In-situ extracellular protein isolation of *yarrowia lipolytica* through membrane technology: A review

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Abstract

The yeast *Yarrowia lipolytica* is one of the most intensively studied non-conventional yeast species. They exhibit a remarkable performance in the efficient secretion of various heterologous proteins. The potential of having an *in-situ* isolation of the proteins would be very much desired since fermentation and product recovery can be carried out simultaneously. Incorporation of membrane technology for such purposes would be beneficial as an initial bulk volume reduction and partial purification. Consideration must be given to membrane as well as the protein as separation may be affected by the MWCO and various interactions possible between solvent, solute and the membrane which would lead to deterioration in efficiency and fouling. Studies related to *Y. lipolytica* cultivation and *in-situ* isolation which have been carried out are compiled and configuration of *in-situ* protein recovery for the yeast is proposed. The prospect for continuous recovery extracellular protein is highlighted.

Keywords: Y. lipolytica, membrane, extracellular proteins, in-situ isolation

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1.0 Introduction

Yarrowia lipolytica (originally classified as Candida lipolytica) is one of the most intensively studied 'non-conventional' yeast species. The interest on this special arose from the fact that this species were able to grow on hydrophobic substrate such as alkanes, fatty acids and triglycerides. This dimorphic yeast naturally secretes several metabolites (i.e. extracellular proteins) such as proteases, lipases, esterases and RNase[1,2]. Among them is alkaline extracellular protease (AEP) which could reach several grams per liter under optimized condition.

The potential of having *in-situ* isolation of the secreted metabolites will be very much desired since the fermentation and separation can be carried out simultaneously. The removal of these extracellular proteins during fermentation with the incorporation of membrane technology would be beneficial as a first step in downstream purification as initial bulk-volume reduction and partial purification. Owing to their rapid separation and high capacity, it is seen as an alternative that is cost effective and can be fine-tuned to achieve high productivity and product purity at the same time[3-6]. Compared to other purification techniques, it provides a high throughput with low cost and it is much easier to scale up[7].

Other than using membrane, in-situ product removal have been accomplished by a number of methods including affinity technique[8], partitioning in an aqueous two-phase system (ATPS)[9], two-liquid-phase system[10] and other purification method provided that they are rapid, specific and high yield.

Besides providing instantaneous separation of secreted metabolites, the incorporation does also bring several advantages. It can enhance the yield of secreted protein with limited stability and help in product inhibition effect[8]. In many cases, the secreted proteins have exhibited limited stability in culture medium[11,12]. The amount of protein decline rapidly from the maximum concentration during batch culture unless appropriate stabilizer is added. For example, monoclonal antibody fragment produce by transgenic *Nicotiana tobacum* cells accumulate at concentration of 20 ng/ml in the absence of stabilizing agent but reach concentration up to 350 ng/ml in the presence of stabilizer such as gelatin and PVP[11].

This review paper is written to evaluate the performance of *in-situ* isolation of extracellular protein from *Y. lipolytica* by comparing it with other related studies that have been carried out.

2.0 The non-conventional yeast, *Y. lipolytica*

This hemiascomycetous yeast can be readily isolated from diary products, shoyu or salads containing meat or shrimps. The yeast is considered as non-pathogenic and their inability to survive under anaerobic conditions permits an easy elimination. Y. *lipolytica* is preferred for heterologous protein expression compared to the widely used *S. cerevisiae* due to higher secretion efficiency especially for high molecular weight protein and in terms of performance reproducibility. The FDA (food and drug administration) has classified several processes based on the yeast as GRAS (generally regarded as safe).

Y. lipolytica has the unique ability to use glucose (but not sucrose), alcohols, acetate and hydrophobic substance (such as alkanes, fatty acid and oils) as carbon sources[2]. It was distinguish for its capacity to secrete naturally several proteins into the culture medium; namely two protease i) alkaline extracellular protease (AEP) ii) acid extracellular protease (AXP), several lipases (present of oil in media) and phosphatases (when media is depleted of inorganic phosphate sources), Rnase and esterase[2]. Under convenient inducing condition, *Y. lipolytica* secretes very large amount of AEP (encoded by the *XPR2* gene).

3.0 Effect of media composition

Although secreted proteins rates and yield are highly dependent on the type of microorganism, the type of substrate and culture condition (i.e. nutrient concentration, mode of operation and so on), some aspects of the processes are common. It has been generally found that *Y. lipolytica* grows in rich YPD medium at pH 6.8 secretes large amount (1-2 g/l) of AEP[13,14] while at pH 4.0, AXP is detected. Interestingly, induction of the AXP and occurs under condition very similar to that of AEP except for the pH of the medium while the extracellular RNase activity is detected in *Y. lipolytica* cultures grown under same condition leading to AEP secretion.

As for extracellular phophatases, it is induced in *Y. lipolytica* when it is cultured in media depleted of inorganic phosphate. It is usually detected in the medium at the end of the exponential growth phase of the yeast[15]. Extracellular lipase can be enhanced significantly by the present of olive or corn oil when used as both carbon and inducer sources in the medium[16]. Urea was found as the best nitrogen source for lipase production. The extracellular thermostable esterase of low molecular weight has also been detected. The summary of the protein secrected is tabulated in table 1.

Since the main objective of this paper is to evaluate the membrane performance for *in-situ* separation, a rich YPD media is sufficient as the medium for the culture to promote the secretion of protein from *Y. lipolytica*.

Table 1
Summary of secreted protein

Secreted protein	MW (kDa)	Remark	Reference
Alkaline extracellular protease (AEP)	32	Protease from the subtilisin family, intracellularly processed from 55 kDA glycosylate precursor	[2]
Acid extracellular protease (AXP)	28-36	Three protein of the same species have been found	[2]
RNase	45	Partially degraded by AEP into 43 kDa and 34 kDa protein	[2]
Phosphateases	90-200	A glycosylated protein	[2]
Lipase	-	Enhanced and induced by the present of oil	[2]

4.0 Membrane for protein separation

4.1. Fractionation of protein

Fractionation of protein to give a moderate to a highly pure component from the protein mixtures is quite a simple task[17-20] when compared to high resolution of pure protein fractions in both permeate and retentate. There are many factors governing membrane retentivity including the shape of solute, fluid mechanics bulk mass transfer limitation and the various interactions possible between solvent, solute and the membrane.

4.2. Molecular weight cut off

A common assumption is that the molecular weight cut off (MWCO) should match closely to the molecular weight of the solute of interest. This is not always true since the MWCO rating is often relative. The MWCO is typically defined as 90% retention level (usually dextran) of the solute in the feed. When the system involves protein as solute, the size of protein molecule, though having the same molecular weight as dextran, may differ considerably due to the natural 3-D structure and folding of the protein structure. Therefore, a better performance is sometimes achieved with membrane having MWCO significantly higher or lower than the molecular weight of the solute which is to be retained[21].

4.3. Solute-solute interaction

It is well known that the filtration can be drastically influence by the nature of solute-solute interaction[20,22,23]. Solute-solute interaction will determine the filtration rate and the rejection of solute when one or more proteins are ultrafiltrated. Such interaction can be achieved by manipulating the IEP of the protein and using a charged membrane. A charged protein will have an increase in hydrodynamic diameter compared an uncharged one and needs a membrane with a bigger pore to pass thorough. The protein will not be easily transmitted through a pore close to the size of the protein when the membrane is having a similar charge. It will only be transmitted at its IEP where it has no charge.

In binary solution, if the protein is charged like the membrane, fouling of the membrane will not occur. This type of fractionation was done by Nakao et al.[24] with myoglobin (MYO) and bovine serum albumin (BSA) on a modified polymeric membrane. Saksena and Zydney[25] used the same membrane to separate BSA and immunoglobin G (IgG) at their IEPs, increasing the separation by factor of 20 by operating at low salt concentration and near the IEP of BSA. Chaufer et al.[26] and Millesime et al.[27] fractionated whey protein using inorganic modified membrane. Effect that is even more dramatic was seen by van Reis et al.[28] for the separation of BSA from antigen binding fragment (Fab) derived from a recombinant DNA antibody. In this case, nearly 1000 fold purification of BSA with 94% yield by operating near the IEP of the Fab (pH 4.8) using a negatively charged membrane in diafiltration mode. The best fractionation was achieved when one of the proteins was at its IEP while the other was refrained due to charge repulsion with the membrane.

5.0 Membrane fouling

5.1. Concentration polarization

A problem when using membrane is as always concentration polarization if high concentration of protein is used. Generally, the solute whose molecular dimension is small enough to pass through the membrane pore is substantially retained by the same membrane with the present of larger molecules. Concentration polarizations can not only leads to irreversible fouling by altering interactions between solvent, solutes and membrane but also the flux and selectivity during the UF[29]. An approach to reduce the effect is by applying diafiltration[30,31].

5.2. Protein adsorption

Protein adsorption is known to play a critical role in many membrane systems. The extend of adsorption have been shown to depend on the solution pH and ionic strength[32]. Adsorption refers to the deposition of foulants on the membrane surface resulting from electrochemical attractions. These attractions may arise from non-covalent intermolecular forces such as Van der Waals forces and hydrogen bonding. Adsorption is always associated with internal pore fouling since most of the surface area of the membrane occurs internally. Photomicrographs of cross section of any UF membrane will show a high internal surface area of membrane. The sponge like structures suggests convoluted and tortuous pore pathways. Adsorption along the pore pathway can lead to

more extensive fouling. A protein might denature upon adsorbing on the surface. The denatured protein attracts other protein and when this process repeats, deposits build up on the membrane.

5.3. Agglomeration

Generally, agglomeration describes colloidal precipitates resulting from solute-solute attractions. When aggregated solute deposits on the membrane surface, it reduces permeability. On the other hand, controlled aggregated of solutes can facilitate UF. For instance, better retention of B-lactoglobulin was achieved by adjusting the pH of the feed solution so that the protein aggregated into clusters of 2 or 8 protein molecules[33]. Maximum protein interaction and maximum flux occurred near the IEP at pH 4.5.

Fouling is not always detrimental. The term dynamic membrane exists and describes that deposits can benefit the separation process by reducing the membrane effective MWCO so that the solute of interest is better retained.

6.0 Experimental Design

A lot of studies have been carried out on the harvesting of the secreted proteins from Y lipolytica. Cultures on solid medium are mostly for basic research purposes especially in protein recognization and recombinant protein expression[34-36]. Larger quantity of metabolites production is usually done in a shake flask (Erlenmeyer or conical flask) and in bioreactor. These are shown in the following table 2.

Table 2
Metabolites production from Y. lipolytica

Targeted metabolites	Working Volume/Volume	Mode of operation	Reference
Lipase	50 ml / 250ml	Shake flask	37
Phosphatase	-	Shake flask	38
γ-decalactone	200 ml / 500 ml	Shake flask (baffled)	39
Phosphatase (excocellular)	-	Shake flask	40
Acyl-CoA oxidase	200 ml / 500 ml	Shake flask	41
Phosphatase 2A	- / 500 ml	Shake flask	42
Protein kinase CK2	- / 500 ml	Shake flask	42
Lipid	0.38 L / 0.6 L	Bioreactor	43
NADH:ubiquinone oxidoreductase (complex I)	10 L / 10 L	Bioreactor	44
Citric acid	9.1 L / 15 L	Bioreactor	45

Membrane application can be seen in one of the studies involving citric acid production[45] using a membrane biomass-recycle pilot-scale bioreactor. The polysulfone membrane has a 200 kDa MWCO with overall filter area of 0.59 m^2. A cross flow microfiltration is applied to concentrate the biomass by eliminating the permeate at the end of every run. James et al.[8] demonstrate an example of in-situ product removal in their concept of affinity chromatography bioreactor (ACBR). The affinity chromatography column is used to remove heavy chain mouse monoclonal antibody (HC MAb) and 6-his tagged granulocyte macrophage colony-stimulating factor (GM-CSF) as it is being form from the suspension culture if genetically modified *N. tabacum* cells (NT-1).

In this paper, the authors would like to highlight a new design for *in-situ* product removal by combining both the concept of membrane fractionation and fermentation simultaneously. A proposed configuration is shown in Figure 1. Cultivation of *Y. lipolytica* will be carried out in a pH 6.8 buffer, YPD rich medium in a 500 ml modified Erlenmeyer flask fitted with filters arms for media outlet. The media will be circulated by a peristaltic pump from the flask through a glass filter frit to an ultrafiltration membrane unit. The membrane unit is operated in a cross-flow mode. The retentate is returned back to the flask and the fractionated product is collected as the permeate.

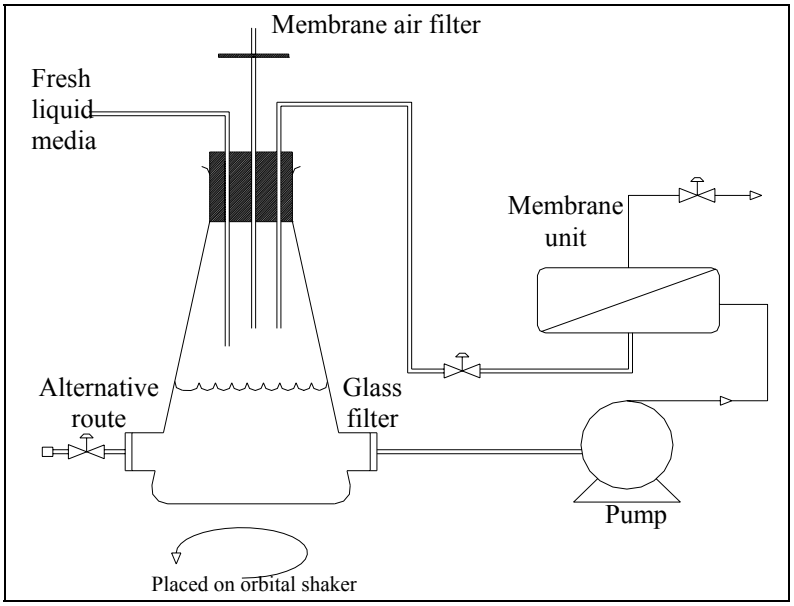

Figure 1: Configuration of in-situ protein isolation for *Y. lipolytica*

7.0 Continuous recovery of secreted proteins

Under appropriate condition where the experiment will be run, the most abundant secreted protein will be the AEP. The concentration of the protein will build up with time. As soon as the culture reaches the late exponential growth phase, the circulation through the membrane unit will start. The media circulation loop will be primed by withdrawing media from the culture and pumping through the tubing until the entire tubing and the membrane unit is filled. Following priming, media will be filtered by the membrane in a cross flow mode to achieve the desired permeate rate. The withdrawal of permeate will be replenished by the fresh media supplied directly into the flask. The retentate will be channeled back into the flask. Samples will be taken from the permeate and retentate to be tested for their protein content accordingly.

The preliminary study will only focus on the MWCO for *in-situ* separation of extracellular protein just to gauge the extend of fractionation and purification by the incorporation of membrane. The scope of study may feature system involving charged membrane and manipulation of IEP and ionic strength through pH and salt concentration. For a live system, changing the pH and salt concentration may be difficult since it could be disadvantageous both to the culture system and metabolite secretion.

8.0 Conclusion

Y. lipolytica has been gaining attention among researches for its heterologous protein expression for basic research as well as for a variety of biomedical and industrial application. The potential of in-situ isolation of the secreted metabolites with membrane technology would enable the fermentation process to be carried out uninterrupted. Besides that, higher yield could be achieved if the secreted protein has limited stability and inhibition effect. The characteristic of the membrane and protein is important in determining the degree of fractionation and purification of the product and prevention of fouling phenomena. Many studies have been carried out on *Y. lipolytica* but not many apply in-situ separation for protein recovery. The configuration proposed for *in-situ* fractionation may open a new prospect of a continuous recovery of secreted protein.

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