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Extraction of forskolin from *Coleus forskohlii* roots using three phase partitioning

Shirish M. Harde, Rekha S. Singhal *

Food Engineering and Technology Department, Institute of Chemical Technology, Matunga, Mumbai 400 019, India

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ABSTRACT

Three phase partitioning (TPP), a technique which is based on partitioning of hydrophilic constituents, proteins, and hydrophobic constituents in three phases comprising of water, ammonium sulphate and organic solvent, was explored for extraction of diterpene, forskolin from *Coleus forskohlii* roots. The process which consists of simultaneous addition of *t*-butanol and ammonium sulphate to the aqueous slurry of *C. forskohlii* roots was optimized with respect to the concentration of ammonium sulphate loading and the ratio of *t*-butanol to slurry. A maximum of 30.83% recovery of forskolin was obtained under the optimized conditions. Ultrasonication and enzyme pretreatment with commercial enzyme preparation of Stargen® 002 and Accellerase® 1500 followed by TPP gave 79.95% and 83.85% recovery when used individually within 4 h as compared to 12 h in Soxhlet extraction. A combination of the two pretreatments increased the yield marginally. Hence enzymatic pretreatment followed by TPP is recommended for extraction of forskolin.

1. Introduction

Forskolin (FSK), a labdane diterpene compound isolated from the roots of *Coleus forskohlii* Briq. [1], is useful in the treatment of health disorders including cardiovascular diseases, hypertension [2,3], asthma, glaucoma [4] and Alzheimer's disease [5]. Its further use in promoting lean body mass, treating mood disorders and its anticancer activities [6] is well known. The pharmacological activities of FSK are mainly due to its role as an activator of adenylate cyclase. FSK increases the amount of cyclic AMP (cAMP) (adenosine monophosphate) in cells by activating adenylate cyclase enzyme. cAMP is an important secondary messengers in the cell, and is considered to be an effective cell regulating compound [7].

The structural complexity of FSK makes the chemical synthesis difficult and uneconomical. Besides, synthetic forskolin is reportedly not as effective as that procured from the natural source [8]. Among the several methods described in the literature for extraction, isolation and purification of FSK from the roots of *C. forskohlii*, refluxing the powdered roots in multiple extraction steps with organic solvents such as benzene, methanol, chloroform and toluene [9] is commonly used commercially. This method gives an extract that is sticky and viscous in nature, besides being time consuming and requiring relatively large quantities of solvents [10]. Supercritical fluid extraction is a potential alternative to conventional extraction methods using organic solvents for extracting biologically active components from plants [11]. However, this method requires expensive high pressure equipment and may also require organic solvents as co-solvent for complete extraction of the bioactives [12]. Other methods reported for extraction of forskolin are hydrotropic extraction [12] and microwave assisted extraction [13], of which the former does not give sufficiently pure FSK.

In this paper, we report an alternative extraction process for the recovery of FSK using three phase partitioning (TPP). TPP is a relatively recent bioseparation technique in which a suitable amount of ammonium sulphate and *t*-butanol is added to an aqueous suspension of the sample. The protein precipitates out in the middle layer between the organic and aqueous phases due to multiple phenomena such as salting out, isotonic precipitation, co-solvent precipitation, osmolyte precipitation and kosmotropic precipitation. Polar compounds such as saccharides separate out in the lower aqueous layer, while non-polar compounds such as oils separate in the upper organic layer. TPP is a simple, inexpensive, scalable, and rapid procedure, works at room temperature, and the chemicals used in the process can be recycled. It does not use polymers which have to be removed later. Tertiary butanol is normally completely miscible with water (b.p. 84 °C) and much less flammable than hexane, methanol or ethanol which are used in conventional extraction.

All developments on TPP so far have used *t*-butanol. TPP has been used for both upstream and downstream processing of enzymes such as bifunctional protease/amylase inhibitor [14], invertase [15], α-galactosidase [16,17], and serine protease [18]. However, literature on use of TPP for extraction of oleaginous material is scant. Sharma et al. [19] showed TPP to extract 82% oil from soybean oil within 1 h, while Shah et al. [20] reported it to extract 97% oil from *Jatropha curcas* L. within 2 h. We reported on successful application of TPP in extraction of oleoresin from turmeric [21].

* Corresponding author. Tel.: +91 22 3361 2512; fax: +91 22 3361 1020.
E-mail address: rs.singhal@ictmumbai.edu.in (R.S. Singhal).

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Pretreatments using enzymes, either alone [22,23] or in combination with sonication [20] has shown increased yields of oil from soybean, rice bran, mango kernel, peanuts and jatropa. Similarly, ultrasonication followed by TPP (UATTP) has shown 87%, 77% and 88% recovery of oil from almond, apricot and rice bran, respectively [24].

Enzyme pretreatment of plant material could help in breakdown of cellulosic structure to increase the extraction efficiency of FSK. Shah et al. [20] used enzyme pretreatment prior to TPP to increase the yield of oil and called this approach as enzyme-assisted three phase partitioning (EATPP). The efficiency of EATPP was concluded to be comparable to solvent extraction with added advantage of being less time consuming.

Since FSK is soluble in polar solvents [25], we hypothesized that it may be possible to extract it from *C. forskohlii* roots by TPP. The present work demonstrates the application of TPP in extraction of FSK from *C. forskohlii* roots. An important further step in the process development has been the pretreatment of the plant material with ultrasonication and enzymes prior to TPP.

2. Materials and methods

2.1. Materials

C. forskohlii roots were procured from Salem, Tamilnadu, India. Dried roots were ground in a mill fitted with 18 mesh sieve to get a particle size below 1 mm and stored in an air tight container for further studies. Standard forskolin (FSK) was a gift sample from Medicinal and Natural Product Research Laboratory, Institute of Chemical Technology, Mumbai, India. Methanol, *t*-butanol and ammonium sulphate were procured from S. D. Fine Chemicals Limited, Mumbai, India. Enzyme samples (Stargen® 002 and Accellerase® 1500) were gifted by Genencor International, Mumbai, India.

2.2. Three phase partitioning (TPP)

TPP was optimized by varying the ammonium sulphate loading and ratio of *t*-butanol to slurry i.e. the quantity of solvent required for maximum yield of extractives. Slurry was prepared by dispersing 5 g *C. forskohlii* root powder in 50 mL distilled water by gentle stirring using a magnetic stirrer. Weighted amount of ammonium sulphate was added to the slurry prepared and vortexed gently, followed by addition of measured amount of *t*-butanol. The extraction was carried out for 1 h by gentle stirring with magnetic stirrer. The mixture was allowed to stand for 1 h for the formation of three phases. The three phases so formed were separated by centrifugation at 5000 g for 20 min. The upper organic layer was collected and the solvent (*t*-butanol) was evaporated on a rotary vacuum evaporator under reduced pressure at 50 °C for 2 min (Buchi Rotavapor, R-124, Switzerland). The extract so obtained was quantified for FSK content by using HPLC. Ammonium sulphate loading was varied from 10 to 50% w/v of slurry while ratio of *t*-butanol to slurry was varied from 0.5:1 to 2:1 with all the other extraction conditions being unchanged. Further evaluation of extraction time from 30 to 120 min was carried out for maximum yield of FSK. To check the effect of pH on extraction of FSK, pH of the system was adjusted to pH 4, 5, 6 and 7 by the addition of 1 N HCl and 1 N NaOH after addition of ammonium sulphate to the slurry. The concentrations of ammonium sulphate and *t*-butanol thus optimized were used further for EATPP and UATPP.

2.3. Enzyme assisted three phase partitioning (EATPP)

One factor at-a-time method was used to study the effect of various parameters on the extraction of FSK. Temperature and pH were kept constant at their optimal levels as determined experimentally (data not shown). The parameters were studied individually for Stargen® 002 (pH 4.5, 50 °C and 2000 U/mL) and Accellerase® 1500 (pH 4.5, 50 °C and 1500 FPU/mL). One unit of enzyme activity was defined as the amount of enzyme that would liberate one mole of reducing sugar per minute under optimized assay conditions. Stargen® 002 is a blend of α-amylase and glucoamylase which hydrolyses starch. Accellerase® 1500 is a mixture of cellulase and glucosidase having action on cellulose.

In EATPP, enzyme pretreatment was given to *C. forskohlii* root slurry prior to TPP using Stargen® 002 and Accellerase® 1500 in McIlvaine's buffer. Specific amount of enzyme was added to the slurry, and the slurry was incubated with gentle stirring with a magnetic stirrer for 1 h. After incubation, FSK was extracted by TPP using the optimized conditions as determined earlier, and the extract was quantified for FSK. For Stargen® 002 and Accellerase® 1500 pretreatment, enzyme concentration (16–80 U/g of substrate and 30–150 FPU/g of substrate, respectively) were optimized.

Effect of combination of these two enzymes was also studied. For this, slurries were incubated for 1 h after addition of Stargen® 002 and Accellerase® 1500 (64 U/g of substrate and 90 FPU/g of substrate, respectively) at pH 4.5, other conditions of extraction by EATPP being the same. Slurries were prepared in McIlvaine's buffer system [26]. Incubation time of combined Stargen® 002 and Accellerase® 1500 pretreatment was optimized by incubating the slurries (pH-4.5, 50 °C) for 30–120 min. The extract so obtained was quantified for FSK content.

2.4. Ultrasound assisted three phase partitioning (UATPP)

In UATPP, ultrasound pretreatment was given to *C. forskohlii* root slurry prior to TPP. Optimization was carried out by using one factor at-a-time method. Optimization of power requirement for the maximum extraction of FSK after TPP was done by varying the power from 18 to 58 W for 10 min. Effect of duty cycle (30–70%) as a pretreatment on extraction of FSK evaluated by subjecting 10% w/v of slurry prepared in distilled water at optimized power for 10 min. Further, optimization of extraction time (5–20 min) of sonication before TPP was carried out for maximum extraction of FSK. The extract was then quantified for FSK content.

2.5. Combination of ultrasonication and enzyme pretreatment followed by TPP

To check the combined effect of ultrasonication and enzyme pretreatment, *C. forskohlii* root slurry was treated with optimized parameters obtained from ultrasonication study. Further, ultrasound treated slurry was subjected to enzyme pretreatment followed by TPP. The extract was then quantified for FSK content.

2.6. Conventional solvent extraction

The *C. forskohlii* root powder was extracted in a Soxhlet apparatus using methanol for 12 h. The extract was cooled and then concentrated by evaporating in rotary vaccum evaporator under reduced pressure at 50 °C (Buchi Rotavapor, R-124, Switzerland). Solvent was recovered from rotavac. The FSK yield was expressed as % w/w of *C. forskohlii* root powder.

2.7. Analytical determination

A Jasco HPLC system fitted with Zorbax eclipse XDB C_{18} column (5μ × 4.6 mm × 250 mm) was used. The column was equilibrated with an acetonitrile-water (50:50) mixture as mobile phase at a flow rate of 1.5 mL/min. FSK was detected by measuring UV absorption at 217 nm [27]. Retention time of standard FSK was 9.7 min.