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Production of (*S*)-2'-fluorophenylethan-1-ol and (*S*)-3'-fluorophenylethan-1-ol by *Alternaria alternata* using ram horn peptone in microbial growth medium

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Abstract: Enantiomerically pure, fluorinated compounds have an important role in medicinal chemistry. *Alternaria alternata* isolates were used for the synthesis of (S)-2'-fluorophenylethan-1-ol 1b and (S)-3'-fluorophenylethan-1-ol 2b. Biocatalytic syntheses of optically active 1b and 2b were achieved by asymmetric reduction of 2'-fluoroacetophenone 1a and 3'-fluoroacetophenone 2a in the batch culture of *A. alternata* containing ram horn peptone (RHP). The reaction conditions (pH, temperature, and agitation) that improve the conversion of the substrates were studied. The optimal reaction conditions were found as pH 6.0, agitation 250, temperature 32 °C and substrate 3 mmol/100 mL. The gram scale productions of 1b and 2b by the most effective biocatalyst *A. alternata* EBK-6 using RHP was carried out in a fermenter with 1-L working volume. The results showed that the yields with >99% enantiomeric excess (ee) of both 1b and 2b reached 75% for 1b and 66% for 2b. The concentrations of products 1b and 2b at the end of fermentation were 3.1 g/L and 2.8 g/L, respectively. As a result, 2 important chiral intermediates for the pharmaceutical industry using *A. alternata* EBK-6 in the submerged culture containing RHP from waste material were scaled up to gram scale with excellent ee.

Key words: *Alternaria alternata*, bioconversion, fluoroacetophenone, fermentation, ram horn, optimization

Mikrobiyal üreme ortamında koç boynuz peptonu kullanılarak *Alternaria alternata* tarafından (*S*)-2'-florofeniletan-1-ol ve (*S*)-3'-florofeniletan-1-ol üretimi

Özet: Enantiyomerik olarak saf florlu bileşikler tıbbi kimyada önemli bir role sahiptir. *Alternaria alternata* izolatları (S)-2'-florofeniletan-1-ol 1b ve (S)-3'-florofeniletan-1-ol 2b'nin sentezinde kullanılmıştır. Optik olarak aktif 1b ve 2b'nin biyokatalitik sentezleri koç boynuz peptonu (KBP) ihtiva eden *A. alternata*'nın sıvı kültüründe 2'-floroasetopepton 1a ve 3'-floroasetopepton 2a'nın asimetrik indirgenmesiyle başarılmıştır. Substratların dönüşümünü artırmak için pH, sıcaklık ve çalkalama gibi reaksiyon şartları çalışılmıştır. Optimal reaksiyon şartları pH 6,0, çalkalama 250, sıcaklık 32 °C ve substrat 3 mmol/100 mL olarak bulundu. KBP kullanılarak en etkili biyokatalist *A. alternata* EBK-6 tarafından 1b ve 2b'nin gram miktarda üretimleri 1 L çalışma hacmine sahip fermentörde gerçekleştirilmiştir. Sonuçlar hem 1b ve hem de 2b'nin % 99 enantiyomerik selektivite (es) ile verimleri 1b için % 75 ve 2b için % 66'ya ulaştığını göstermiştir. Fermentasyon sonunda 1b ve 2b ürünlerinin konsantrasyonları sırasıyla 3,1 g/L ve 2,8 g/L olarak bulunmuştur. Sonuç olarak, atık materyalden üretilen KBP ihtiva eden sıvı kültürde *A. alternata* EBK-6 kullanımıyla ilaç endüstrisi için iki önemli kiral bileşik mükemmel es ile gram miktara kadar üretilmiştir.

Anahtar sözcükler: *Alternaria alternata*, biyodönüşüm, floroasetopepton, fermentasyon, koç boynuzu, optimizasyon

Introduction

Optically active fluorinated chiral alcohols play a key role in medicinal chemistry. There is currently considerable interest in asymmetric syntheses of fluorinated organics, particularly for potential use in pharmaceuticals. These alcohols are versatile intermediates for the synthesis of anti-ferroelectric liquid crystalline molecules (1,2). Recently, we reported the production and gram scale production of acetophenone and its derivatives alcohols as enantioselective reducing agents (3). Some methods on how to make various kinds of fluorinated chiral alcohols have been reported, but we found this to be very difficult to achieve with high enantiomeric excess (*ee*). Enantioselective reductions of fluorinated compounds often proceed with relatively low selectivities (compared to other acetophenone derivatives) (4). Nevertheless, some high selectivities have been reported, i.e. using a chemical reagent 2'-fluoroacetophenone 1a was reduced to the corresponding alcohol in 93% *ee* (5). Using a Tailor-made recombinant whole cell as catalyst, 4-fluoroacetophenone was reduced with high *ee* (>99%) (6). Using resting cells of *Candida tropicalis*, 1a and 2a could be reduced with 97% *ee* (7). Moreover, the literature (8) indicated that the most efficient reduction methods were enzymatic with >99% *ee*.

The culture medium must supply all the essential elements for microbial growth. Certain microorganisms are capable of biosynthesizing all of their cellular constituents from glucose and ammonium sulfate. However, most industrial microorganisms require some source of micronutrients, such as amino acids, trace elements, vitamins, and nucleic acids. In addition, fermentation medium represents almost 30% of the cost for a microbial fermentation, with micronutrients representing the most significant cost of production. Byproducts can supply unique micronutrients to replace expensive peptone and yeast extract. A significant increase in product yield or cost reduction is critical for industrial fermentation utilization of any byproduct (9). In previous studies, we demonstrated that ram horn peptone (RHP) could be obtained from slaughterhouse waste. This peptone is excellent for microbial growth. The important of RHP in microbial media was discussed in our previous studies (10-15). Herein, the performance of *A. alternata* strains using RHP in the batch culture was evaluated for asymmetric reductions of 2'-fluoroacetophenone 1a to (S)-2'-fluorophenylethan-1-ol 1b and of 3'-fluoroacetophenone 2a to (S)-3'-fluorophenylethan-1-ol 2b. We also describe a preparative scale example for the production of 1b and 2b.

Materials and methods

Microorganisms and chemicals

A. alternata strains isolated during our previous study from plant samples, such as sunflower (Hasankale), grapefruit (from a market) and apple (Tortum), were collected around Erzurum, Turkey (10). In this study, 2'-fluoroacetophenone 1a and 3'-fluoroacetophenone 2a were bought from Acros Organics (New Jersey, USA). Reproduction of RHP was carried out with the method described by Kurbanoglu and Kurbanoglu (11). The racemic 2'-fluorophenylethan-1-ol and 3'-fluorophenylethan-1-ol were produced through reductions of 1a and 2a catalyzed with sodium borohydride. The other components of the culture media and the chemical reagents were obtained from Merck and Sigma in the highest purity available.

Cultivation of fungi cells and inoculation

A. alternata strains were maintained at 4 °C on PDA slants. The cultures were transferred to new media at bimonthly intervals. These strains were pre-cultured on PDA medium for 10 days at 28 °C. The conidia from 10-day-old cultures were used for inoculation. The conidial suspension was prepared in 10 mL of sterilized physiological saline by gently scratching conidia with a sterile wire loop and then it was shaken vigorously for breaking up the clumps of conidia. For the next steps, 10 or 1 mL of inoculation was used (15).

Culture medium and reduction reactions

The per liter fermentation medium contained (g/L): glucose 30, yeast extract 3, and ram horn peptone 5. This medium was termed GYRHP. The initial pHs of the culture media were adjusted to the appropriate level with 1 N HCl and 1 N NaOH and sterilized at 121 °C for 15 min. The experiments were carried out in 250-mL Erlenmeyer flasks containing

100 mL of GYRHP. One milliliter of conidial suspension counted microscopically (3.0×10^9) was added to each flask. The flasks were incubated on a reciprocal shaker at 150 rpm and 25 °C for 48 h. After the growth of the fungus, 2'-fluoroacetophenone 1a (1 or 3 mmol) was directly added to each culture. Then, incubation of the flasks continued on a reciprocal shaker at 150 rpm, 25 °C for 48 h.

Preparative scale studies

Preparative scale studies in our lab were conducted in a 2-L fermenter (Biostat-M 880072/3, Germany) with a working volume of 1 L. Ten milliliters of the spore suspension was inoculated into the fermenter containing 1 L of sterile GYRHP. To prevent foam formation, sterilized silicone oil (0.001%, w/v) was added twice, at the beginning and after 24 h of fermentation. After 48 h of incubation, 1a and 2a (30 mmol) were directly added to the fermentation culture. Agitation, pH, aeration (0.4 vvm), and temperature were automatically controlled during the fermentation. The reaction time (6-92 h) was optimized for the reduction of substrates (1a and 2a) to products (1b and 2b) in a submerged system. At regular intervals (6 h) during fermentation, the conversion and the *ee* were determined and the yields were calculated (Figure) (10).

Purification of products and analytical methods

After reduction, the mycelium was separated by filtration, and the filtrate was saturated with sodium chloride and then extracted with ethyl acetate. The mycelia were also extracted with ethyl acetate. The ethyl acetate extracts were combined; the ethyl acetate was dried with $MgSO_4$ and evaporated. For analysis, a small fraction of the product was separated by preparative silica-gel TLC. The *ee* of the product was determined by HPLC with an OB column using eluent n-hexane-i-PrOH, 90:10, flow rate of 0.6 mL/min, detection performed at 220 nm. The crude product was purified by silica gel column chromatography. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian 400 spectrometer in $CDCl_3$. Purified 1b and 2b were identified by spectral data (^{1}H- and ^{13}C-NMR). The purities of (S)-1b and 2b produced via fermenter were also checked with HPLC analysis. The absolute configurations of the compounds were determined by comparing the sign of its specific rotation with that in the literature. The conversion for flask cultures was determined by ^{1}H-NMR analysis with diphenylmethane as internal standard; error ca. ±5% of the stated values (15).

Results and discussion

There is currently considerable interest in asymmetric syntheses of fluorinated organics, particularly for potential use in pharmaceuticals. In addition, asymmetric reductions of the fluorinated acetophenone 1a and 2a have received relatively little attention (compared to other carbonyl compounds) (1-5). Garrett et al. (5) developed a non-enzymatic catalytic process for the enantioselective reduction of 1a with 97.8% (R) isomer *ee*. Biocatalytic asymmetric synthesis is an important research field in organic chemistry due to its high *ee*, mild reaction conditions, and environmental friendliness. Therefore, we designed the (S)-enantiomeric reduction of the fluorinated acetophenones 1a and 2a using a biocatalyst. Ten different strains of *A. alternata* isolated for the asymmetric reduction of acetophenone in a previous study were screened. As a result of screening fungi, *A. alternata* EBK-4 strain was found to be the most efficient to produce (*S*)-1-phenylethanol with a high optical purity of >99% (S) isomer *ee* and yield of 86% (10,15). In this study, *A. alternata* strains were screened for the reduction of 1a to 1b in flask cultures. Shake-flask cultures are very useful in screening many media for target product formation in relatively short periods (9). Firstly, the reduction of 1a to 1b was performed in the flask culture in order to find the optimum fermentation conditions and the most effected biocatalyst, and we initially based this on the asymmetric reduction of 1a to 1b. Secondly, the preparative scale production of both 1b and 2b under the optimum conditions found was performed in the fermenter. *A. alternata* isolates were used for the reduction of 1a to 1b in a medium containing ram horn peptone (RHP), yeast extract and glucose. These results are shown in Table 1. We screened the fungi strains for the reductions of 1a. All strains produced optically active 1b from 1a. However, it is noteworthy that EBK-2,-3,-4,-5,-6,-8, and-10 strains gave high selectivity (>99%). We compared the quantities of (*S*)-1b from each strain. The maximum conversion (100%) was obtained from EBK-7, but the

Table 1. Screening of *A. alternata* isolates for the asymmetric reduction of 1a.

Isolates	Conversions (%)	*ee* (%)-Config.
EBK-1	44	92-*S*
EBK-2	51	99-*S*
EBK-3	34	99-*S*
EBK-4	75	99-*S*
EBK-5	85	99-*S*
EBK-6	94	99-*S*
EBK-7	100	78-*S*
EBK-8	88	99-*S*
EBK-9	65	86-*S*
EBK-10	54	99-*S*

Reaction conditions: substrate 1 mmol, temperature 25 °C, reaction time 48 h, pH 7, agitation 150 rpm

product's *ee* (78%) was rather low. The best strain, *A. alternata* EBK-6, produced (*S*)-1b with >99% *ee* and 94% conversion from 1a. We have previously reported that the practical applications of biocatalysts for the manufacturing of (S)-1-phenylethanol using *A. alternata* EBK-4 strain.

In a previous report, the best isolate for the reduction of acetophenone to (S)-1-phenylethanol was *A. alternata* EBK-4. Moreover, 99% *ee* was not achieved under the same reaction conditions (10). The results showed that the conversion and optical purity are strongly dependent on both the type of ketone used and the different strains of the same species. On the basis of these results, we recommend a careful control of the strain used as biocatalyst for optical purity in the asymmetric reduction. However, the good conversions and *ee* allow their use in future scaling up processes. It has not been considered suitable for large-scale production or industrial application due to the low concentration of substrate in the reaction (16). *A. alternata* EBK-6, due to both its high *ee* and high conversion, was selected for further studies. Various reaction parameters such as pH, agitation, and temperature were examined in order to establish the best conditions for the high conversion with 99% *ee*. The aim was to achieve a high conversion with high optical purity. In this way, we added 3 mmol 1a instead of 1 mmol 1a to submerged culture of *A. alternata* EBK-6.

Table 2 shows the effect of pH on the product's *ee* and conversion of 1a. The microbial production can be performed on a large scale, so that it can be applied in an industrial process using an engineered microorganism and designing the reaction conditions (17,18). Therefore, we decided to determine the performance of microorganisms with optimum reaction conditions. The result showed that the *ee* and conversion of the reaction will depend on a number of parameters. The organism showed a positive effect for 4 different pH values (4-7). The highest conversion (50%) with >99% *ee* was obtained from pH 6.0. This pH indicated that the bioactivity of the EBK-6 isolate was greater for the reduction of 1a. All further studies were carried out at pH 6.0 only. These results were found to be promising for further scale-up.

The effect of reaction temperature on the reduction of 1a is shown in Table 3. The reaction temperature was varied (28-40 °C). The optimum temperature was around 32 °C while the reaction activity decreased rapidly above 36 °C. The enantioselectivity with an increase in reaction temperature increased and then decreased. The results show that the biocatalyst is inactive at high temperature, and the conversion of 1a at 38 and 40 °C is 32 and 16%, respectively. Similarly, high temperature had a negative effect on the product's *ee* as well. The *ee* fell to 75 or 59% from >99%. Both the conversion and *ee* at other temperatures (28-36 °C) remained almost the same. It was found that temperature variation considerably influenced both the conversion and *ee*. The suitable temperature for the reduction of 1a was about 32 °C due to the fairly high conversion (67%). Hence, all the subsequent

Table 2. Effects of different pHs on the reduction of 1a by *A. alternata* EBK-6.

pH	Conversions (%)	*ee* (%)-Config.
3	Weak growth	-
4	26	>74-*S*
5	37	>99-*S*
6	50	>99-*S*
7	31	>99-*S*
8	Weak growth	-

Reaction conditions: substrate 3 mmol, temperature 25 °C, reaction time 48 h, agitation 150 rpm

reduction experiments were carried out at 32 °C. The reduction experiments were performed at different agitations.

The effect of agitation on the reaction rates of the biocatalyst is presented in Table 4. The results showed that the conversion of 1a was greatly affected by all agitation rates. The enantioselectivities of the product obtained in this study were also affected by the change in agitation rate. The *ee* values for the reaction were >99% in the agitation range of 100-300 rpm. However, at the highest agitation rate, both the conversion and *ee* were significantly decreased. This decrease is due to the effect of shear stress on fungus cells as well as on the enzyme structure. A significant increase in the conversion of 1a was observed at 250 rpm (87%) as compared to other agitation rates. Therefore, this rate was used for subsequent studies.

The effect of incubation time on the conversion and enantioselectivity for preparative scale production was investigated in this study. The conversion of 1a continued in incubation until it reached its maximum value. These results are given in Table 5. The combination of optimum fermentation conditions reached the highest values up to 100% conversion with maximum enantioselectivity (>99%). The conversion of 1a was completed after 72 h incubation. The optimum incubation time was 72 h after addition of 1a. The *ee* (>99%) for all fermentation times did not vary. The conversion could be increased by increasing incubation time. This is a satisfactory result. It was found that there was no inhibitor effect on organism activity of the product from substrate up to 3 mmol/100 mL concentration. In an attempt to increase the substrate concentration, we carried out the reaction with 4 mmol/100 mL substrate, but after a reaction time of 86 or 98 h the desired conversion was not achieved.

Although there was an increase in substrate concentration, the enantioselectivity of the product remained the same throughout the reaction (data not shown). Pfruender et al. (19) reported that whole cell biocatalysis can effectively be used for the production of enantiomerically pure compounds, but efficiency is often low. Toxicity and poor solubility of substrates and products are the main obstacles. A reason for the lack of increase in conversion may be product inhibition on the cells as a result of increased substrate

Table 3. Effects of different temperatures on the reduction of 1a by *A. alternata* EBK-6.

Temperatures (°C)	Conversions (%)	*ee* (%)-Config.
28	54	>99-*S*
30	62	>99-*S*
32	67	>99-*S*
34	60	>99-*S*
36	57	>99-*S*
38	32	75-*S*
40	16	59-*S*

Reaction conditions: substrate 3 mmol, pH 6.0, time 48 h, agitation 150 rpm

Table 4. Effects of different agitations on the reduction of 1a by *A. alternata* EBK-6.

Agitation (rpm)	Conversions (%)	*ee* (%)-Config.
100	30	>99-*S*
150	67	>99-*S*
200	78	>99-*S*
250	87	>99-*S*
300	60	>99-*S*
350	40	65-*S*

Reaction conditions: substrate 3 mmol, pH 6.0, time 48 h, temperature 32 °C

Table 5. Effect of incubation time on the reduction of 1a by *A. alternata* EBK-6.

Time (h)	Conversions (%)	*ee* (%)-Config.
24	56	>99-*S*
48	87	>99-*S*
60	93	>99-*S*
72	100	>99-*S*
86	100	>99-*S*
98	100	>99-*S*

Reaction conditions: substrate 3 mmol, pH 6.0, temperature 32 °C, agitation 250 rpm

concentration. Therefore, the optimum substrate concentration for this bioprocess was determined to be 3 mmol/100 mL. Under optimum reaction conditions (pH 6.0, agitation 250, temperature 32 °C and substrate 3 mmol/100 mL), the production of 1b and 2b from 1a and 2a substrates in preparative scale was carried out using a fermenter.

The bioreductions of 1a and 2a for the gram scale production of 1b and 2b using *A. alternata* EBK-6 are shown in the Figure. The reactions were carried out on preparative scale at 30 mmol/L substrate concentrations (1a and 2a) with 1-L working volume in a 2-L fermenter. The *ee* values for both 1b and 2b products at all reaction times were >99%. These high *ee* values are satisfactory results in organic synthesis. A popular method of obtaining enantiomerically pure fluoro-substituted secondary alcohols is enantioselective reduction of the corresponding ketones (6). Therefore, it is noteworthy that both substrates, 1a and 2a, gave high selectivities. After 66

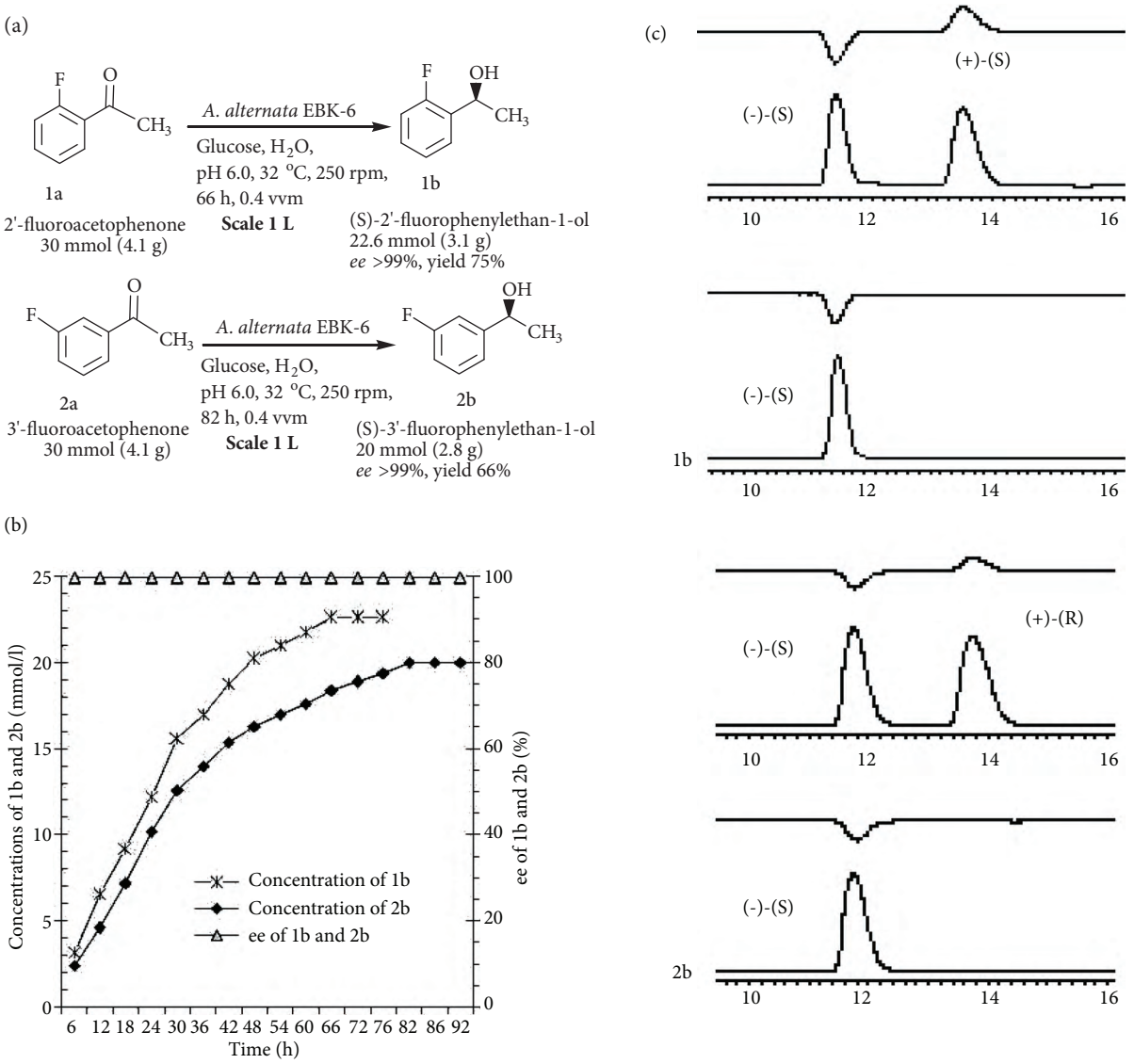

Figure. Scheme of the asymmetric reduction of 1a and 2a (a). Production in the preparative scale of 1b and 2b via fermenter by *A. alternata* EBK-6 (b). Chiral analyses of the 1b and 2b products by HPLC (c). Conversion and yield values after maximum incubation times. Yield (%) = 100 × b/a, Conversion (%) = b/b + a × 100, a and b are the concentrations of initial substrate and product, respectively.

h reaction time, the yield of 1b (75%) reached a stable value with 100% conversion, whereas the bioreduction of 2a for the same reaction time continued. The conversion of 2a compared to 1a was complete after a longer period of time. The highest yield (66%) of 2b was achieved after 82 h with complete conversion. We can see that the process is more sensitive to the fluoro in the *ortho* position of the aromatic ring of the ketones. When the reaction time was changed from 36 h to 42 h, the conversions of 1a and 2a substrates were obviously decreased, although the suitable reaction conditions were automatically controlled. Further extension of the reaction time would lead to a serious decrease in substrate conversion. This clearly demonstrates that the conversion decrease may be caused by product inhibition in the fermenter.

Accordingly, the decline in conversion must be due to inhibition of the enzyme responsible for the bioreduction by the product concentration formed in fermentation culture (20). On the other hand, a slight decrease in fermentation time in the reduction of 1a to 1b was observed in the fermenter as compared to reduction at shake flask level. The reaction time was reduced from 72 to 66 h. All parameters optimized at flask level were used in fermenter studies. In addition, air was supplied at 0.4 vvm rate to fungus culture. This time decrease could be due to oxygen, uniform distribution of medium components, and automatic control of the culture conditions. As a result, the concentrations of products 1b and 2b at the end of fermentation were 3.1 g/L and 2.8 g/L, respectively. The synthesis of (R)-4-fluorophenylethan-1-ol as reported by Groger et al. (6) is based on a reduction of the corresponding 4-fluoroacetophenone in the presence of a tailor-made whole-cell biocatalyst, containing an alcohol dehydrogenase and a glucose dehydrogenase. We applied the submerged culture of *A. alternata* isolate using RHP from waste material in order to make (*S*) *ortho* and *meta* chiral fluorinated aryl alcohols in good yields and in >99% *ee*. An important aspect of the microbial process is the development of a suitable culture medium to obtain the desired product with a low-cost substrate. For example, sugar cane molasses, beet molasses, cheese whey, plant origin liquid waste, and fish protein hydrolysate have been used as carbohydrate and protein sources for microbial fermentation (21-24). The utilization of a waste material with optimization of culture conditions in organic synthesis via a microbial process has been demonstrated to be an efficient strategy.

In this development an optimized process for commercial production of microbial products, selecting a suitable low-cost culture medium and establishing the most favorable fermentation conditions are the 2 most important components (25). We have found an efficient process to prepare *ortho* and *meta* fluorinated aryl alcohols in good yield and in >99% enantiomeric excess using *A. alternata* fungus under optimized fermentation conditions with RHP from waste material. This study has a positive economic impact due to the utilization as substrate for microbial growth of a waste material instead of commercial nitrogen and mineral sources.

Conclusion

Gram scale production of both (*S*)-2'-fluorophenylethan-1-ol and (*S*)-3'-fluorophenylethan -1-ol was shown for the first time to occur by a batch fermentation process when *A. alternata* biocatalyst was used. In this work, (*S*)-1b and (*S*)-2b chiral alcohols were successfully produced on a gram scale. The bioreduction protocol is demonstrated to be applicable to the production of some enantiomerically pure alcohols. *Alternaria alternata* was used for the first time as a biocatalyst for efficient production of 1b and 2b.

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