Çukurova Üniversitesi Mühendislik Mimarlık Fakültesi Dergisi, 31(ÖS 2), ss. ÖS 43-ÖS 52, Ekim 2016
Çukurova University Journal of the Faculty of Engineering and Architecture, 31(SI 2), pp. SI 43-SI 52, October 2016

Whole Cell Biotransformation of Fusel Oil into Banana Flavour by *Lindnera saturnus*

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Geliş tarihi: 10.08.2016 Kabul tarihi: 05.10.2016

Abstract

An efficient biotransformation of isoamyl alcohol obtained from fusel oil to isoamyl acetate in a molasses based medium via in situ product removal (ISPR) with macroporous adsorption resin was carried out with *Lindnera saturnus*. Nine types of macroporous adsorption resins with different polarities and surface areas were tested with synthetic medium and then in batch and fed-batch cultivations. H103 resin had the best adsorption capacity because of its large and nonpolar surface areas. The isoamyl acetate concentration was increased with 42 and 30 folds in batch and fed-batch cultivations, respectively, compared to the biotransformations without addition of adsorbent resin. When 1 g H103 resin (wet w/v) was added to 50 mL of the biotransformation medium, the total isoamyl acetate concentration achieved was 1.9 g/L, of which 123 mg/L remained in the aqueous phase and 1787 mg/L was adsorbed onto the resin, within 120 h in fed-batch system. This was the highest isoamyl acetate yield by biotransformation until now and was remarkable for making the process more feasible for industrial application.

Keywords: Isoamyl acetate, Fusel oil, Biotransformation, *Lindnera saturnus*

Lindnera saturnus Kullanılarak Hücresel Biyodönüşüm Yolu ile Fuzel Yağından Muz Aroması Eldesi

Öz

Bu çalışmada, fuzel yağından elde edilen izoamil alkolün şeker pancarı melası içeren ortamda izoamil asetata *Lindnera saturnus* mayası ile biyodönüşümü, makrogözenekli adsorpsiyon reçeineleri ile Yerinde Ürün Kazanımı tekniği kullanılarak etkin bir şekilde gerçekleştirilmiştir. Farklı polarite ve yüzey alanlarına sahip dokuz farklı makrogözenekli adsorpsiyon reçinesi sentetik ortamda test edilmiş ve daha sonar kesikli ve kesikli-beslemeli biyodönüşüm denemelerinde kullanılmıştır. H103 reçinesi nonpolar özelliği ve geniş yüzey alanına sahip oluşu nedeniyle adsorpsiyon kapasitesi en yüksek reçine olarak bulunmuştur. Kontrol denemesi ile karşılaştırıldığında, reçine ilevesi yapılan kesikli ve kesikli-beslemeli biyodönüşüm denemelerinde izoamil asetat konsantrasyonunda sırasıyla 42 ve 30 kat artış gözlemlenmiştir. 120 saatlik kesikli-beslemeli biyodönüşüm denemesinde, 1 g H103 reçinesi (ıslak w/v) 50 mL biyodönüşüm ortamına ilave edildiği zaman, elde edilen izoamil asetat miktarı 1,9 g/L (123 mg/L sulu ortamda + 1787 mg/L reçineye adsorplanmış vaziyette) bulunmuştur. Bu miktar, biyodönüşüm

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yoluyla şimdiye kadar elde edilmiş en yüksek izoamil asetat miktarı olup ve prosesin endüstriyel uygulamaya aktarılması açısından kayda değer veriler olarak değerlendirilebilir.

Anahtar Kelimeler: İzoamil asetat, Fuzel yağı, Biyodönüşüm, *Lindnera saturnus*

1. INTRODUCTION

Flavour compounds found widespread application in food, beverages, cosmetics, detergents and pharmaceutical products with a world-wide industrial size estimated at US $ 24 million in 2015 [1]. Most of the available flavour compounds (84 %) are now produced via chemical synthesis, although extraction from natural material continues [2]. However, recent market surveys have shown that c onsumers prefer foodstuff that can be labelled as "natural". Although flavours may be produced by chemical transformation of natural substances, the resulting products cannot legally be labelled as natural [3]. An alternative route for natural flavour synthesis which is based on employment of new biotechnological processes, de novo microbial processes (fermentation) or bioconversions of natural precursors using microbial cells or enzymes (biocatalysis), have increased considerably in the past two decades [4-6].

Esters are commonly used flavouring agents, very appreciated for the fruity aromas they provide. They are employed in fruit-flavoured products, baked goods, wines, and dairy products. Acetate esters, such as ethyl acetate, hexyl acetate, isoamyl acetate and 2-phenylethyl acetate are recognised as important flavour compounds [3]. Especially, isoamyl acetate, the characteristic compound of banana flavour, is produced with an amount of 74 tonnes per annum [7]. Several yeasts are capable of producing large amounts of fruity ester flavours. The genus *Lindnera* (formerly *Williopsis*) synthesizes high amounts of volatile esters, e.g. isoamyl acetate [8-9]. The most efficient biotechnological approach used to obtain natural isoamyl acetate is the growth-associated biotransformation of isoamyl alcohol to isoamyl acetate using the yeast *Lindnera* [7,10,11]. However substrate and product inhibition during biotransformation limits the final isoamyl acetate concentrations in conventional biotransformations. In our previous study, isoamyl acetate formation was affected by the addition of fusel oil at the ratios of more than 1% to the medium. The toxicity of fusel oil was the main reasons for low isoamyl acetate productivity [7].

Fed-batch cultivation is an industrial technique where the process is started with a relatively low substrate concentration. As the substrate is consumed, it is replaced by adding a concentrated substrate solution at a low rate while keeping the substrate concentration in the medium below the toxic level [12]. Fed-batch fermentation is advantageous in cases where an initial high substrate concentration and product accumulation is toxic to the culture. However, when applied to systems such as biotransformation of isoamyl alcohol to isoamyl acetate, an ISPR technique should be applied in combination with the fed-batch cultivation. By feeding the medium at a slow and controlled rate, isoamyl alcohol toxicity can be kept below inhibitory levels, while the ISPR technique can be applied simultaneously to remove isoamyl acetate toxicity.

Stark et al. [13] listed, in a complete review, all the projects on ISPR on flavour production in the last 20 years. The wide variety of flavour compounds amenable of microbial production permits the application of various separation methods. For example, organic solvent extraction [14], ionic liquid extraction [15], solid-liquid sorption [16], supercritical CO_2 extraction [17], immobilized solvent extraction [18], and pervaporation [19], have been employed to remove 2-phenylethanol from the medium following its production. Although some of these techniques are more efficient, they have some shortcomings. For example, the viscosity of organic solvents causes problems during fermentation and product separation [16]. The choice of the right solvent is a critical point, since many solvents can be toxic towards the biocatalyst. At the industrial scale, safety becomes an important issue, if large

volumes of "critical" solvent are to be used [20]. ISPR using adsorption processes have been mostly applied for the concentration and separation of various bioproducts like proteins, flavours, secondary metabolites etc. Especially, adsorbent resins are used in bioproduction of natural flavours and antibiotics, which are toxic to the producing organisms [20,21]. Adsorption resins have many advantages including high loading capacity, concentration of targeted components, higher adsorption specificities and easier desorption, better mechanical strength and re-uses [21]. In the fermentation of kirromycin and rubradirin, adsorbent resins have been successfully applied to decrease the toxicity and repression of the fermentation products [22,23]. Solid adsorbents also have been found useful in the fermentation of benzaldehyde, which shifted the biotransformation pathways of L-phenylalanine into benzaldehyde and greatly enhanced benzaldehyde production [24,25]. Some biotransformations improved by in situ product recovery have been studied by different researchers [16,26-33].

In this study, production of isoamyl acetate by biotransformation of isoamyl alcohol using the yeast *Lindnera saturnus* was investigated with using ISPR by macroporous adsorption resins in a molasses-based medium. Batch and fed-batch biotransformations were carried out in order to determine the effect of substrate and product inhibition on microorganism during the production.

2. MATERIALS AND METHODS

2.1. Chemicals and Macroporous Resins

Isoamyl acetate of 99% purity and moleculer sieve were purchased from Sigma Chemical (St. Louis, MO, USA). Na_2SO_4 and absolute ethanol were supplied by Merck (Darmstadt, Germany). Sugarbeet molasses was supplied from Malatya Sugar Factory (Malatya, Turkey), while isoamyl alcohol 98.8% purity for the bioconversion was sourced from the distillation of fusel oil obtained from Mey Suma Factory (Tarsus, Turkey). The macroporous resins tested included H103, AB8, NKA2 and NKA9 were purchased from the Chemical Plant of Nankai University (Tianjin, China) with the exception of XAD4, XAD1180, XAD7HP, DAX8 and HP2MG, which were purchased from Sigma Chemical (St. Louis, MO, USA). Detailed properties of these resins were shown in Table 1. All other chemicals were of analytical grade and were commercially available.

2.2. Strain, Medium, Culture Conditions and Biotransformation

Lindnera saturnus HUT 7087 was obtained from HUT Culture Collection (Higashihiroshima, Japan). This strain was chosen in a previous study for the highest isoamyl acetate production [9]. Yeast was routinely maintained in slant medium containing 10 g/L glucose, 5 g/L peptone and 3 g/L yeast extract and re-cultured monthly.

Fusel oil was distilled by Vigreux column in order to obtain a pure isoamyl alcohol fraction (98.8%) as precursor for biotransformation. The water content of fusel oil was reduced by Na_2SO_4 and moleculer sieve before distillation [34].

Sugarbeet molasses was diluted with deionized water to 10° Brix and pretreated according to Yilmaztekin et al. [7] to separate heavy metal ions that could affect yeast growth. The molasses solution was then adjusted to pH 5.0 with NaOH and used as preculture and biotransformation medium.

The strain was inoculated into 50 mL of preculturing medium, and incubated at 25°C and 160 rpm for 48 h. Then a 3 mL cell suspension was transferred to 250 mL Erlenmeyer flasks containing 50 mL of biotransformation medium. After inoculation, 1 g of macroporous resins, without prior sterilization, were added to the submerged culture and incubated at 25°C and 100 rpm. In the batch biotransformation, 50 µL isoamyl alcohol was directly added to the medium after 48 h of biotransformation. In the fed-batch biotransformation, 50 µL isoamyl alcohol was directly added to the medium at 48, 72 and 96 h of biotransformation. All biotransformations were terminated at 120 h. Triplicate experiments were

performed under the same conditions. Concentrations of isoamyl acetate and isoamyl alcohol were analysed by gas chromatography (GC).

Table 1. Typical properties of macroporous adsorption resins

Resins	Polarity	Surface area ($m^2 g^{-1}$)	Average pore diameter (A°)	Particle size (mm)
XAD-1180N	Nonpolar	≥ 450	400	0.35-0.60
XAD-4	Nonpolar	≥ 750	55-80	1.20-6.80
H103	Nonpolar	1000-1100	85-95	0.30-1.25
DAX-8	Moderately polar	160	225	0.30-1.25
XAD-7HP	Moderately polar	≥ 380	90	0.56-0.71
AB-8	Moderately polar	480-520	130-140	0.30-1.25
HP2MG	Polar	500	200	0.30-1.25
NKA-2	Polar	160-200	145-155	0.30-1.25
NKA-9	Polar	250-290	155-165	0.30-1.25

2.3. Testing of Adsorption Capacity, Adsorption Ratio and Desorption Recovery

Nine macroporous adsorption resins with different polarities and surface areas were used. Before use, the resins were washed with distilled water and soaked in ethanol to remove impurities and then washed with distilled water sufficiently to remove the solvent. The affinity of isoamyl acetate and isoamyl alcohol for these adsorbents was measured as follows: 1 g of the hydrated macroporous resins and 50 mL of biotransformation medium containing 2.78 g/L isoamyl acetate and 1,98 g/L isoamyl alcohol were introduced into a 250 mL Erlenmeyer flask, and the flask was then shaken in a rotary shaker at 25°C and 100 rpm for 2 h. The isoamyl acetate and isoamyl alcohol concentrations in solution after adsorption were analysed by GC. The resins were collected and eluted with absolute ethanol in 250 mL Erlenmeyer flasks and shaken at 25°C and 100 rpm for 2 h. The desorption solutions were analysed by GC to determine the concentration of isoamyl acetate and isoamyl alcohol. The adsorption capacity, adsorption ratio and desorption recovery were calculated by equations 1, 2 and 3 as follows:

Adsorption capacity:

$$Qe = (C0\text{-}Ce)\frac{Vi}{W} \quad (1)$$

Adsorption ratio:

$$E\ (\%) = \frac{(C0\text{-}Ce)}{C0} x\ 100 \quad (2)$$

where *Qe* is the adsorption capacity (mg/g resin), which represents the mass of adsorbate on 1 g of resin at adsorption equilibrium; *E* is the adsorption ratio, which represents the percentage of total adsorbate at adsorption equilibrium; *C0* and *Ce* are the initial and equilibrium concentrations of isoamyl acetate and isoamyl alcohol in the solution, respectively (mg/L); *Vi* is the volume of the initial feed solution (mL) and *W* is the weight of the macroporous resins (g).

Desorption recovery:

$$D\ (\%) = \frac{Cd\ Vd}{(C0\text{-}Ce)\ Vi} x\ 100 \quad (3)$$

where *D* is the desorption recovery (%); *Cd* is the concentration of isoamyl acetate in the desorption solution (mg/L); *Vd* is the volume of the solution; *C0*, *Ce* and *Vi* are the same as defined as above.

2.4. Analytical Methods

After the cultivation period, the macroporous resins in the biotransformation culture were separated into a 250 mL Erlenmeyer flask by filtration, and were eluted with absolute ethanol (volume equal to the biotransformation medium) at

25°C and 100 rpm for 2 h to recover the isoamyl acetate. The eluant was analysed by GC to determine the isoamyl acetate concentration adsorbed on the resin. Isoamyl acetate in the aqueous phase was analysed by GC after yeast cells were removed by centrifugation. GC was carried out using DBWAX fused silica capillary column (0.25 mm i.d. x 30 m x 0.25 µm film thickness) (J&W Scientific, CA, USA), flame ionisation detection (180°C) and split/splitless injection (160°C) with a gas chromatograph, Agilent 7080 (Palo Alto, USA). GC settings were as follows: oven temperature: 4 min at 40°C, then increased by 1.8°C per minute up to 94°C and 40°C per minute up to 180°C and finally 4 min at 180°C; carrier gas: He (1.3 mL/min); split rate: 1:50. The quantification was performed by using internal standard (3-pentanol) method. The results given represent the means for three determinations each, with their standard deviations.

3. RESULTS AND DISCUSSION

3.1. Selection of Macroporous Adsorption Resins

The choice of the most suitable adsorption resin was based on two criteria: a strong ability to adsorb the product and in contrast a low ability to adsorb substrate [30]. Adsorption capacity is also one of the main factors influencing process cost. The higher the adsorption capacity, the smaller the amount of adsorbent required in order to keep product concentration under the toxicity level, and thus the smaller the necessary adsorption unit. Expressed in terms of cost, the higher the capacity, the lower the investment costs involved [20].

The adsorption capacity (*Qe*), adsorption ratio (*E*) and desorption recovery (*D*) of the resins tested for both isoamyl alcohol and isoamyl acetate were listed in Table 2. The affinities of the selected adsorption resins for isoamyl alcohol and isoamyl acetate were determined in a molasses based aqueous solution containing known amounts of isoamyl alcohol and isoamyl acetate. All adsorbents showed an acceptable affinity for both isoamyl alcohol and isoamyl acetate. The resins could be ordered, regarding to their isoamyl acetate *Qe* as follows:

H103 > NKA2 > XAD1180 > XAD4 > XAD7HP > DAX8 > AB8 > NKA9 > HP2MG. The *Qe* of resins H103, NKA2 and XAD-1180N were close and considerably higher than the other resins. Highest *Qe* is shown by H103, followed by NKA-2 with a slightly lower capacity. XAD-1180N has a quite smaller capacity. This could be explained based on the differences in the surface areas and polarities of the resins. Since the surface area of H103 is greater than those of XAD-1180N and NKA-2, hence H103 showed a better *Qe*. The selection of the proper resin should be in accordance with their polarities, surface areas and the features of the adsorbates. In the present work, H103 was chosen as the best resin for its high surface area and nonpolarity, which in nature have good affinity for the nonpolar İsoamyl acetate molecule. However, because of close *Qe* values of H103, NKA2 and XAD-1180N, we decided to test them also in biotransformation trials.

The recovery of the isoamyl acetate from the resins was conducted with absolute ethanol. Isoamyl acetate is used in food industry as a fragrant ingredient, therefore some solvent with toxic nature or non-acceptable odour such as methanol, acetone and butanol were not considered. Ethanol was chosen in desorption for its nontoxic and wide acceptance in perfumes and flavours. However, the results of the desorption tests carried out with a volume of ethanol equal to biotransformation medium for only one cycle were not satisfactory for all the resins having 12.9-29.6% desorption yields. The desorption recoveries of the resins followed the order:

NKA2 > H103 > XAD1180 > XAD4 > XAD7HP > HP2MG > DAX8 > AB8 > NKA9. NKA2, H103 and XAD1180 had the best desorption recoveries among the other resins. It is predicted that repeated cycles of desorption with ethanol would increase the recovery of the isoamyl acetate from the resins.

Table 2. Results of adsorption capacity (*Qe*), adsorption ratio (*E*) and desorption recovery (*D*) for isoamyl acetate (IAc) and isoamyl alcohol (IAl) with different macroporous resins

Resins	Q_e IAc (mg/g resin)	Q_e IAl (mg/g resin)	*E* IAc (%)	*E* IAl (%)	*D* IAc (%)
XAD-1180N	129.3 ± 1.5	29.4 ± 2.0	97.7 ± 1.6	30.9 ± 1.9	27.3 ± 1.5
XAD-4	127.4 ± 1.7	10.3 ± 1.4	95.6 ± 1.3	10.8 ± 1.4	24.3 ± 1.7
H103	132.2 ± 0.9	46.3 ± 1.7	99.1 ± 1.7	48.6 ± 1.5	27.3 ± 0.6
DAX-8	121.8 ± 2.1	14.9 ± 1.2	91.3 ± 1.2	15.7 ± 1.7	16.3 ± 1.1
XAD-7HP	125.5 ± 0.6	17.4 ± 0.8	94.1 ± 1.8	18.2 ± 1.6	24.3 ± 1.2
AB-8	121.3 ± 1.1	11.2 ± 1.0	91.0 ± 2.2	11.8 ± 1.3	16.0 ± 0.9
HP2MG	111.5 ± 1.9	10.1 ± 0.4	83.6 ± 0.9	10.6 ± 0.6	19.0 ± 0.8
NKA-2	131.5 ± 0.7	47.5 ± 0.9	98.6 ± 2.1	49.9 ± 0.9	29.6 ± 1.8
NKA-9	118.0 ± 1.3	5.4 ± 0.3	88.4 ± 0.7	5.7 ± 0.5	12.9 ± 0.6

3.2. Batch Biotransformation with Adsorption Resins

As shown in Fig. 1, with the addition of the macroporous resins to the biotransformation medium, the isoamyl acetate concentration exceeded that without the addition of resin. The process consists of a logarithmic growth phase which took 48 h, followed by biotransformation of added isoamyl alcohol during the stationary phase. The isoamyl alcohol was added in small amounts due to substrate toxicity and it was completely converted to isoamyl acetate after 120 h of biotransformation. The highest isoamyl acetate concentration reached was 1204 mg/L with resin H103, which was 42 times higher than the isoamyl acetate concentration without resin addition (28.5 mg/L). Surprisingly, the addition of NKA-2 resin did also improve the isoamyl acetate concentration, although it has a polar nature and small surface area. It was concluded that H103 resin was the best adsorbent in batch biotransformation.

3.3. Fed-Batch Biotransformation with Adsorption Resins

The concentrations of isoamyl acetate produced in fed-batch biotransformations carried out with different macroporous adsorption resins were given in Fig. 2. Isoamyl alcohol was added to the medium at 48. h of biotransformation and continued with 24 h intervals up to the end of biotransformation. The strategy for fed-batch biotransformation was to avoid the inhibition of isoamyl alcohol on the yeast, which was observed in our previous work [7]. Addition of small amounts of isoamyl alcohol at intervals of 24 h led to a 2.2 fold increase in isoamyl acetate concentration in fed-batch biotransformation (64 mg/L) compared to batch transformation (28.5 mg/L). This indicated that large amounts of isoamyl alcohol in the medium negatively affected yeast growth. Since the biotransformation of isoamyl alcohol to isoamyl acetate is strictly growth-associated, yeast growth could act as an indicator of isoamyl acetate production during the process [7]. In the experiments carried out with addition of resins, the total isoamyl acetate concentration (isoamyl acetate in aqueous phase plus adsorbed onto the resin) exceeded that without addition of resin. When the biotransformation was finished, the highest total isoamyl acetate concentration reached 1.9 g/L with H103 resin added biotransformation (123 mg/L in the aqueous phase and 1787 mg/L adsorbed onto the resin) which was 30 times higher than the control. To our knowledge, this is by far the highest isoamyl acetate production from isoamyl alcohol by biotransformation. About 1374 mg/L isoamyl alcohol was not converted and remained in the biotransformation medium carried out with H103 resin, which was the lowest amount of isoamyl alcohol detected in the medium after biotransformation among the other resins. It seemed that better results could be achieved in fed-batch biotransformation with macroporous adsorption resins than in batch biotransformation.

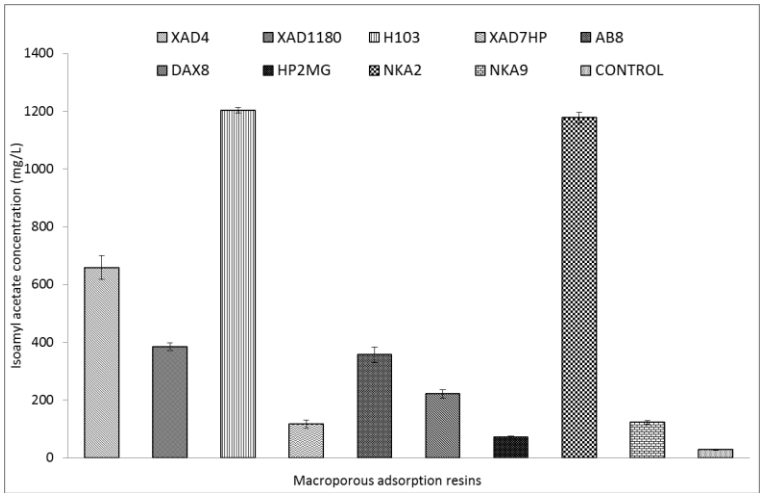

Figure 1. Isoamyl acetate production from isoamyl alcohol by *Lindnera saturnus* HUT 7087 in batch cultivation with the addition of nine types of macroporous resins to the biotransformation medium. The amount of macroporous resin was 1 g/50 mL. The isoamyl alcohol was added at 48th hour of biotransformation. Cultivation was carried out in an orbital shaker at 25°C and 100 rpm for 120 h. The control experiment was carried out without the adding of resin. Desorption was also carried out on a rotary shaker with 100 rpm at 25°C with absolute ethanol for 2h. The concentrations of isoamyl alcohol and isoamyl acetate were analysed by GC. The values were means of three replicates, and the error bars indicated standard deviations

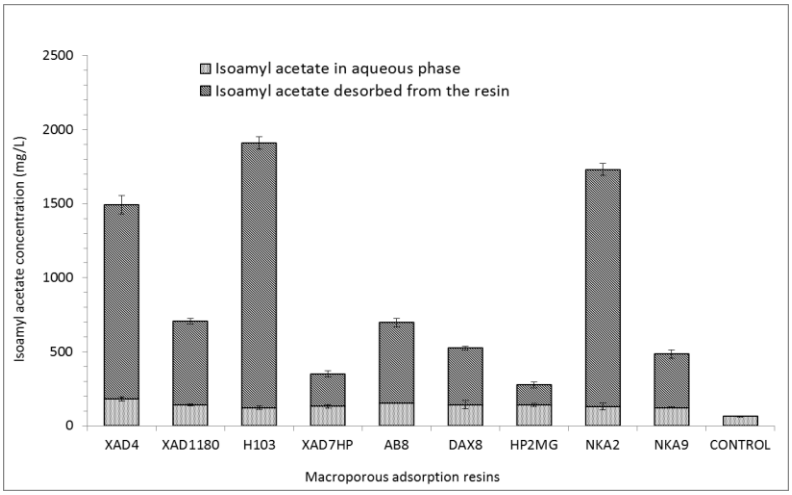

Figure 2. Isoamyl acetate production from isoamyl alcohol by *Lindnera saturnus* HUT 7087 in fed-batch cultivation with the addition of nine types of macroporous resins to the biotransformation medium. The amount of macroporous resin was 1 g/50 mL. The isoamyl alcohol was added at 48, 72 and 96th hour of biotransformation. Cultivation was carried out in an orbital shaker at 25°C and 100 rpm for 120 h. The control experiment was carried out without the adding of resin. Desorption was also carried out on a rotary shaker with 100 rpm at 25°C with absolute ethanol for 2h. The concentrations of isoamyl alcohol and isoamyl acetate were analysed by GC. The values were means of three replicates, and the error bars indicated standard deviations

4. CONCLUSION

In conclusion, critical strategies of improving biotransformation of isoamyl alcohol to isoamyl acetate are to limit the amount of added isoamyl alcohol below the inhibitory level with continuous addition at 24 h intervals and to use macroporous adsorption resin for ISPR. A fed-batch biotransformation with ISPR technique demonstrated very promising results for isoamyl acetate production by *Lindenera saturnus* in this work. By combining the fed-batch technique with product recovery, inhibition for both substrate and product was eliminated. 1.9 g/L isoamyl acetate concentrations obtained with the process presented in this paper have been never achieved before. However, according to us the isoamyl acetate productivity could be improved further by a continuous bioprocess using external adsorption columns for ISPR. Efforts are underway to build a continuous adsorptive bioprocess for isoamyl acetate production.

5. ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of The Scientific and Technological Research Council of Turkey (Project No. 111 O 369) (Ankara, Turkey) and Inonu University Scientific Research Center (Project Number 2012/177) (Malatya, Turkey), the HUT Culture Collection for providing yeast strain (Higashihiroshima, Japan), Malatya Sugar Factory (Malatya, Turkey) for molasses and Mey Suma Factory (Tarsus, Turkey) for fusel oil. The authors also would like to acknowledge the contribution of the COST Action FA0907 (BIOFLAVOUR).

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