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Aroma compounds recovery from mycelial cultures in aqueous two-phase processes

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Abstract

This paper presents the evaluation of the potential use of aqueous two-phase systems (ATPS) for the recovery of 6-pentyl-α-pyrone (6PP) produced by *Trichoderma harzianum*. The partition behaviour of 6PP and *Trichoderma harzianum* mycelium (biomass) in polyethylene glycol (PEG)–salt (phosphate and sulphate) and PEG–dextran ATPS was investigated. The influence of defined system parameters (e.g. molecular mass of PEG and dextran, volume ratio, etc.) on the partition behaviour of 6PP and *Trichoderma harzianum* mycelium was evaluated to select under which conditions 6PP and mycelium partition to opposite phases. In PEG–dextran systems either large extraction phases were required or mycelium and 6PP partitioned to the same phase. ATPS comprising V_r=0.23, PEG 8000 6.6% w/w and sulphate 14.0% w/w provided the best conditions to satisfy the process requirement of biomass accumulation into the bottom phase and 6PP concentration in the top phase.

Keywords: Aqueous two-phase systems; Aroma compounds; 6-Pentyl-α-pyrone

1. Introduction

Flavour and aroma chemicals used in food, cosmetic and detergent industries are products of great commercial significance. Existing technologies of flavour and aroma production by micro-organisms have opened up an alternative source in comparison with the chemical synthesis [1]. In this context, the production of 6-pentyl-α-pyrone (6PP) represents a very interesting case because the concentration of the product of interest (aroma) in the fermentation broth causes inhibition of the growth of the micro organism and, as a result the production of the aroma is inhibited [2]. Such a situation definitively raises complications upon downstream processing.

Recovery of the products exploiting extractive fermentation or in situ removal of products represents an alternative technology to remove the product from the fermentation broth as it is formed. Therefore, the productivity of the processes can be increased. In the case of the production of 6PP, several attempts to remove the molecule have been published (e.g. using adsorbent, pervaporation and immobilization). However, these processes have disadvantages as complications and negative effects upon the performance of the micro-organism [3,4]. In this context, reviews on in situ removal [5,6] of

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products from fermentation broth suggest the application of an aqueous two-phase system (ATPS) as an alternative for extractive fermentation of biological products [7]. This technique has several process advantages including; biocompatibility, easy to scale up, low cost, etc. Extractive fermentation using ATPS for the recovery of products has been addressed particularly for protein products. However, reports discussing the use of the ATPS process for the recovery of aroma compounds produced by mycelial cultures are not common.

The present research is concerned with the potential use of ATPS for the recovery of aroma compounds. A representative model (production of 6PP by *Trichoderma harzianum*) has been chosen to evaluate the recovery of aromas using ATPS. A practical approach which exploits the known effect of the system's parameters upon protein partition was used to examine the partition behaviour of 6PP from *Trichoderma harzianum* in polyethylene glycol (PEG)–dextran and PEG–salt aqueous two-phase systems.

2. Experimental

2.1. Characterisation of aqueous two-phase systems

Aqueous two-phase systems were compounded for convenience on a fixed mass basis on a top-loading balance. Predetermined quantities of solid poly-(ethylene glycol) (PEG, Sigma Chemicals, St. Louis, MO, USA) of nominal molecular mass of 1000, 1450, 3350 and 8000 g/gmol, dextran (nominal molecular mass 10 000, 40 000, 70 000, 500 000 g/gmol, Sigma Chemicals, St. Louis, MO, USA), sodium sulphate or potassium phosphate (Sigma Chemicals, St. Louis, MO, USA) were mixed with broth from *Trichoderma harzianum* fermentation to give a final weight of 15 g. Solid components (PEG, dextran or salts) were dissolved and phases dispersed by gentle mixing for 30 min at 25°C. Complete phase separation was achieved by low-speed batch centrifugation at 1500 *g* for 20 min at 25°C. Visual estimates of the volumes of top and bottom phases and solids, were made in graduated centrifuge tubes. The volumes of the phases were then used to estimate the volume ratio (V_r=volume of the top phase/volume of the bottom phase). Samples were taken from the phases and diluted for biochemical analysis and subsequent estimation of 6PP and biomass partition coefficient (K=concentration of solute in the top phase/concentration of solute in the bottom phase). Results reported are the average of three independent experiments and errors were judged to be ±5% of the mean value.

2.2. Strain and cultivation conditions

Trichoderma harzianum IMI 206040 was used. Stock cultures were kept at 4°C for no longer than one month. The stock culture was used to inoculate freshly prepared PDA medium and incubated at 29°C for 5 days. After sporulation was evident, spores were recovered with 3 ml of salt solution [NaCl, 0.9% (w/v) and Tween 40, 0.05% (w/v)] by mild agitation. This spore suspension was used to inoculate the culture medium. Erlenmeyer flasks (500 ml) containing 100 ml of medium (malt extract 20 g/l and glucose g/l; pH 5.6) were inoculated using an initial spore concentration of 5×10^3 spores per ml. Cultures were carried out at 200 rpm, and 29°C. Results presented are the average of two independent runs.

2.3. Analytical procedures

Mycelial biomass was evaluated by dry weight measurements. Broth samples (25 g) were washed three times in a salt solution (NaCl, 0.9% w/v), filtered through 0.45-μm Millipore membranes (Millipore; Massachusetts, Boston, USA) and oven-dried until a constant weight at 85°C was achieved. The extraction of the aroma compounds from the samples (50 ml) was carried out using methylene chloride (20 ml) as reported earlier [8]. The concentration of aroma was estimated using a Hewlett-Packard chromatograph (HP model 6890, Palo Alto, CA, USA), equipped with flame ionisation detector and Hewlett-Packard (HP-25M) capillary column (internal diameter 0.32 mm and 30 m of length). The oven temperature rise was set from 125°C to 220°C at a rate of 5°C/min. Temperature of the injector and detector were 200°C and 250°C, respectively. Helium was used as carrier gas at 1.5 ml/min.

3. Results and discussion

3.1. Partition behaviour of 6PP from Trichoderma harzianum in PEG–salt ATPS

The design of aqueous two-phase processes is limited by the poor understanding of the mechanism governing the behaviour of molecules in ATPS. Consequently, for each extraction process, once general conditions have been selected on the basis of experience or process limitations (e.g. polymer and salt type) more specific partition conditions (polymer, salt concentration, volume ratio, etc.) need to be empirically established. In the present research, before designing the aqueous two-phase process in which the aroma (6PP) and the solids (biomass) concentrate in opposite phases, on the basis of the potential use of ATPS for in situ recovery of 6PP produced by *Trichoderma harzianum,* general process conditions were defined. The polymer-rich phase was defined as the extractive phase and the salt-rich phase as the fermentative phase. Thus, for the initial experiments, a V_r less than one was selected (i.e. 0.3; estimated from non-biological ATPS). System pH was set according to the fermentation pH (i.e. 5.6–6.0). Additionally, in order to add the minimum amount of chemical-forming phases (PEG and salt) ATPS close to the binodal (tie line length; TLL<35% w/w) were selected. As a consequence of the previous definition of the values of the majority of the systems parameters, it was decided to examine the effect of molecular mass of the polymer in PEG–phosphate ATPS.

Table 1 illustrates the impact of increasing molecular mass of PEG on the biomass and 6PP partition coefficient (K_B and K_{6PP}, respectively). The results showed that for all the different nominal molecular mass of PEG studied, the K_B was less than one, which implied that the majority of biomass loaded to the ATPS was concentrated in the phosphate-rich bottom phase (i.e. 59–80%). In the case of the 6PP partition coefficient, it was greater than one (i.e. 1.1–2.6) which although it implied that 6PP concentrated mainly in the top phase, the recovery of the aroma compound from such a phase was less than 43%. This reduced recovery of the 6PP can be explained by the small extractive top phase (V_r=0.3). In this context, reports that discuss the partition behaviour of molecules of low molecular weight in ATPS (in comparison with proteins) are not common. Rogers et al. [9] reported the partition behaviour of metal ion in PEG-based aqueous biphasic systems and proved the potential of ATPS for the recovery of such a metal ion from different suspensions. However, the partition behaviour of aroma compounds in ATPS has not been previously discussed. The partition behaviour of 6PP in ATPS is reported here for the first time.

In the partition experiments using PEG–phosphate, ATPS problems of phase formation (and phosphate precipitation) associated with the system pH (5.6–6.0) were observed in some cases. Thus, it was decided to use PEG–sulphate ATPS to study the partition behaviour of biomass and 6PP in these systems. Table 2 illustrates the partition behaviour of 6PP produced by *Trichoderma harzianum* fermentation broth in PEG–sulphate ATPS. The results showed that for all the ATPS studied, the biomass partitioned predominantly to the lower phase (i.e. 71–85%). However, the recovery of 6PP from the

Table 1
Partitioning of 6PP from *Trichoderma harzianum* fermentation broth in PEG–phosphate ATPS[a]

System	PEG (%w/w)	Salt (%w/w)	V_r	K_B	Y_B (%)	K_{6PP}	Y_{6PP} (%)
PEG 1000–phosphate	7.5	17.4	0.3	0.26	80	1.1	24
PEG 1450–phosphate	7.4	16.4	0.3	0.40	72	2.42	41
PEG 3350–phosphate	6.9	14.7	0.3	0.34	74	2.61	43
PEG 8000–phosphate	6.2	12.0	0.3	0.38	59	2.27	40

[a] For all systems, volume ratio was estimated from non-biological experimental systems as described in Section 2. The partition coefficient (K) represents the ratio of the 6PP and biomass concentrations in the phases. The recovery of 6PP and biomass (Y_{6PP} and Y_B) from the top and bottom phase (respectively) are expressed relative to the initial amount of 6PP and biomass content in the fermentation broth and loaded to the systems.

Table 2
Partitioning of 6PP from *Trichoderma harzianum* fermentation broth in PEG–sulphate ATPS[a]

System	PEG (%w/w)	Salt (%w/w)	V_r	K_B	Y_B (%)	K_{6PP}	Y_{6PP} (%)
PEG 1000–sulphate	7.0	14.6	0.26	0.41	71	2.42	39
PEG 1450–sulphate	7.2	16.6	0.22	0.24	81	3.5	44
PEG 3350–sulphate	7.4	10.6	0.3	0.36	74	1.95	39
PEG 8000–sulphate	6.6	14.0	0.23	0.17	85	3.77	45

[a] Conditions and assumptions as in Table 1.

opposite phase (top phase) was relatively low (i.e. 39–45%). The partition behaviour of 6PP produced by *Trichoderma harzianum* in ATPS cannot be fully explained. It can be suggested that the relatively low recovery of the 6PP from the PEG-rich phase may be associated with the reduced volume of that phase (V_r equal or less than 0.3) to accommodate the 6PP and the presence of other solutes characteristic of the fermentation broth (e.g. proteins, organic acids, etc.). In contrast, for the recovery of biomass, a large volume of the bottom phase is available. In general, from the ATPS studied here, the system comprising V_r=0.23, PEG 8000 6.6% w/w and sulphate, 14.0% w/w provided the best conditions to satisfy the process requirement of biomass accumulation into the bottom phase (i.e. 85%) and concentrate the 6PP in the opposite phase (i.e. 45%). A 45% recovery of 6PP from the top phase may be considered relatively low for an conventional recovery process. However, such a value has to be interpreted within the context of an integrated process for the recovery of 6PP from the fermentation broth of *Trichoderma harzianum*. In this circumstance, the culture medium could be detoxicated by lowering the accumulation of 6PP in the fermentation broth.

3.2. Partition behaviour of 6PP from Trichoderma harzianum in PEG–dextran ATPS

The use of PEG–dextran ATPS was considered in the present research to further characterise the partition behaviour of 6PP and biomass in ATPS. In general, the development of extraction processes for the recovery of macromolecules involving the use of PEG and dextran are limited for economic reasons. Thus, the application of PEG–dextran ATPS has been concentrated for the recovery of high value products (e.g. cultures of hybridoma cells; [10]) in which the cost of the chemicals used in the extraction process is justified by the price of the final product. In the case of the recovery of 6PP using PEG–dextran systems, economic aspects were not considered at this stage. In the PEG–dextran ATPS characterised by a V_r less than 1.0 (i.e. 0.25–0.33), 6PP and biomass from the fermentation broth partition to the same phase except the ATPS with dextran 40 (Table 3). In this latest system, biomass concentrated in the top PEG-rich phase and the 6PP in the bottom dextran rich phase. Such partition behaviour anticipates the use of a reduced top fermentative phase and a large extractive bottom phase. Although such a situation can be alleviated by increasing V_r (from 0.31) it may present process complications (phase separation after the fermentation) for the potential use of ATPS in an in situ recovery of 6PP.

In contrast, for PEG–dextran ATPS with V_r greater than one, biomass and 6PP partition to opposite phases (Table 4), except in the case of the systems comprising PEG 1450 and dextran 70. In this particular system, both the 6PP and the biomass partition coefficient (K_{6PP} and K_B) exhibited values greater than one, which implied that both biomass and 6PP concentrate predominantly in the top PEG-rich phase. For the majority of the PEG–dextran systems used here, 6PP and biomass concentrated in opposite phases. From these results it is important to consider that the practical application of such systems may be limited by the V_r greater than one (i.e. 3.0–8.3). In this context, further experimentation using the same TLL can be pursued to reduce the ATPS V_r and conserve the partition properties of the system. However, it can be anticipated that the economic aspects associated with the use of dextran will have an impact on the potential commercial application of the ATPS process. In general, at this stage the partition behaviour and the mechanism

Table 3
Partitioning of 6PP from *Trichoderma harzianum* fermentation broth in PEG–dextran ATPS with $V_r<1.0$[a]

System	V_r	Phase	K_B	Y_B (%)	K_{6PP}	Y_{6PP} (%)
PEG 1450–dextran 10	0.33	PEG 1450	0.04	4.0	0.44	13
		Dextran 10		96		87
PEG 1450–dextran 40	0.31	PEG 1450	8.0	89	1.37	30
		Dextran 40		11		70
PEG 1450–dextran 70	0.26	PEG 1450	8.2	89	1.65	30
		Dextran 70		11		70
PEG 1450–dextran 500	0.25	PEG 1450	12.6	93	1.17	23
		Dextran 500		7.0		77

[a] For all systems, the use of 10, 40, 70 and 500 represents the molecular mass of dextran; 10 000, 40 000, 70 000 and 500 000 g/gmol respectively. Volume ratio (V_r) was estimated from non-biological experimental systems as described in Section 2. The partition coefficient (K) represents the ratio of the 6PP and biomass concentrations in the phases. The recovery of 6PP and biomass (Y_{6PP} and Y_B) from the top and bottom phase (respectively) are expressed relative to the total amount of 6PP and biomass content in the phases. Possible losses at the interface were not considered for these estimations.

Table 4
Partitioning of 6PP from *Trichoderma harzianum* fermentation broth in PEG–dextran ATPS with $V_r>1.0$[a]

System	V_r	Phase	K_B	Y_B (%)	K_{6PP}	Y_{6PP} (%)
PEG 1450–dextran 10	4.6	PEG 1450	0.01	1.0	3.1	93
		Dextran 10		99		7.0
PEG 1450–dextran 40	6.0	PEG 1450	0.16	14	5.8	97
		Dextran 40		86		3.0
PEG 1450–dextran 70	3.0	PEG 1450	15.1	94	4.0	92
		Dextran 70		6.0		8.0
PEG 1450–dextran 500	8.3	PEG 1450	0.1	8.0	2.8	96
		Dextran 500		92		4.0

[a] Conditions and assumptions as in Table 3.

governing the partition of 6PP in ATPS is not clear and cannot be fully explained. Thus further characterisation is required to extend knowledge of such phenomena.

4. Conclusion

It has been shown that 6PP and biomass from fermentation broth of *Trichoderma harzianum* partition in opposite phases in PEG–phosphate ATPS. However, these systems presented problems of phase stability and phosphate precipitation associated with the system pH. The PEG–dextran ATPS proved to be unsuitable for the recovery of 6PP since both biomass and the aroma partitioned to the same phase or involved the use of a large extraction phase. The use of PEG–sulphate systems resulted in the selection of operating conditions for the potential recovery and concentration of 6PP produced by *Trichoderma harzianum*. It can be concluded that the results reported here demonstrated the practical application of ATPS processes for the potential recovery of 6PP in particular, and for aroma compounds, in general.

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