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Research Article

Development of aqueous two-phase systems comprising poly ethylene glycol and dextran for purification of pullulan: Phase diagrams and fiscal analysis

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Pullulan is a commercially important Exopolysaccharide (EPS) with wide-spread applications which is produced by *Aureobasidium pullulans*. The alternative α (1 4) & α (1 6) configuration in pullulan provides it the specific structural and conformational properties. Pullulan is currently being exploited in food, health care, pharmacy, lithography, cosmetics. The fermented broth is processed by organic solvent precipitation for isolation and purification of pullulan. In this study, we have tried to analyze the potential of aqueous two phase system as an alternate technique to extract pullulan from fermented broth. Including this viability of ATPS was also compared with conventional organic solvent precipitation system in terms of cost and time. It was found that ATPS process produced a higher yield of pullulan (80.56%) than organic solvent precipitation method (71.6%). ATPS was also found more economical and less time consuming method.

Keywords: Aqueous two phase separation (ATPS) / *Aureobasidium pullulans* / Fiscal analysis / Organic solvent precipitation / Phase diagram

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1 Introduction

Aureobasidium pullulans is a black yeast like fungus predominantly found in many ecological niches. This microbe is reported for its ability to produce a high amount of pullulan via fermentation [1]. Pullulan is an extracellular product of the microbe, thus can be rescued from the leftover medium after 168 h of fermentation. After fermentation, many steps of downstream processing are employed to extract pullulan from the broth. As described in the line diagram for organic solvent precipitation (Fig 1), the whole process is quite labor intensive that runs for long hours with constant observations. Thus, an alternate downstream process is required to increase the efficacy. Moreover, this novel technique in concern shall also reduce the capital quotient of the whole system.

ATPS is one such process, which may be used to remove all the process constraints while obtaining a high yield of pullulan. All the processes of extraction and purification of biological products by ATPS occur in single or two steps. It is suggested to use ATPS processes in place of other conventional chromatographic operations to reduce steps of downstream processing [2]. This method offers advantages of being versatility, cost effective, reproducible and environmental responsive as compared to the conventional methods. ATPS consists of two immiscible polymers or a polymer and a salt in water. The common polymers used for ATPS are Polyethylene glycol (PEG) and dextran [3, 4]. Besides them, ATPS systems also include poly-propylene glycol (PPG) [5], poly-ethylene oxide sulfide (PEOS) [6], alcohols [7], surfactants [8], biopolymers [9], ionic liquids [10] and organic acids [11]. Including these, multiple inexpensive polymers are also present such as ethylene oxide/ propylene oxide (EOPO) [12, 13]. Another more economical option in system selection and experimental design is picking up a polymer-salt scheme i.e. citrate [14], sulfates [15, 16], and phosphates salts [17] for ATPS extraction techniques. One component ATPS or a single polymer in water is one of the most contemporary innovation in the field of aqueous separation. Such compounds are termed as Smart polymers [18] and have been studied for phase formation with a slight amendment in temperature, pH, and protons [19]. A single suitable system of ATPS provides integrated concentration, clarification and purification of the target product all at once.

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Abbreviations: AS PUL, Pullulan produced via ATPS; ATPS, Aqueous Two Phase Separation; CCS, cooled concentrated supernatant; EPS, Exopolysaccharide; INR, Indian National Rupee; OS PUL, Pullulan produced via Organic solvent Precipitation; PEG, Polyethyleneglycol; STL, Slope of Tie Line; TLL, Tie Line Length

Figure 1. Process line diagram showing pullulan production through comparative representation of organic solvent precipitation (conventional) and Aqueous Two phase Separation (proposed).

ATPS has been successfully applied in recovery of proteins such as recombinant viral coat protein L₁ from recombinant *E. coli* [20], cyclic-oligosaccharides e.g. cyclodextrins (CDs) from *Bacillus cereus* cyclodextrins glycosyltransferase [21, 22], and for various other biopharmaceutical products for instance interleukin, insulin-like growth factor, human growth hormone (hGH) and monoclonal antibodies [23].

In this work, the fundamentals of liquid-liquid aqueous two phase extraction, a method is applied for separation, concentration, and purification of fermentative produced pullulan from *A. pullulans*. The kinetics of this novel technique are studied

with the help of partition coefficient, phase diagram as well as a comparative study of material balance economy with respect to the conventionally used organic solvent precipitation.

2 Materials and methods

2.1 Materials

All the reagents used in this study were of analytical grade. All the reagents and culture medium components were

procured from HiMedia (Mumbai, India) and Sigma Aldrich (India). *Aureobasidium pullulans* was procured from Microbial Type Culture Collection (MTCC 1991) Chandigarh, India.

2.2 Methods

2.2.1 Growth and cultivation of microorganism

The microorganism was grown and maintained in YPD medium (Yeast extract 10 g/L, Peptone 20 g/L, Dextrose 20 g/L and Agar 15 g/L at pH 7.0) and incubated at 28°C. The grown culture was subcultured every 3 weeks.

2.2.2 Fermentation

The strain of *A. pullulans* with less pigmentation was selected to perform the fermentation process. The inoculum was prepared by growing the strain in growth medium (Sucrose 50 g/L, K_2HPO_4 2 g/L, $(NH_4)_2SO_4$ 1 g/L, $MgSO_4 \cdot 7H_2O$ 0.05 g/L, NaCl 0.5 g/L, $FeSO_4$ 0.01 g/L, $MnSO_4$ 0.01 g/L, $ZnSO_4$ 0.01 g/L at pH 7) in incubator shaker at 28°C and 200 rpm for 48 h. The resulting culture was used to inoculate (10% v/v) the production medium (Sucrose 50 g/L, $(NH_4)_2SO_4$ 1 g/L, Yeast extract 2 g/L, NaCl 1.5 g/L and K_2HPO_4 5 g/L at pH 6.5). The fermentation was carried out in a shake flask at 200 rpm and 28°C for 168 h.

2.2.3 Down-stream processing through organic solvent precipitation

The fermented broth was centrifuged at 10 000 rpm for 20 min at room temperature to separate biomass. The separated cell pellet was washed three times with suspension into 10 mL distilled water and centrifuged at 10 000 for 20 min. All the obtained supernatants were pooled and processed further. The pellet was autoclaved and discarded. The supernatant was cooled at 4°C for 3 h. Cold ethanol was added to cooled supernatant in a stepwise manner (addition of 10 mL after every 10 min) till the double volume ethanol was mixed. Mixing was performed by magnetic stirrer [23]. Pullulan was precipitated at the bottom of the flask. The precipitate was left at 4°C till a layer of pullulan accumulated at the bottom. The precipitate was dried on a pre-weighted filter paper at 50°C. The amount and yield of pullulan were calculated in g/L and as per 100 g of sugar consumed.

2.2.4 Pullulan extraction via aqueous two phase separation (ATPS)

The fermented broth was centrifuged at 10 000 rpm to remove biomass and supernatant was cooled and processed by ATPS technique. For ATPS, stock solutions of solid polyethylene glycol (PEG) (50% w/w) and dextran (40% w/w) were used. Different volumes of phase forming polymers were added to the supernatant and the mixture was centrifuged at 3000 rpm for 15 min at 4°C to obtain distinct and perfect phases. Both the phases were analyzed for the presence of pullulan.

2.3 Analytical procedure

2.3.1 Determination of pullulan by pullulanase hydrolysis/enzyme assay

Pullulan recovered via organic solvent precipitation (OS PUL) and ATPS processes (AS PUL) was dissolved in distilled water at pH 5.0. Pullulanase (10 ASPU/g) was added to 10 mL pullulan solution. The mixture was gently shaken at 45°C for 6 h and 50 strokes/min, which resulted in the hydrolysis of pullulan to maltotriose. The released reducing sugar was subjected to Somogyi-Nelson test [24]. For this assay; 1 mL of the hydrolysate was mixed with 1 mL of Nelson-Somogyi reagent (NS reagents) and boiled at 100°C for 20 min. The solution was cooled and kept at room temperature for 10 min. To this cooled solution, 1 mL of Nelson's color reagent was added and incubated at 37°C for 5 min. The final volume was made to 5 mL using distilled water and the absorbance of this solution was measured at 540 nm. Absorbance was measured after each hour to estimate the pullulanase activity on all the three samples (Standard Pullulan 10% w/v, OS PUL 20% w/v and AS PUL 20% w/v). All the solutions were prepared in triplicate to minimize the errors and any prospect of contamination.

2.3.2 Phase diagram

The phase diagram consists of the binodal curve, critical point, and a tie line. The binodal data is crucial for design and development of ATPS extraction protocol. The phase diagram can be easily constructed by considering these pivotal elements. The binodal curve is a convex curve simply formed by joining binodal points which are in turn determined by three methods; turbidometric titration method, cloud point method and node determination method [25]. In this experiment, turbidometric titration method was adopted to obtain the binodal points. Appropriate stock solutions of 50% (w/w) of PEG and 40% (w/w) dextran were prepared at 4°C, pH 7.0. Dropwise titration was performed with the two polymers until the system turned clear

Figure 2. Diagram of Pullulanase enzyme assay: Standard Pullulan (pullulan), Pullulan obtained from Organic Solvent precipitation (OS PUL) and from Aqueous Two phase Separation (AS PUL).

or the phases were formed. The system was then centrifuged at 3000 rpm for 10 min to obtain clear and distinct phases. The number of systems titrated is proportional to the number of points on the binodal, thus a greater accuracy is achieved. The critical point on the curve represents the point above which the volume of both the phases is theoretically equal. A tie line connects the two points laying on the binodal curve. Like component concentration, tie line length is also expressed in % (w/w) and is determined as

$$TL L = \sqrt{(B_{Dextran} - T_{Dextran})^2 + (B_{PEG} - T_{PEG})^2}$$

Here, the top and the bottom phase composition of dextran and PEG derives the tie line length. For the tie line determination all the two phase systems were made in triplicates. Generally, all the tie line positioning in a single phase diagram are parallel to each other and hence slope of tie line (STL) can be intended as

$$STL = \frac{\Delta PEG}{\Delta Dextran}$$

2.3.3 Analysis of purity of pullulan and material balance comparison

Purity analysis of purified pullulan was conducted using the enzymatic hydrolysis of pullulan by pullulanase enzyme. The standard pullulan was used as a reference. Including this yield of the process was calculated with respect to consumed sugar. To compare and study the cost effectiveness of both the processes thorough the scrutiny of the cost (reagents) and time consumed for individual operations (Organic solvent precipitation and ATPS) were calculated. This elementary evaluation of currency consumption and their comparison in terms of time expense has also been provided in a tabular arrangement. The prices of reagents used in fermentation, organic solvent precipitation, and ATPS were

obtained from HiMedia (Mumbai, India) and Sigma Aldrich (India).

3 Results and discussion

Investigators had followed numerous downstream approaches such as conventional organic solvent precipitation [26, 27], ATPS [2, 4], azeotropic methods [28], etc for extraction of pullulan and other metabolites from fermented broth. In this study, our aim was to validate the proposed method i.e. ATPS. To prove its cost-effectiveness and significance in isolating pullulan from the fermented broth of *A. pullulans*. Fermentation was carried out and the medium was then treated to obtain maximum amount of pullulan. Both the processes were analyzed on the terms of yield, purity, performance and on the scale of economies.

3.1 Isolation of pullulan

In this experiment, the pullulan extraction from fermented broth was performed using conventional organic solvent (OS) precipitation and ATPS approach. Alike organic solvent precipitation, ATPS also produced pullulan in the form of precipitates [27, 29]. In OS precipitation, the precipitates tend to settle down after overnight resting. The filtration and drying process produced 35.8 g/L of OS PUL. The yield of this process was found to be 71.6%. The similar results have been reported by [30–32].

In the ATPS process, pullulan was precipitated in the bottom phase. After the precipitation it was washed with acetone to remove the traces of dextran from pullulan [33]. The isolated pullulan was dried and weighed to be 40.28 g/L.

Figure 3. (A) Phase diagram of PEG 6000 and Dextran at 4°C. The evolution of PEG-dextran phase diagram was studied with 1% and 2% pullulan in (B) and (C), respectively. Point (a), (b) and (c) represents in graph are the critical point of the respective phase diagram.

To the best of authors knowledge no literature has had reported pullulan productivity and yield till date. The yield of this process was found to be 80.56%. Thus it was analyzed that ATPS approach produced more yield than conventional method.

3.2 Pullulan detection by enzyme bioassay

Pullulan-6-glucanohydrolase also known as Pullulanase is a de-branching enzyme. The enzyme is particularly known for its ability to hydrolyses α -1,6-glucosidic linkage in pullulan. Pullulanase activity on pullulan chiefly produced maltotriose [34]. The enzymatic bioassay of pullulan by pullulanase reproduce results analogous to those formerly determined by HPLC or any other analytic method [35]. The enzymatic methods are highly specific in nature thus a sole pullulanase bioassay was capable of discriminating pullulan from any other sugar that might be present in the fermented broth. Pullulanase bioassay was also instrumental in deriving pullulan purity [36]. With respect to the standard pullulan sample obtained from Sigma-Aldrich, purity for OS PUL came out to be 52.24% whereas AS PUL was found to be 56.83 % pure. Absorbance of the reducing sugar obtained after enzyme hydrolysis was monitored at an interval of 1 hour

each. Moreover, a correlation of pullulanase action was established for both the samples (OS PUL & AS PUL) and was compared with standard pullulan (Fig. 2). It was also observed that after 6h incubation with enzyme there was no enhancement in the absorbance which may be due to complete hydrolysis of substrate. It was also observed that AS PUL exhibited higher purity than OS PUL.

3.3 ATPS evaluation with the help of phase diagram

Phase diagram is considered as the blueprint of the ATPS technique under precise conditions. Each phase diagram is unique under a given set of parameters (e.g. temperature, pH, phase forming components and their concentration) and illustrates the functional zone of the whole process [37]. A phase diagram of PEG 6000 and dextran was established at 4°C (working temperature for pullulan separation, [38]). A 1 and 2% pullulan were subsequently added to the PEG-Dextran system to study its effect on binodal curve, critical point and tie line length (Fig. 3). A separate phase diagram for 1 and 2% pullulan was also constructed, Point (a) in Fig. 3 (A) represents the critical point of PEG-dextran system where as (b) and (c) in Fig. 3 (B & C)

Figure 4. The FTIR absorption spectrum of Pullulan (A), OS PUL (B) and AS PUL (C) for characterization and identification of major bonds and configuration.

portrays critical point of PEG-dextran system with 1 and 2% respectively. Apparently, critical point on the binodal curve divides the phase diagram in the monophasic and biphasic zone. With the addition of pullulan, the critical point was readily achieved with the lesser concentration of the phase forming components. This implies the capability of pullulan in aiding phase formation. This characteristic of the EPS is further exploited in ATPS systems with pullulan as one of the phase forming component [39, 40]. Here the system achieves equilibrium at critical point, thus the value of TLL equals to zero. The ratio of TLL of the three systems comes out to be 25.7:22.9: 21.6. The declining drift in the length of Tie line signifies the tendency of a system to achieve equilibrium with the addition of pullulan. TLL, as well as the slope of the line, are well modeled in the phase diagram itself.

3.4 Characterization of pullulan and FTIR assessment

FTIR analysis of pullulan obtained from organic solvent precipitation (OS PUL) and ATPS (AS PUL) was studied in comparison to reference pullulan commercially available from Sigma-Aldrich (Fig. 4A). The strong absorption in the range of 3200–3550 indicated the presence of –OH repeating units like that of sugars. OS PUL (Fig. 4B) as well as PUL (Fig. 4C) both shows strong peaks in this range. Absorption in the range of 2000–2400 cm^{-1} indicates a SP^3 hybridization of C–H bond, both the sample along with standard pullulan showed strong peaks in this range [29]. A strong stretch for O–C–O bond of the alkane compound was found in the range of 1540–1870 cm^{-1} , standard pullulan showed a peak at 1692.33 cm^{-1} , absorption peak at 1636.56 cm^{-1} for AS PUL (Fig. 4C) and 1665.56 cm^{-1} for OS PUL (Fig. 4B) was also observed. A 1300–1600 cm^{-1} range is fervent for C–O–H

bending, both the sample as well as standard pullulan showed peaks in this range though at different intensities and absorption peaks.

A broader range of absorption peaks in-between 1400 and 1000 cm^{-1} was observed for C–O stretching. Alpha configuration considered as the distinguishing feature of pullulan exists in the absorption range of 650–1000 cm^{-1} . Absorption peaks at 660 cm^{-1} , 686.81 cm^{-1} and 936.28 cm^{-1} for standard pullulan, OS PUL and AS PUL respectively was observed. Peaks in the range of 900 and 650 cm^{-1} proved the presence of α -1, 6 and α -D-glucopyranosid units, respectively (Fig. 4).

3.5 Analysis of the proposed method

Economic calculation of processes is used to analyze the viability of a process. For industrially favorable process it must be cost effective. In this study, time and cost constraints of both the processes were considered important and analyzed (Table 1). Seed culture and fermentation were the elementary processes in the pullulan production. Fermentation was followed by downstream processing, which comprised of basic unit operations to separate the cell mass and processing of supernatant by Organic solvent precipitation and ATPS. Thus downstream processing was considered major value defining steps. The cost of reagents and time consumed during each precipitation process was calculated. It was observed that cost incurred in organic solvent precipitation (ethanol) was significantly towering (3930 INR) in comparison to cost of ATPS (dextran and polyethylene glycol) (835 INR). In terms of time consumption, ATPS involved overnight (12 h approximately) for phase formation post simple unit operation, whereas organic solvent precipitation required constant vigilance throughout the 34 h of

Table 1. Fiscal analysis of the organic solvent precipitation and aqueous two phase separation (ATPS)

Serial number	Process	Chemical requirements	Cost (₹)	Time (H)	Total cost (₹)
1.	Seed culture	Sucrose Magnesium sulphate Sodium chloride Ferrous sulphate Manganese sulphate Zinc sulphate Dipotassium hydrogen Phosphate Ammonium sulphate	31.2 0.0096 0.1 0.0052 0.0065 0.0035 2.76 0.38	48 h	34.46
2.	Fermentation	Sucrose Yeast Sodium chloride Dipotassium hydrogen phosphate Ammonium sulphate	31.2 4.328 0.303 6.9 0.38	168 h	43.111
3.	Organic Solvent Precipitation	Ethanol	3930	33 h 20 mins	3930
4.	ATPS	Poly ethylene glycol Dextran Acetone	144 18.43 672	24 h	834.43

operation. Thus in terms of time and cost constraints, ATPS proves to be a more efficient process. This may aid in reducing the high process cost of pullulan separation from fermented broth.

4 Concluding remarks

The present investigation found aqueous two phase extraction process to be suitable and more economic for extraction of pullulan. This method provides an easy, efficient and scalable system to produce pullulan of higher purity (56.83%) in comparison to customary organic solvent precipitation (52.24%). The pullulan produced by ATPS was free from traces of organic solvent, thus doesn't require re-precipitation with acetone or any other organic solvent. ATPS eliminates the need for precipitation, constant vigilance and high cost of organic solvent. This process may possibly be further refined by choosing more suitable phase forming components. Easy operation, high scalability, and consistent results are other important features that make it more reliable to adopt in research as well as for industrial purposes.

Practical application

- (1) Pullulan is a lucrative biopolymer with a wide industrial application which has been commercially produced by a finite number of diligence such as, Hayashibara Ltd. (Japan), NOF Corporation (Japan), Advanced Enzyme Technologies Ltd. (India), Yushiseihin Co. (Japan) etc. The long tedious down-stream processing step forbids the product from gaining business acceptability.
- (2) In this study, an Aqueous Two Phase Extraction system (ATPS) has been proposed as an alternative harvesting technique to obtain pullulan. From the last two decades this process has been constantly perceived by both academia and industries. Besides, this process is also suitable for the separation and purification of various particles and molecules. Moreover, the potential of this process also has been explored in the production of monoclonal antibodies, human recombinant proteins and enzymes, natural colorant, whole cell partitioning and even virus like particles.
- (3) The liquid-liquid extraction in ATPS is an integrative process where extraction, concentration, and partial purification occur simultaneously. Density gradient phenomenon is the driving force behind all the unit operations occurring in a single experimental step.

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5 References

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