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Optimisation of Extractive Bioconversion for Green Polymer via Aqueous Two-Phase System

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Being renewable and biodegradable, the “green-polymers”, polyhydroxyalkanoates (PHAs) have been extensively studied as a potential substitute for conventional plastic due to increasing concern towards the environment. However, high raw material cost and downstream recovery cost has always been the bottleneck for wide applications of PHAs. Among PHAs recovery methods, the main principle for aqueous two-phase system (ATPS) is to promote the accumulation of biopolymers in favour of one phase using environmental-friendly phase-forming components. Having the advantage of providing mild environment for bioseparation together with capability to handle high operating capacity and reducing downstream processing volume, extractive bioconversion via ATPS which integrates upstream fermentation and downstream purification can be the perfect solution. Extractive bioconversion of PHAs by *Ralstonia eutropha* H16 via ATPS has been studied by investigating effects of pH and addition of salts and modelling by using Design Expert™. The optimum result obtained in this study is PHA concentration and recovery yield of 0.139 g/L and 65 % using ATPS of polyethylene glycol 8,000/sodium sulphate with conditions of pH 6 and addition of 0.5M NaCl.

1. Introduction

Due to increasing concern towards resource depletion and environmental issue, biodegradable polyesters from renewable resources such as polyhydroxyalkanoates (PHA) have gained much attraction as potential substitutes for conventional plastics (Leong et al., 2014). PHAs are thermoplastics which are biocompatible and completely biodegradable, non-toxic, water-insoluble, inert, indefinitely stable in air, have high structural diversity and good processibility on equipment (Reddy et al., 2012). Due to its properties, PHAs have wide range of applications, such as packaging, machinery housings, disposable utensils, accessories and in medical field (Keshavarz and Roy, 2010).
However, productions of PHAs are much more expensive than conventional petrochemical-based plastics. Thus, a cost-effective and green recovery method for PHA is essential to stay competitive among others bio- and synthetic plastics. The most conventional and most extensively used method in the laboratory to recover PHA is by using solvent extraction due to its rapidity and simplicity (Fiorese et al., 2009). Not mentioning this method consumes large amount of volatile and toxic solvents such as chloroform, it disrupt the native orders of PHA granules’ polymer chains.
An aqueous two-phase system, ATPS is a coexisting two immiscible phases that form when two structurally different polymers or an inorganic salt and one polymer are mixed in water beyond critical concentration (Prinz et al., 2012). ATPS serves as primary recovery step of isolation and purification by

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partly remove the target product from impurities or substrates and reduce the subsequent downstream processing volume. The application range of ATPS are wide and includes proteins, microorganisms, nucleic acids, animal and plant cells, amino acids, lipids, antibiotics, antibodies, small organic species, metal ions, micro and nano-solid particulates (Guo et al., 2009). Moreover, this system has been further applied on delignification of paper pulp processing (Li et al., 2001) and as green reaction media (Chen et al., 2005).

Compared to traditional isolation and purification methods such as solvent extraction and others (Keshavarz and Roy, 2010), aqueous two-phase systems (ATPSs) have many distinguishable advantages and unique characteristics that attract the interests of researchers and industries. Consisting of high water content (70 - 90 % wt/wt), ATPS provide mild environment for separation of sensitive biomaterials (Ng et al., 2012). Moreover, the phase-forming components of ATPS are generally safe and relatively environmentally-friendly unlike conventional solvent extraction (Li and Peeples, 2004). Besides ability to handle high capacity and reducing volume of subsequent purification steps (Rabelo et al., 2004), large-scale purification using ATPS can be easily and reliably predicted from laboratory experiments' data. ATPS is a readily solution for industrial demand of cost-effective and highly efficient large-scale bioseparation technology with short processing time (Ferreira et al., 2008).

Extractive bioconversions which integrate fermentation together with downstream processing such as clarification, concentration, and partial purification using a continuous process has gained increasing interests (Show et al., 2012). The main principle of extractive fermentation using ATPS is promoting the partitioning of the target product in one phase, while cell and substrates accumulated in the other phase. The quality, yield and production cost of bioproducts can be optimised as different partitioning behaviours of product-degrading agents from target biomaterials would overcome the hydrophobic and/or electrostatic product-debris interactions, while eliminate denaturation and hydrolysis of the products.

Design of experiment (DOE) is a well-developed statistical method which has wide range of application across many industries and disciplines. Not only being as one of the most powerful DOE software, Design Expert™ is also simple enough to use (Stat-Ease, 2015). This papers aims to study the novel extractive bioconversion of PHA from *Ralstonia eutropha* H16 via ATPS, as well as optimise the design factors of pH and salt addition using Design Expert™ modelling software.

2. Methodology

2.1 Microorganisms and culture conditions

Polyethylene glycol (PEG) 8000 and salts were supplied by LGC Scientific Sdn Bhd, while nutrient broth and yeast extract were obtained from Sigma-Aldrich (M) Sdn Bhd. *R. eutropha* H16 was cultivated using defined medium with the glucose as carbon source (30 g/L). The composition of defined medium per Lcontained: 6.7 g Na_2HPO_4, 1.5 g KH_2PO_4, 2.5 g $(NH4)_2SO_4$, 0.2 g $MgSO_4$-$7H_2O$, 10 mg $CaCl_2$, 2 g yeast extract, 5 mL trace mineral solution (TMS). The trace mineral solution is composed of 6.0 g Na_2EDTA, 0.29 g $FeCl_3$-$6H_2O$, 6.84 g H_3BO_3, 0.86 g $MnCl_2$-$4H_2O$, 60 mg $ZnCl_2$, 26 mg $CoCl_2$-$6H_2O$, and 2 mg $CuSO_4$-$5H_2O$ per L. Seed culture was maintained at nutrient broth agar plate at 30 °C for overnight and transferred to culture tube containing nutrient broth for incubation at 150 rpm for 24 h. It was then inoculated into a 250 mL shake flask of 100 ml working volume at 5 % (v/v) and cultivated for 72 h.

2.2 Extractive Bioconversion of PHA experiments

R. eutropha culture of different pH ranging from 5 to 9 were fermented in PEG 8000/sodium sulfate ATPS to investigate the effect on bacteria growth rate and partitioning behaviour of PHA. In the other hands, addition of salts affects the recovery of biomolecules in ATPS due to the differential distribution of the salt ions between the phases. Therefore, the effect of adding NaCl in the concentration of 0 - 0.5 M was examined. For qualification and quantification purpose, PHA was undergone acidified methanolysis to form methyl ester followed by gas chromatography (Perkin Elmer Clarus 500) analysis.

2.2 Partitioning behaviors of PHA in aqueous two-phase system

The partitioning of PHA can be described by their respective partition coefficients, K_{PHA}, which defined as the ratio of PHA concentration in the top to that in the bottom phase:

$$K_{PHA} = C_{top}/C_{bottom} \quad (1)$$

The phase volume ratio, V_r is defined as:

$$V_r = V_{top}/V_{bottom} \quad (2)$$

where V_{top} and V_{bottom} are top and bottom phase volume.

Recovery Yield (Y), % of PHA in top phase was evaluated using:

$$Y = 100/(1+[1/(V_r \times K_{PHA})]) \quad (3)$$

where V_r was denoted by volume ratio and K_{PHA} was denoted by partition coefficient of PHA.

Purification factor (PF) was defined as the ratio of top phase PHA concentration to the overall PHA concentration using:

$$PF = (\text{Top Phase PHA Concentration})/(\text{Overall PHA Concentration}) \quad (4)$$

2.4 Optimisation Model

Optimization of parameters pH and salt addition concentration was done through the application of software called Design Expert™. Model selected was factorial D-optimal design with two factors and 4 responds (concentration of dry cell, concentration of PHA, recovery yield and purification factor). These responds were then analysed by using analysis of variance (ANOVA) method; A, B, AB model followed by definition of optimisation criteria or constraint for each responds. The weightage of concentration of dry cell, concentrations of PHA, recovery yield and purification factor are 3, 3, 2 and 1 respectively. All the responds are set to maximize. The solution with desirability closest to unity indicates the most optimised conditions or parameter.

3. Results and discussions

3.1 Effect of pH on bacteria growth

As one of the key determining factors in microorganism's growth, each bacteria has its own optimum pH for optimal growth. The growth of *Ralstonia eutropha* H16 under different pH were investigated and presented in Figure 1. From the figure, it can be clearly observed that *R. eutropha* grow better in a slightly acidic condition and has an optimum growth at pH 5 to 6. The bacteria has highest DCW of 0.850 g/L when fermented under pH 5 at 72th. In the other hand, it can be seen that the lag phase of bacteria growth curve become longer as the pH increase. In most cases, inhibitions of bacterial growth occur at extreme pH. At pH 9, the bacteria has stop growing which may be caused by denaturation where the proteins folding properties are destroyed and followed by loss of biological activity due to the protein's active site is no longer suitable for biological activity. According to Garland (1977), the growth inhibitions that observed at high pH could be caused by a direct effect of the OH^- ion on cellular components.

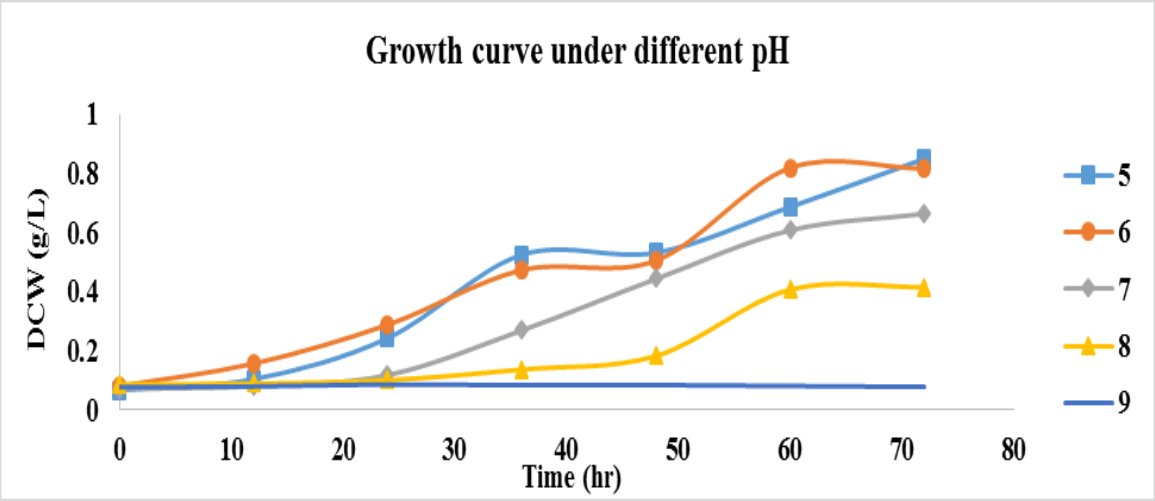

Figure 1: Growth curve of Ralstonia eutropha H16 under different pH

3.2 Combined effect of salt addition and pH to partitioning behavior of PHA

The addition of salt influences the partitioning of biomolecules in ATPS by modifying the electrostatic properties of the phases. The hydrophobic ions force the partitioning of their counter ions to the more hydrophobic phase and vice versa. From Table 1, it can be observed that the partition coefficient increase as the concentration of sodium chloride in the system increases from 0 to 0.5M and this apply to every pH. According to da Silva et al. (2014), salts such as NaCl distribute predominantly into PEG-rich phase at

high concentration. Thus, the salting-out effect promotes the partitioning of biomolecules to move from salt-rich phase to the PEG-rich phase (Zhi et al., 2004).

Coincidentally, pH also has a great influence on the partitioning behavior of biomolecules in ATPS. In partitioning of biomolecules, pH alters the electrostatic interactions between biomolecules and phase-forming components, while also influenced the phase composition (Asenjo et al., 1994). Table summarize the result of extractive bioconversion and partitioning behaviour of PHA at different pH with 0 to 0.5M concentration of salt addition. From the table, it can be observed that PHA content increase as the pH increase. In the other hand, highest partition coefficient and recovery yield can be obtained at pH 7, while the value of purification factor is the largest at pH 9. It can be concluded that the partitioning of PHA shifted towards PEG-rich top phase at neutral pH.

Table 1: Results of extractive bioconversion of PHA under different pH and salt concentration

pH	Salt concentration (M)	PHA concentration (g/L)	Partition coefficient (K_{PHA})	Purification factor (PF)	Recovery Yield (%)
5	0	0.116	1.230	1.220	55.2
	0.2	0.121	1.421	1.274	58.7
	0.5	0.124	1.728	1.310	63.3
6	0	0.098	1.361	1.154	57.6
	0.2	0.118	1.544	1.392	60.7
	0.5	0.139	1.854	1.644	65.0
7	0	0.110	1.441	1.165	59.0
	0.2	0.112	1.688	1.182	62.8
	0.5	0.129	1.967	1.360	66.3
8	0	0.080	1.312	1.085	56.7
	0.2	0.085	1.564	1.158	61.0
	0.5	0.089	1.673	1.212	62.6
9	0	0.024	1.017	1.282	50.4
	0.2	0.250	1.117	1.326	52.8
	0.5	0.026	1.321	1.379	56.9

3.3 Optimisation of pH and salt addition parameters using Design Expert™

Using the experimental data of the design factors, the optimisation is proceeded using Design Expert™. The parameter's responds and their weightage are presented in Figure 2. After running the D-optimal design model, the best solution obtained is pH 6 with addition of 0.5 M of NaCl with desirability of 0.968. Despite having lower recovery yield than that of pH 7 with addition of 0.5 M NaCl, pH 6 with addition of 0.5 M NaCl still comes out at top as shown in Figure 3. This is because the concentration of dry cell and PHA outweigh the advantage of recovery yield. Therefore, pH 6 with addition of 0.5 M NaCl is the optimum condition based on the data from the experiment.

Table 2: Optimisation constraint

Constraints	Goal	Lower limit	Upper limit	Lower weight	Upper weight	Importance
pH	Is in range	5	9	1	1	3
Salt concentration	Is in range	0	0.5	1	1	3
DCW	maximize	0.077	0.850	1	1	3
PHA concentration	maximize	0.024	0.221	1	1	3
Recovery yield	maximize	50.415	66.399	1	1	2
Purification factor	maximize	1.100	1.632	1	1	1

Table 3: Optimisation solution

Number	pH	Salt concentration	DCW	PHA concentration	Recovery yield	Purification factor	Desirability
1	6	0.5	0.816	0.221	65.203	1.632	0.968
2	7	0.5	0.663	0.181	66.399	1.494	0.818
3	6	0.2	0.816	0.184	58.455	1.359	0.728
4	5	0.5	0.850	0.188	59.938	1.200	0.697
5	7	0	0.663	0.167	55.571	1.382	0.595

4. Conclusions

For extractive bioconversion of PHA, slight acidic pH is more suitable for growth of *R. eutropha* H16 and accumulation of PHA, while the partitioning behaviour of PHA shift towards PEG-rich top phase as the concentration of NaCl increase. From Design Expert™ optimisation model, pH 6 with addition of 0.5M NaCl is the optimum condition for this preliminary study of extractive bioconversion of PHA via ATPS. This can be further applied using other ATPS systems, such as thermoseparating-based, alcohol-salt, ionic liquid and others.

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