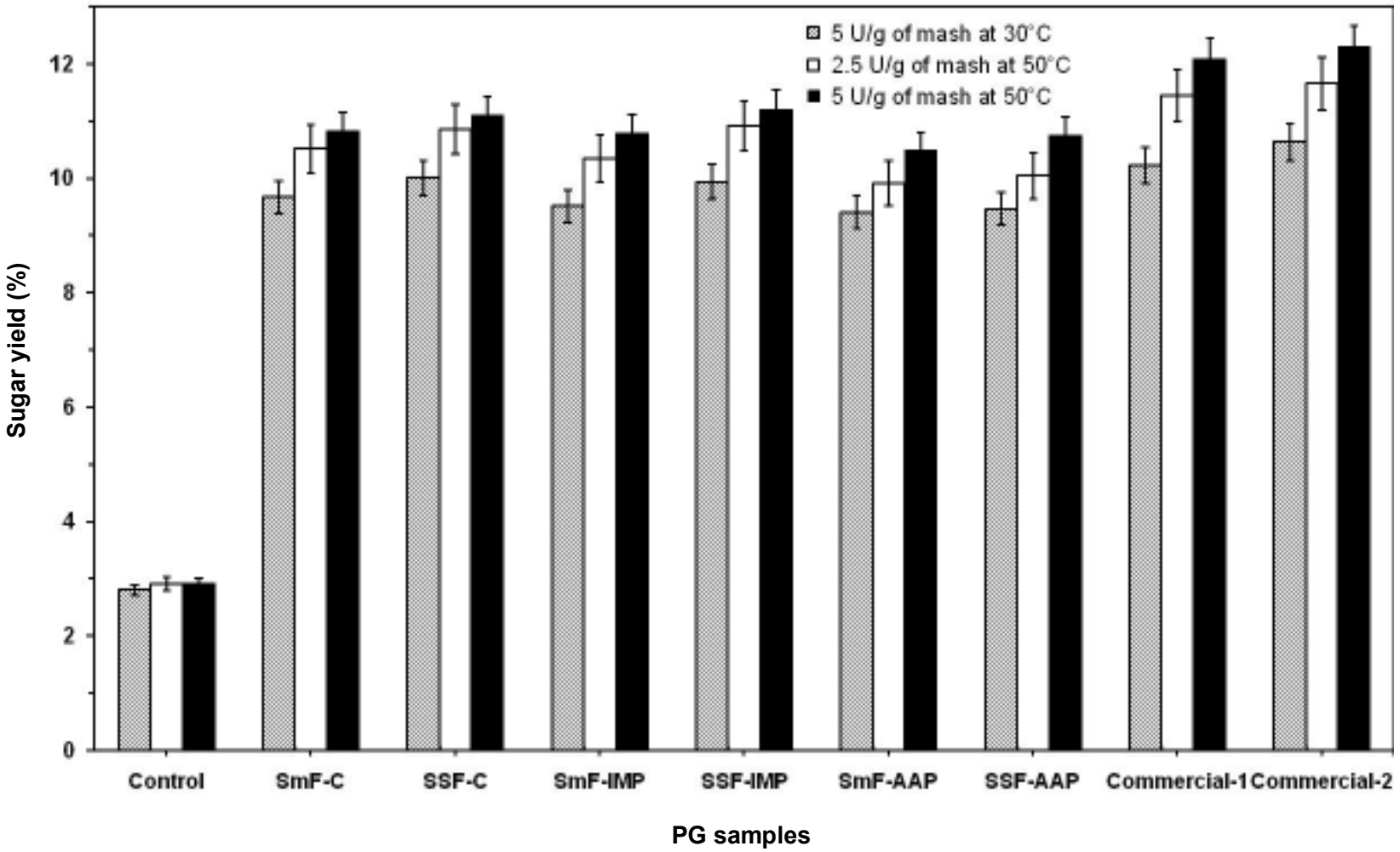


Fig. 8.2 Comparison of *A. carbonarius* PG with commercial PGs on sugar yield (Incubated for 2 h)

not significantly different (Table 8.2). The sugar yields obtained with commercial PGs were greater and significantly different from the yields obtained with AAP processed PGs, however, not significantly different from crude and IMP processed PGs. The trend of juice yield obtained was similar to that of sugar yield since the differences among TSS values obtained with various PGs were only marginal. Juice yield requires hydrolysis of cell wall polymers like pectin, cellulose and hemicellulose. In addition to PG, pectin transeliminase and pectin esterase also aid in pectin hydrolysis. Since the commercial PG preparation of Sigma contained these enzymes (www.sigmaaldrich.com), they contributed to increase in juice yield.

The juice clarity was measured in terms of turbidity; lower the turbidity value higher is the clarity and vice versa. Enzymatic treatment with all the PGs resulted in greater juice clarity compared to the control (Table 8.2). Higher PG addition as well as higher incubation temperature increased the juice clarity with all the PGs (Fig. 8.3). The clarity of juice obtained with SSF crude, SSF-IMP-PG and SmF-IMP-PG (96-102 NTU) was closer to the values obtained with the commercial PGs (56-76 NTU) compared to other PGs. As in the case of sugar yield, the juice clarity values obtained with AAP processed PGs (219-238 NTU) were lower compared to other PGs tested (Fig. 8.3). AAP exhibited a phenomenal selectivity for purifying PG under the optimized conditions and thus almost completely eliminated the contaminant proteins present in the SmF- and SSF-cultures. IMP employing MF and UF membranes eliminated larger and smaller impurities present in the cultures to a greater extent owing to size

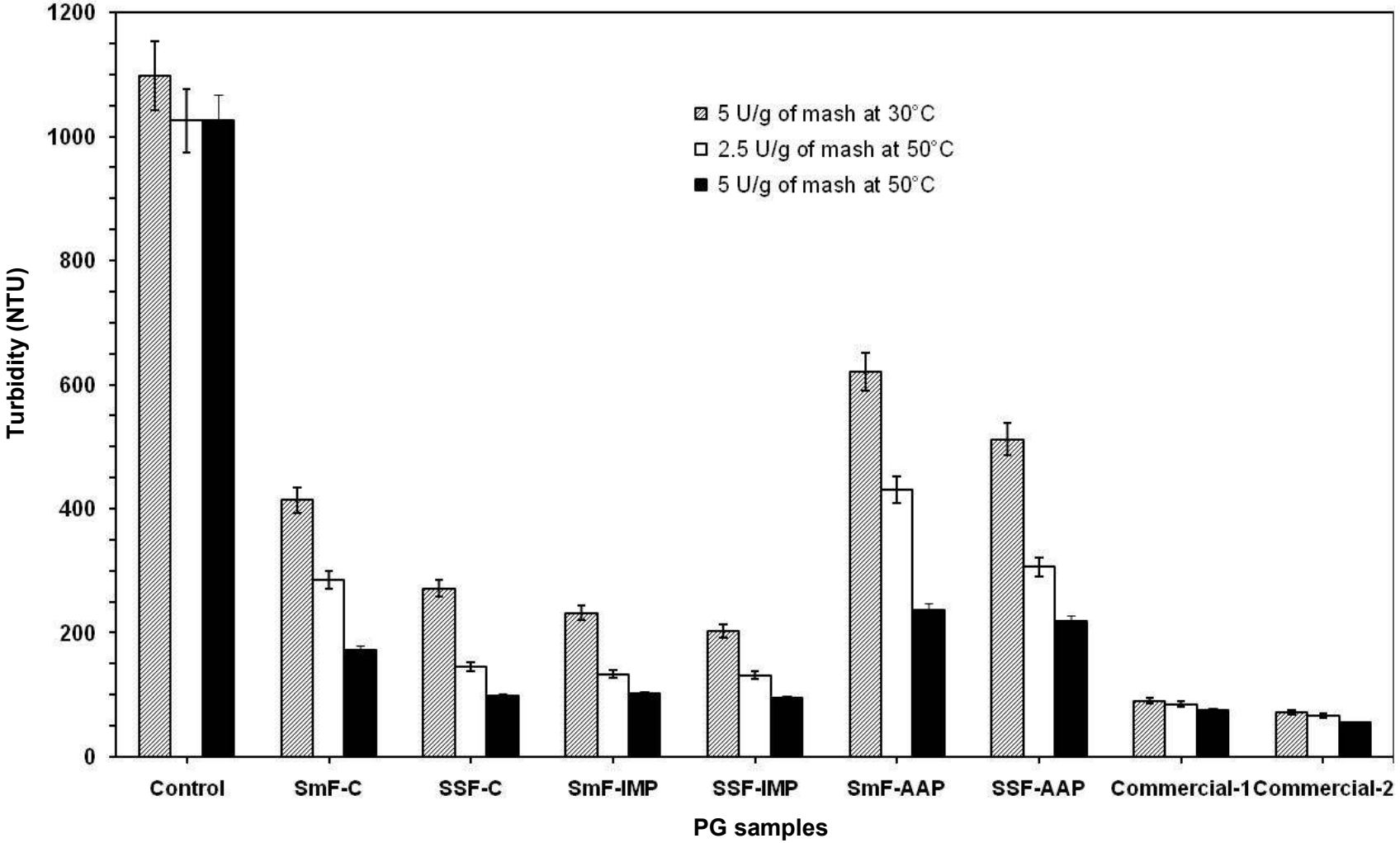


Fig. 8.3 Comparison of *A. carbonarius* PG with commercial PGs on juice clarity (Incubated for 2 h)

exclusion, but did not eliminate various other contaminant proteins of similar molecular mass (protease and amylase) as that of PG present in the cultures. Accordingly, purity of AAP-processed PGs was much higher compared to IMP-processed PGs due to high selective purification ability of AAP. The secretion of other contaminant proteins was much lower in SmF cultures while it was higher in SSF cultures, which helped to achieve greater purity during IMP processing SmF cultures. For the same reasons, IMP was not very effective for processing crude SSF, but helped SSF-IMP-PG to perform slightly better in applications such as extraction and clarification as the contaminant proteins acted in a beneficial manner. The PG, protease and amylase activities of crude and AAP- and IMP-processed PGs are presented in Table 8.3. The results revealed that the differences in hydrolysing performances observed with crude and laboratory purified PGs have a bearing on the presence of other cell wall digesting enzymes along with PG.

The enzymatic treatment reduced the viscosity, AIS and pH of the juice as compared to the control (Table 8.2). The pectin content in the juice measured in terms of AIS is largely responsible for the viscosity of the juice. Upon enzyme treatment, degradation of pectin leads to a reduction of water holding capacity and as a consequence free water is released along with other juice components to the system, resulting in reducing the viscosity of the juice as well. In the present study, a reduction in viscosity of juice to the extent of ~66% was observed with all the PGs after enzyme treatment of mash. Singh and Gupta (2004) reported a viscosity-drop of ~36% during the enzymatic clarification of commercial apple juice. Reduction in juice viscosity as high as 82-91%, has been reported during enzymatic treatment of apple

Table 8.3 Enzyme activities of various PG samples[1]

Type of PG	PG (U/g of mash)	Protease ($x10^{-3}$ U/g of mash)	Amylase ($x10^{-2}$ U/g of mash)	PG specific activity (U/mg)
SmF-Crude	5	0.4	0.7	1190
SSF-Crude	5	2.6	4.1	530
SmF-IMP	5	0.8	0.9	5590
SSF-IMP	5	5.8	4.6	640
SmF-AAP	5	ND	ND	9770
SSF-AAP	5	ND	ND	2450
Commercial-1	5	3.5	37	330
Commercial-2	5	4.1	44	120

[1]ND – Not detected; For other abbreviations see Tables 8.1 and 8.2.

mash with different commercial pectolytic enzymes (Oszmianski *et al.*, 2009). Busto *et al.* (2006) reported the viscosity reduction of ~35% during enzymatic treatment of apple pectin. The differences in performances could be ascribed to many factors, such as type of enzyme preparation, apple variety, treatment and pressing conditions. There was no considerable difference in pH, colour and density of juice obtained from treated apple mashes with various PGs (Table 8.2).

Although, the overall performances of SSF crude PG, SmF-IMP-PG and SSF-IMP-PG were comparable, SmF-IMP-PG may be preferred owing to the presence of various contaminants in the SSF crude and relatively higher downstream processing costs involved with processing lower strength of SSF culture extracts. Further, IMP of SmF cultures improved the PG specific activity to a greater extent of 5590 U/mg protein (4.7 fold), while SSF cultures

improved the specific activity only to an extent of 640 U/mg protein (1.2 fold), which revealed that other contaminant proteins also recovered along with PG during IMP processing of SSF cultures (Table 8.3). The performance of commercial PG was much better than all other crude and laboratory purified PGs. However, formulations of commercial PGs are reported to contain pectin esterase, pectin lyase and hemicellulase besides PG. Catalysis of the substrate with pectin esterase is associated with methanol release and the presence of methanol in food products is undesirable (Semenova *et al.*, 2006). Hence, it is necessary to control the amount of pectin esterase and other undesirable contaminant enzymes in industrial enzyme preparations for food applications. It may be desirable to use a relatively pure PG such as SmF-IMP-PG, formulated with other suitable and defined cell wall digesting enzymes for improved performance in extraction and clarification of fruit juices since most countries do not restrict the use of these enzymes (Ribeiro *et al.*, 2010).

8.3 Conclusions

The study revealed that the performance of SmF-IMP-PG and SSF-IMP-PG of *A. carbonarius* was reasonably good in terms of sugar yield and clarity. The better performance exhibited by commercial PGs could be attributed to the presence of other cell wall digesting enzymes in their preparations which may include esterase that need to be preferably avoided owing to methanol production. The results showed that SmF-IMP-PG is suitable for formulating as a commercial enzyme with other desirable cell wall digesting enzymes for extraction and clarification of fruit juices.

Enzymes are industrially produced either by SmF or SSF. Since microorganisms produce a mixture of metabolites along with the target protein (enzyme) during fermentation, purification and concentration are important for downstream processing which include centrifugation, precipitation, membrane filtration and chromatographic separation. Pectinases are one of the major food enzymes obtained from microbial sources. In order to meet the increasing demand for pectinase, membrane purification of crude enzymes appears both cost effective and efficient over other processes. Although membrane based approaches have contributed towards improving methodologies for enzyme purification, the potential of membrane technology has not been completely exploited to achieve greater purity as well as recovery. Therefore investigations were carried out on the application of membrane technology for the purification of *A. carbonarius* PG produced by SmF and SSF.

PG production and elimination of carbohydrate impurities

Studies on PG production using mutant *A. carbonarius* showed the ability of the fungus to produce the enzyme both in SmF and SSF using corn flour and wheat bran, respectively as main substrates. Enzyme titre of SmF showed 2.6 times higher PG (U/ml) yield than SSF. In absolute measure, one gram of starch yielded 13,100 U by SmF as against 4940 U obtained in SSF process. Higher yield together with shorter fermentation period resulted in 6.6 times greater productivity favoring SmF for PG production over SSF. The higher PG productivity obtained by SmF could be attributed to the use of mutant *A. carbonarius* that was more effective in glucose regulation.

Elimination of carbohydrates from culture broth is essential since their presence interferes with purification processes and lead to enzyme loss. A set of MF (70-450 nm) and UF (10-500 kDa) membranes were examined to eliminate carbohydrates and other non-protein impurities from *A. carbonarius* SmF culture broth containing PG. Of the various membranes tested, 10 kDa membrane exhibited near complete rejection of PG and protein leading to higher PG recovery. Further, diafiltration with 10 kDa membrane resulted in near complete elimination of carbohydrates, salts and acids with 96% PG recovery. However, there was no improvement in PG specific activity due to rejected proteins. Hence, subsequent studies were focused on developing an IMP using MF and UF membranes to improve the specific activity of PG.

Integrated membrane process for improving PG specific activity

A protocol for an integrated membrane process developed employing 450 nm MF and 50 kDa UF membranes revealed its suitability for the purification of PG from SmF. During MF, the specific activity increased to 1.5 fold purity due to elimination of larger contaminating proteins and resulted in a recovery of 85% PG. In the subsequent step, 50 kDa UF membrane with diafiltration enhanced the specific activity of PG to 5590 U/mg protein (4.7 fold) by eliminating smaller contaminating proteins with an overall recovery of 76% PG. IMP not only improved the specific activity but was also effective in near complete elimination of carbohydrates and other impurities.

In contrast, IMP resulted only in 1.2 fold purification of PG from SSF due to the presence of contaminating proteins similar to the molecular mass of PG such as protease and amylase leading to low membrane selectivity.

PG recovery of 72% and carbohydrates elimination obtained were comparable to processing SmF culture broth. The presence of contaminating proteins in the SSF culture extract similar to the molecular mass of PG was confirmed by SDS-PAGE. Therefore, an alternate method of purification was attempted for processing crude microbial broths.

Alginate affinity purification of PG

This separation process 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. Under optimum conditions of elution, using 1 M NaCl, the specific activity of SSF-PG enhanced to 2450 U/mg protein (4 fold) with 61% recovery and resulted in almost complete elimination of carbohydrates. The results evidenced alginate binding to SSF-PG, significantly improving its purity in the process. SDS-PAGE confirmed the presence of only the PG protein. In the case of SmF-PG, the specific activity enhanced to 9770 U/mg protein (8.7 fold), much higher than that was achieved with IMP (5590 U/mg protein), with 74% recovery. These results suggested that AAP was a suitable method for purification of PG.

Techno-economic analysis for PG production and purification

An economic analysis of SmF and SSF processes for PG from *A. carbonarius* along with IMP and AAP for downstream processing was performed to make an appropriate process selection. The study on upstream processes emphasized that it is more prudent to evaluate the enzyme yield as well as

productivity in terms of carbon source. Cheap raw materials used as the main substrate in the SSF process are often considered advantageous for the SSF process. However, the analysis suggested that substrate cost need not be an influencing factor in the process selection as it formed only 2-7% of total product cost of upstream process. Further downstream process costs are far more expensive than upstream process costs and application of simpler methodology like IMP could make a difference in the production process. For a production scale of 30 kl purified PG concentrate per year, the SmF-IMP process required a total capital investment that was 15-24% lower than the SmF-AAP and SSF-AAP processes. The corresponding unitary product cost was also lower by 24-36% in SmF-IMP process. Thus the SmF-IMP process proved to be very attractive from a techno-economic point of view.

Scale-up of integrated membrane process

Scale-up studies on IMP were carried out in cross-flow membrane systems as they offered several advantages over the dead end filtration system used for developing IMP. Stirring and pumping employed in membrane processing systems impart shear leading to loss of enzyme activity. Assessing the shear sensitive nature of PG revealed that there is a critical speed of stirring (400 rpm) corresponding to a shear stress of 2.1 Pa which should not be exceeded in self-stirred membrane cells. Likewise, peristaltic pump showed its suitability for processing culture broth over shear intensive gear pump, recording a negligible loss of PG.

IMP (450 nm followed by 50 kDa) scheme attempted in a cross-flow laboratory system (VCR 5 fold) for processing SmF-PG resulted in slightly

lower PG purity (4.1 fold) and overall recovery (64%) compared to stirred membrane cell (4.7 fold; 76% recovery; VCR 10 fold). The results suggested that matching performance could be achieved in the cross-flow membrane system by maintaining the same VCR as that of the self-stirred system with a larger membrane area. The permeate flux after normalizing with respect to feed - membrane area in the cross-flow membrane system, was 1.6 and 15 fold higher during MF and UF, respectively. In the pilot scale system, the specific activity of PG obtained with 200 nm MF followed by 20 kDa UF membranes was comparable with self-stirred system which provided evidence that the performance could be replicated in the scale up.

The flux decline in the proposed IMP was analyzed using a resistance-in-series model which revealed that the fouling resistance was predominant with MF while it was the cake resistance in UF with both self-stirred and cross flow membrane systems. The results also showed that the membrane, fouling, cake and total resistances were reduced in a cross-flow filtration system, thereby improving the productivity in the process.

Application of purified PG for fruit juice clarification

PGs are extensively used in fruit and vegetable processing industries to improve juice yield and clarity. The efficacy of SmF- and SSF-PG purified using IMP and AAP was tested for the extraction and clarification of apple juice. Juice clarity obtained with SSF crude, SSF-IMP-PG and SmF-IMP-PG (96-102 NTU) was lower than commercial PGs (56-76 NTU) but much greater compared to other laboratory PGs (173-238 NTU). The juice yield measured in terms of sugar yield was ~12% lower than formulated commercial PGs.

The study suggested the suitability of SmF-IMP-PG for formulating with other desirable cell wall digesting enzymes for the application in extraction and clarification of fruit juices.

Studies on the purification of SmF- and SSF-PG showed downstream process needs are specific for individual cases and warrants careful considerations backed up with a techno-economic analysis. Membrane technology was found suitable for its potential in concentrating and purifying PG from *A. carbonarius* culture broth by eliminating carbohydrates, salts and contaminating proteins with greater overall enzyme recovery and lesser processing steps. SmF followed by IMP (450 nm followed by 50 kDa) revealed their suitability for the production and purification of PG from *A. carbonarius* in terms of process performance, economics and product application.

The knowledge obtained from this study would enhance the efforts towards application/integration of membrane technology with other downstream processing methods for the purification and concentration of various enzymes in order to obtain higher purity and overall recovery with lesser processing cost.

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