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Full Length Research Paper

Microbial biocatalytic preparation of 2-furoic acid by oxidation of 2-furfuryl alcohol and 2-furanaldehyde with *Nocardia corallina*

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The preparation of 2-furoic acid via oxidation of 2-furfuryl alcohol and 2-furanaldehyde was studied by two procedures using whole cells of *Nocardia corallina* B-276. We describe a comparison using a batch cultivation (3-L) versus resting cells in potassium phosphate buffer (0.1 M, pH 7.0). In the 3-L bioreactor, 2-furfuryl alcohol (1.35 g/L) was biotransformed after 24 h, to the acid with 81% yield. The bioconversion of 2-furfuryl alcohol using resting cells leads to and almost quantitative yield (98%) of 2-furoic acid in 21 h. 2-furanaldehyde was also oxidized to the 2-furoic acid in 88% yield in 8 h, the ratio substrate:cells was 1:3.5 (w/w). This method was scaled up to 9 g/L of 2-furanaldehyde.

Key words: Biocatalyst, 2-furoic acid, 2-furanaldehyde, 2-furfuryl alcohol, *Nocardia corallina*, oxidation.

INTRODUCTION

Furan compounds are commonly found in plants, the most widespread simple furan in environment is furfural, 2-furanaldehyde (Belay et al., 1997). Since furfural is produced from lignocelluloses by treatment with strong acids such as sulfuric acid, then wastewaters from the pulp and paper mill industries (Brune et al., 1982) and oat and corn-processing industries contain furfural as one of the waste products (Boopathy et al., 1993).

There is an interest in the enzymatic synthesis of carboxylic acids from natural, renewable aldehydes, such as furfural (Villa et al., 1996) or vainillin (Tanaka and Hirokane, 2000). For example 2-furoic acid can be prepared from the corresponding aldehyde or alcohol by chemical oxidation, but there are problems to be solved with respect to waste treatment or productivity in this methodology. Enzymatic process could be an alternative in the syntheses of carboxylic acids from natural renewable aldehydes, such as furfural. Strains of *Acetobacter* (Füchtenbusch et al., 1998; Wandel et al., 2001; Molinari et al., 2003; Mitsukura et al., 2004), *Burkholderia* (Tanaka and Hirokane, 2000), *Gluconobacter* (Molinari et al., 1999), *Nocardia* (Pérez et al., 1999), *Pseudomona* (Villa et al., 1996) and *Serratia* (Mitsukura et al., 2004) have been reported to oxidize alcohols and aldehydes.

Our previous work with *Nocardia corallina* in culture media (Luna et al., 1997) was resumed to study their potential application in resting cells and a bioreactor of 3-L with cells in the culture media; the final aim of our ongoing study is to produce 2-furoic acid from the corresponding alcohol or aldehyde with a high yield and the highest concentration possible with this microorganism. In view of the fact that the furfural and 5-hydroxymethylfurfural produced during acid hydrolysis of lignocellulosic material are potent inhibitors of the microorganisms that produce bioethanol (Liu et al., 2004, 2005), it is important to consider this problem since the oxidations we are going to study are biocatalyzed by a microorganism (Villa et al., 1996; Taherzadeh et al., 2000).

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MATERIALS AND METHODS

2-Furfuryl alcohol, and 2-furanaldehyde were purchased from Al-

drich and were analyzed by their infrared spectra, recorded on a Perkin-Elmer Paragon 1600 FT, as liquid films; hydrogen and carbon nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR), were recorded on a varian 400 MHz instrument, in $CDCl_3$ using tetramethylsilane as internal reference and by TLC on silica gel 60 GF_{254} (Merck). HPLC analysis was performed on an Agilent 1100 liquid chromatograph, equipped with a diode array detector, using a Hypersil BDS-C18 (5 µm, 250 x 4.6 mm) column, the mobile phase was methanol-water (90:10), 0.7 mLmin^{-1}, detected at 230 nm, at 24°C. The GC analysis was performed on a Hewlett-Packard HP 6890 gas chromatograph, equipped with a flame ionization detector, a HP-5 column (30 m x 0.33 mm), at 100°C, N_2 as carrier gas, at 0.6 mLmin^{-1}.

Growth experiments and biotransformation with *N. corallina* were performed in a stirred 3-L bioreactor (2.2 L working volume) equipped with an ADI 1030 Bio Controller (Applikon, Schiedam and The Netherlands), at atmospheric pressure and at 226 rpm, with an aeration rate of 0.9 vvm (air only). The pH and oxygen percentage of fermentor were monitored continuously, samples were withdrawn every hour and the optical density (at 660 nm) and the dry cell weight (gL^{-1}) were measured.

Organism and growth

N. corallina B-276 (ATCC 31338) was grown at 28 - 30°C on agar plates (15 g agar L^{-1}, 3 g beef extract L^{-1} and 5 g peptone L^{-1}).

Liquid cultures were incubated in an orbital shaker, the broth composition was Solution A. 0.05 g $FeSO_4 \cdot 7H_2O$ L^{-1}, 1.74 g K_2HPO_4 L^{-1} , 2 g $(NH_4)_2SO_4$ L^{-1} and 1 g yeast extract L^{-1}; Solution B. 1.5 g $MgSO_4$ L^{-1}, Solution C. 2 g glucose L^{-1}. Each solution was sterilized.

Procedure A: Biotransformation using bioreactor 3-L

The cells of *N. corallina* B-276 (ATCC 31338) were grown as indicated. Preculture I. A 500 mL Erlenmeyer flask containing sterile culture mediun (200 mL) was inoculated from an agar plate (three days old) at 28 - 30°C on an orbital shaker 170 rpm for 40 - 44 h (duplicated). The content of both pre-culture I flasks (400 mL) was aseptically poured into a 3-L bioreactor (Pre-culture II) containing 1800 mL culture medium, at 28 - 30°C and 0.9 vvm (air only) at 126, 226 and 300 rpm. Cellular growth was monitored every hour by optical density determination (660 nm). After 6 - 8 h, 1.35 g/L of furfuryl alcohol in 5 mL of N,N-dimethylformamide and 80 mL of n-octane was added, and incubated in the same conditions for 24 h. The reaction mixture was acidified to pH 1 with 0.5 M HCl, then saturated with NaCl and centrifuged at 4500 rpm for 15 min and the 2-furoic acid was extracted with ethyl acetate (4 X 200 mL).

Procedure B: Biotransformation using resting cells

The cells of *N. corallina* B-276 (ATCC 31338) were grown as indicated above. Pre-culture a 500 mL Erlenmeyer flask containing 200 mL of sterile culture. A medium was inoculated from an agar plate (three days old) and incubated at 28 - 30°C on an orbital shaker (170 rpm) for 48 h. The cells were collected by centrifugation at 4500 rpm for 15 min. Cells were washed twice with potassium phosphate buffer (0.1 mol L^{-1}, pH 7.0).

The cells were incubated in 50 mL phosphates buffer, 0.1 mol L^{-1}, pH 7.0, for 30 min at 28 - 30°C on an orbital shaker (170 rpm), then the substrates were added to the whole cells, with a substrate:cells ratio (w/w) 1: 3.5, using 0.6% (v/v) of N,N-dimethylformamide, and shaked in the same conditions for 8 - 21 h, depending of the substrate (furfural or furfuryl alcohol). After this time, the reaction mixture was centrifuged at 4500 rpm/15 min. The reaction mixture was acidified to pH 1 with 0.5 M HCl, and then saturated with NaCl, the 2-furoic acid was extracted with ethyl acetate (4 x 15 mL).

In both methods the residue was dissolved in 0.5 mL of MeOH, HPLC grade, and filtered (millipore filter 22 mµ). Samples were analyzed by HPLC, using a C-18 column, detected at 230 nm, 0.7 mLmin^{-1} and MeOH:H_2O (90:10) as the eluent to determine the conversion degree of furfuryl alcohol and furfural to the carboxylic acid; t = 4.11 min, t_r = 4.32 min. and t_r = 3.82 min. To determine the conversion degree of furfuryl alcohol and furfural to the carboxylic acid by GC; t_r = 1.96 min, t_r = 1.86 min. and $t_{r(respectively)}$ = 4.21 min.

The product was identified by IR (film) ν: 2925, 1685, 1472 and 1302 cm^{-1} respectively and RMN ^{1}H: δ= 7.65 (dd, J=0.87 Hz, J=1.75 Hz, 1H), 7.34 (dd, J=0.87 Hz, J=3.6 Hz, 1H), 6.57 ppm (dd, J=1.75 Hz, J=3.6 Hz, 1H) y RMN ^{13}C: δ=162.98, 147.08, 143.38, 119.83 y 112.00 ppm, melting point, 125 - 127°C and thin layer chromatography against an authentic sample.

RESULTS AND DISCUSSION

The main product of this microbial conversion of furfural or 2-furfuryl alcohol is the 2-furoic acid. This is very interesting because other species of microorganism induce two products in this biotransformation, the acid and the alcohol by an oxidation and a reduction promoted by the enzymatic system, like *Neuroespora ascopores* (Eilers and Sussman, 1970). The destruction of the aromatic ring is also absent in our conversion, in contrast some other microorganisms like *Pseudomonas putida* catalyzes the conversion of furan-2-carboxylate to 2-oxo-glutarate (Kitcher and Trudgill, 1970), and *Methanococcus deltae* degrades the furfural (Belay et al., 1997). So, the analysis of the conditions of this kind of biotransformation is very important to avoid the aforemention problems.

Biotransformation using 3-L bioreactor

Aeration and agitation in aerobic fermentation process are very important, because it is known that they can create shear forces, causing morphological changes, variation in their growth and damage to the cell structure, also changes in the product formation (Kim et al., 2003). Then the measurement of OTR (oxygen transfer rate from the gas phase into the broth, mol m^{-3} h^{-1}), K_{La} (volumetric mass transfer coefficient for O_2, h^{-1}) and vvm (aeration rate), can be correlated with the microbial growth.

In this study the vvm was kept constant at 0.9, the agitation rates were 126, 226 and 300 rpm, the values of OTR and K_{La} are shown in Figure 1. The lowest OTR and K_{La} were at 126 rpm, and the higher values occurred at 226. Then a K_La, and OTR of 78.84 h^{-1} and 14.78 mol m^{-3} h^{-1} were required to avoid oxygen limitation.

When we studied the oxidation of 2-furfuryl alcohol under the mentioned bioreactor conditions, it was observed that the biotransformation was greatly influenced by the agitation rate. After 24 h of reaction at 126 and 226 rpm, at 1.35 g/l of substrate, the isolated yields of 2- furoic acid, (Figure 2) were 76 and 81 % res-

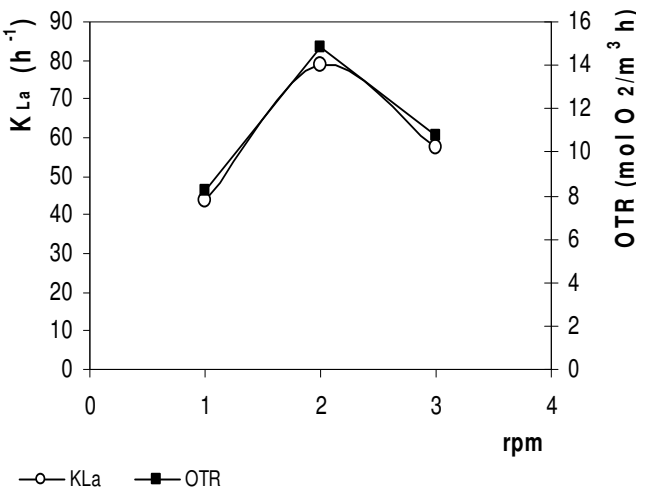

Figure 1. Volumetric mass transfer coefficient for O_2, h^{-1}, and OTR, mol m^{-3} h^{-1}, at different agitation rates (1, 2 and 3 on the x-axis represents 126, 226 and 300 rpm, respectively).

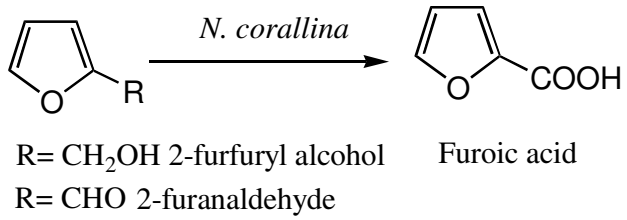

Figure 2. Biotransformation by *Nocardia corallina*.

pectively, but at 300 rpm the yield dropped to 23% (Figure 3). These results are in agreement with those found by Techapun et al. (2003). We also observed morphological changes in the manner *N. corallina* growth at 300 rpm, because at that speed pellets were formed, and at lower speed the microorganism was completely dispersed in the media.

In previous experiments we found that the logarithmic growth under these conditions, 0.9 vvm and 226 rpm, was around 5 h (Manjarrez et al., 2007).

Biotransformation using resting cells

Another interesting way to carry out the oxidation is using the resting cells, with the advantages of avoiding dilution, employment of higher concentrations of the biocatalyst and the control of pH is easier. Then the oxidation using resting cells in a ratio of 1:3.5 (w/w) and 3 g/L of substrate, leads to an almost quantitative conversion of 2-furoic acid from furfuryl alcohol in 21 h (98%), and 88% of the same acid from furfural in 8 h.

Next, we tried to find out the maximum substrate con-

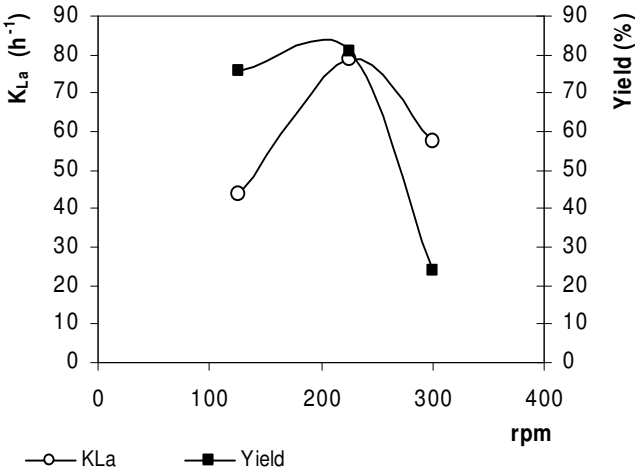

Figure 3. Volumetric mass transfer coefficient for O_2, h^{-1}, and yield, atdifferent agitation rates.

centration that the resting cells of *N. corallina* can oxidize under the reaction conditions studied, increasing the concentration of the aldehyde. Beginning with 3 g/l of furfural we increased it until 9 g/L, through a fed-batch procedure with two more additions of furfural (0.15 g each) and a total 12 h of biotransformation. At the end of the process some furfural remained unchanged (2-furoic acid/ furfural of 92:8). After more additions of 2-furanaldehyde, we did not observe an increase in the oxidation product, this could be due to an inhibition effect.

Then 9 g/L represents the upper limit of the procedure. Recently a significant inhibitory effects of substrate and product was reported with another actinomycete, *Rhodococcus erythropolis* in the biotransformation of (-)-trans-carveol to (*R*)-(-)-carvone (Morrish and Dauglis, 2008). Due to the fact that some authors have taxonomically classified *N. corallina* B-276 as *Rhodococcus rhodochrous* (Matsui et al., 2007), this result is relevant.

Conclusion

Biocatalysis is a valuable alternative to classical organic synthesis, the recent successful applications for the preparation of active pharmaceutical ingredients are promising (Tao et al., 2007). In particular whole cell catalyzed process applied to the commercial synthesis of fine chemicals (Ishige et al., 2005; Woodley, 2006) do not need the addition of cofactors since those are provided by the cell. Here, with whole cells of *N. corallina* the efficient production of 2-furoic acid from the corresponding aldehyde or alcohol by two procedures was achieved. It is to worth mentioning that the use of resting cells improved substantially our previous results (Luna et al., 1997), since the oxidation to 2-furoic acid was almost quantitative (98%).

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