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# FUMARIC ACID PRODUCTION BY *RHIZOPUS NIGRICANS* AND *RHIZOPUS ORYZAE* USING APPLE JUICE*

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The aim of this work was to compare the abilities of *Rhizopus nigricans* and *Rhizopus oryzae* cells to produce fumaric acid in free and immobilized cultures in a polyurethane foam. The cultivations were conducted in conical flasks in a rotary shaker as well as in a fermentor (7.5 L of volume). Apple juice containing 5% of reducing sugar was used as a source of carbon for fumaric acid biosynthesis and 5% of pure glucose. The immobilised cells of *R. nigricans* produced about 1.8%–2.5% of fumaric acid in the presence of glucose, and that was about 2-6-fold higher when compared with the free cells. About 2.2%–2.9% of fumaric acid were determined in the culture filtrates of *R. oryzae* and these values did not differ both for the free and immobilized cells because the free cells showed a pattern of self-immobilization. Experiments conducted under the same conditions using apple juice biosynthesis of fumaric acid gave an average of 2.8% after 6 days of cultivation by the examined strains, and after changing the medium, the concentration of the acid increased to an average of 3.2%, which was about 28% higher than in the glucose-containing medium.

## INTRODUCTION

Fumaric acid has a broad spectrum of applications in many branches of industry, from synthetic resins and biodegradable polymers possessing special properties and surfactants, including chemical synthesis of many compounds. It is also widely used in food industry. Because of its powerful acidifying properties, 2 parts of this acid can replace 3 parts of citric acid. As a component of fruit jellies, this acid exhibits a very useful property – it increases gelling power, so the addition of gelatin during the production can be diminished [Krajewska *et al.*, 1983].

The only disadvantage of this acid is its weak solubility in water, though this problem was solved by gaining a modified CWS acid (Cold-Water-Solubility), *i.e.* a mixture containing fumaric acid and additionally some compounds facilitating its solubility.

At present, fumaric acid is gained through chemical synthesis by the isomerization of maleic acid obtained from the oxidation of benzen [Petruccioli *et al.*, 1996]. The raw materials originate from petroleum, whose price is continuously rising, and from by-products of the processes which contaminate the environment. A future method would be the biotechnological production of fumaric acid; cheap raw materials could be successfully used as a source of carbon. Apple juice is such a raw material, and obtaining this juice in Poland is relatively cheap and easy.

The aim of the study was therefore to utilise, together with the synthetic broth, apple juice for the fumaric acid biosynthesis with the use of *Rhizopus nigricans* and *Rhizopus oryzae* strains.

## MATERIALS AND METHODS

Two strains of microorganisms were used for the production of fumaric acid: *Rhizopus nigricans* 3/2 and *Rhizopus oryzae* 3/6 DSM 905 from the collection of the Department of Food Technology and Storage, Agricultural University of Lublin, Poland. The spores, cultivated in a potato broth containing agar, were flushed with a salt solution. In such an inoculum, the cells were counted in order to introduce the same amount of cells to the flasks with the growing broth and to provide the same growth conditions. On average, $1.5\times10^9$ of spores were introduced into 100 mL of the growing broth each time.

The composition of the growing broth was as follows [Kautola & Linko, 1989]: 5% of glucose, 0.236% of $(NH_4)_2SO_4$, 0.030% of $KH_2PO_4$; 0.025% of $MgSO_4\times7H_2O$; 0.007% of $ZnSO_4$; 0.001% of $Fe_2(SO_4)_3$; 0.30% of malt extract and 2% of $CaCO_3$.

The production broth had the following composition: (i) 5% of glucose and 2% of $CaCO_3$; (ii) apple juice containing 5% of reducing sugars, obtained by diluting the apple juice concentrate (produced by ZPOW "Agros-Fortuna" in Milejów) and 2% of $CaCO_3$.

The cultivations of the microorganisms were conducted in conical flasks (0.5 L) in a rotary shaker at 28°C. Some of the flasks were provided with polyurethane foams in a form of a ring (8-cm diameter; thickness 1 cm) in order to immobilize the microorganism. Simultaneously, under the same conditions, control cultivations with free cells were conducted.

During the fumaric acid production, a Gallenkamp fermentor (7.5 L of volume), equipped with polyurethane

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foams in a form of rings on three perforated teflon discs, was also used. The cultivation conditions were: temperature – 28°C, stirring speed – 300 rpm, airflow – 22 $m^3$/h per L of the cultivation medium.

The cultivations in the growing medium were carried on for 3 days, and when the glucose was exhausted, the medium was removed and a sterile production medium was introduced. After 7 days of cultivation, when a lack of carbon source was observed, a new sterile production medium was introduced and this procedure was repeated three times. During the production phase, the following measurements were made every 24 h: pH value, reducing sugars concentration measured with 3,5-dinitrosalicylic acid [Miller, 1959], and fumaric acid concentration.

Unicam UV/VIS spectrophotometer was used in order to evaluate the fumaric acid concentration; the solutions were 200-fold or 400-fold diluted prior the analysis. The absorbance was measured at 255 nm. This wavelength was previously selected as an optimal wavelength for pure fumaric acid solutions. Simultaneously, HPLC measurements of the fumaric acid content were conducted, using the following equipment: Waters C18 column, mobile phase – water: butanol: acetic acid (93.8:6.0:0.2), UV detector preset at 255 nm.

## RESULTS AND DISSCUSION

In the first part of the experiment, the cultivations of *Rhizopus nigricans* and *Rhizopus oryzae* in conical flasks were conducted. The obtained results of fumaric acid concentrations on the production medium containing pure glucose are shown in Figure 1. These results suggest that immobilization of *R. nigricans* cells increased the fumaric acid concentration several times in comparison to the free--cultures. After two days, the immobilized cells in the first production medium produced 1.29% (w/v) of fumaric acid, and after 6 days the acid concentration was 1.78%. The free cells produced at the same time 0.5% and 0.95% of fumaric acid, respectively. After the change of the production medium, the immobilized cells produced after the second day 0.8% and after the sixth day 1.79% of the fumaric acid. At the same time, the free cells produced 0.41% and 0.65% of the acid, respectively. The ratio of the fumaric acid concentration in the culture filtrates of immobilized cells in comparison to the medium with free cells was 2.3. During the cultivation with the third production medium, after two changes of the medium, the immobilized cells produced after the second day 1.67% and after the sixth day 2.54% of fumaric acid. The concentration of the acid in the culture filtrates with free cells was 0.33% and 0.43%, respectively. This time, the ratio of the fumaric acid concentration produced by immobilized cells on the polyurethane foam in comparison to the concentration in the culture filtrates of free cells was higher reaching 5.4. Such a high ratio was a result of the diminishing of fumaric concentration after subsequent broth changes in the cultivations containing free fungal cells.

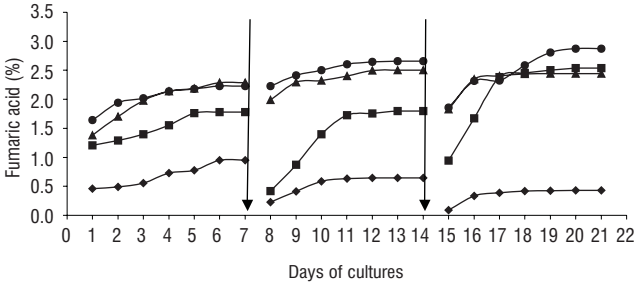


FIGURE 1. Fumaric acid production by *Rhizopus nigricans* and *Rhizopus oryzae* using broth containing pure glucose: ◆—◆ – *R. nigricans* non-immobilized biomass; ■—■ – *R. nigricans* immobilized biomass; ▲—▲ – *R. oryzae* non-immobilized biomass; ●—● – *R. oryzae* immobilized biomass; ↓ – Change of the cultivation broth.

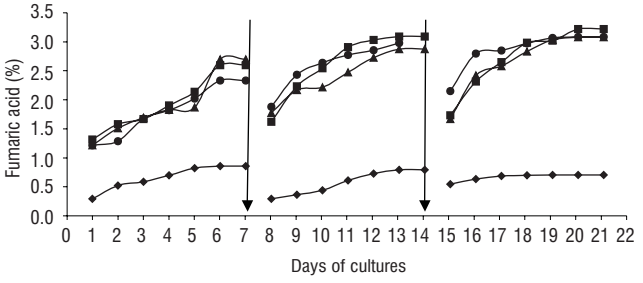


FIGURE 2. Fumaric acid production by *Rhizopus nigricans* and *Rhizopus oryzae* using broth containing apple juice after three changes of the production broth: ◆—◆ – *R. nigricans* non-immobilized biomass; ■—■ – *R. nigricans* immobilized biomass; ▲—▲ – *R. oryzae* non-immobilized biomass; ●—● – *R. oryzae* immobilized biomass; ↓ – Change of the cultivation broth.

There were no differences in the fumaric acid production between free and immobilized biomass of *R. oryzae* (Figure 1). In the case of the immobilized biomass, fumaric acid content in the medium was 1.94% after two days and 2.23% after 6 days, whereas the free-cells produced 1.51% and 2.29% of fumaric acid, respectively. In the second production broth, fumaric acid concentration for the immobilized cells was 2.41% after 2 days and 2.66% after 6 days, and in the case of free-cell cultivations fumaric acid concentrations were 2.29% and 2.5%, respectively. After subsequent changes of the production medium, the concentration of the acid was 2.32% after the second day of cultivation and 2.87% after the sixth day, whereas in the case of the free-cells the concentrations were 2.35% and 2.44%, respectively. The similar results obtained for the free-cells and immobilized-cells seem to be clear, because the biomass of *R. oryzae*, under the conditions applied, was growing in the form of the sphere, so self-immobilization occurred. However, the cells of *R. nigricans* formed minute spheres and the change of the broth in flasks with free cells was more difficult to obtain. The results of the maximal fumaric acid production using free and immobilized cells of *R. nigricans* and *R. oryzae* in the production medium containing glucose as a source of carbon are shown in Figure 3.

In the next part of the experiment, the apple juice was utilised (Figure 2). In the cultivation with the immobilized *R. nigricans* cells, after 6 days of the process, the concentration of the fumaric acid was 2.8%, and after the change of the broth into a new one, after the same time, the concentration was 3.2%. Under the same conditions of study, free cells produced after the same time 0.83% and 0.68% of fumaric acid, respectively. After the next change of the broth, the concentration of the fumaric acid in the medium with immobilized cells was 3.31%, and in the cultivations with free fungal cells was 0.6%. It can be stated that fumaric

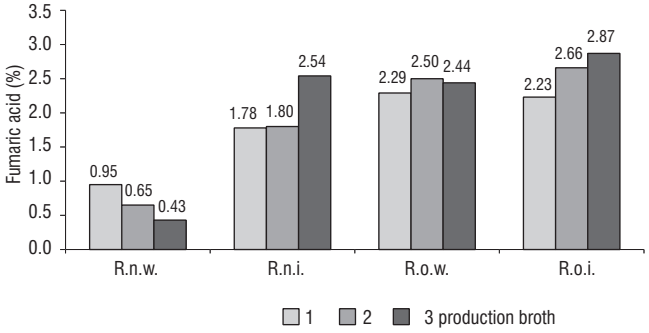


FIGURE 3. Maximal yields of fumaric acid produced by immobilized and non-immobilized *Rhizopus nigricans* and *Rhizopus oryzae* cells using production broths containing glucose: R.n.w. – *R. nigricans* non--immobilized biomass; R.n.i. – *R. nigricans* immobilized biomass; R.o.w. – *R. oryzae* non-immobilized biomass; R.o.i. – *R. oryzae* immobilized biomass.

acid concentration was about 28% higher when the substrate was apple juice for the immobilized-cells cultivation in comparison to the results obtained on pure glucose as the source of carbon (Figure 2).

When conducting experiments with *R. oryzae* in the cultivation broth containing apple juice, similar fumaric acid concentrations were recorded no matter if the biomass was immobilized in polyurethane sponge or was growing in large conglomerates. Fumaric acid concentration in the first broth containing the apple juice after two days was on average 1.4%, and after 6 days it reached 2.3%. After replacement of the medium, after 2 days, the acid content was up to 2.4%, and after 6 days it was 3.0%. After the next change of the medium, there was 2.8% and 3.2% of the acid, respectively (Figure 2).

In the fermentor cultivations, lower fumaric acid concentrations were recorded. After the three days of *R. nigricans* cultivation using the broth containing pure glucose, the fumaric acid concentration was 1.13%. When using the apple juice, the corresponding concentration was 0.68%, and after 5 days the concentration was 0.8%. Maximal fumaric acid concentrations in cultivation broths, containing either free or immobilized cells of *R. nigricans* and *R. oryzae* (in the three broths containing apple juice) are shown in Figure 4.

The results obtained for the fumaric acid concentration

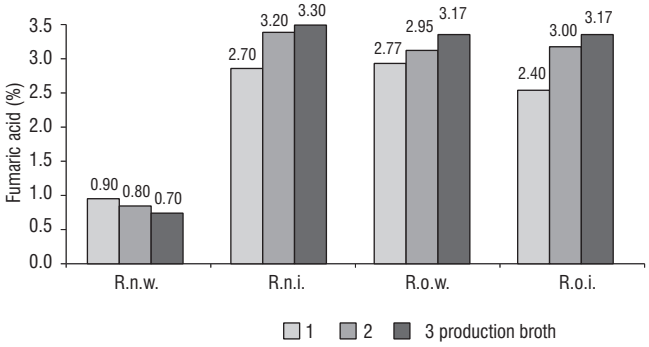


FIGURE 4. Maximal yields of fumaric acid produced by immobilized and non-immobilized *Rhizopus nigricans* and *Rhizopus oryzae* cells using the first, second and third production broth containing apple juice: R.n.w. – *R. nigricans* non-immobilized biomass; R.n.i. – *R. nigricans* immobilized biomass; R.o.w. – *R. oryzae* non-immobilized biomass; R.o.i. – *R. oryzae* immobilized biomass.

using spectrophotometric method and HPLC system were very convergent. Considering the HPLC as a reference method, the coefficient of 1.02 was obtained. It can be therefore concluded that the spectrophotometric method, which is simple and fast, can be applied for fumaric acid measurements under the conditions described in this experiment.

The growing medium used in the experiments was complete, considering its composition, but the production medium was very poor and contained only glucose as a source of carbon and $CaCO_3$ neutralizer.

Investigations conducted by many authors [Kautola & Linko, 1989; Moresi *et al.*, 1991; Romano *et al.*, 1967] showed that production media containing nitrogen sources, and other mineral components and a source of carbon, cause the growth of biomass, and broths lacking a source of nitrogen give higher fumaric acid yields. When apple juice was introduced to the cultivation growth, the yields of fumaric acid were about 28% higher than in broths containing pure glucose. Fumaric acid biosynthesis by *Rhizopus* strains can be stimulated by the chemical composition of the production medium. After 5 days of cultivation in a fermentor, using the immobilized cells of *R. nigricans*, a concentration of 8 g/L of the acid was gained, and in the shaken cultivations after the same period of time 22 g/L of the acid was produced. Many authors [Buzzini *et al.*, 1992, 1993; Gobbetti & De Vincenzi, 1993; Petruccioli & Angiani, 1995; Petruccioli *et al.*, 1996] used, except monomeric sugars, different sources of carbon for the fumaric acid production: potato flour [Moresi *et al.*, 1991], hydrolysate of corn flour [Federicci & Petruccioli, 1996], lyophilised orange peels [Buzzini *et al.*, 1992], molasses [Petruccioli & Angiani, 1995; Petruccioli *et al.*, 1996], and grape must [Buzzini *et al.*, 1993; Gobbetti & De Vincenzi, 1993]. The results of these experiments varied, depending on the microorganisms used, the conditions of the experiments, carriers, types of fermentor, *etc*.

When using grape must and the cultures of *Candida hydrocarbofumarica* for the fumaric acid production, Gobbetti and De Vincenzi [1993], gained a yield of 7.5 g/L of the fumaric acid after 7 days of cultivation, and 13.5 g/L after 14 days of the experiment. Petruccioli and Angiani [1995] tested different carriers for *Rhizopus arrhisus* NRRL 1526 cultivations and showed that immobilization of the biomass in calcium alginate was most effective among all the carriers tested. The yield of the fumaric acid was 21.7 g/L after 5 days of cultivation, with the glucose from molasses as a source of carbon. The cells absorbed on polyurethane foam excreted 24% less fumaric acid. In spite of that, in subsequent studies, Petruccioli *et al.* [1996] utilized polyurethane foam to immobilize the *R. arrhizus* biomass in semi-continuous cultivations in a fermentor. They utilized molasses as a source of carbon, and after 48 h of such an experiment the yield was 22–28 g/L. Kautola and Linko [1989], using *R. arrhizus* TKK 204-1-1a immobilized in polyurethane foam, and xylose as a source of carbon, gained a very promising result – a 3.5-fold higher yield of the fumaric acid concentration in comparison to the cultivation with non-immobilized cells.

In the experiments conducted in the described work, polyurethane foam was applied in order to immobilize the biomass. In the broth containing immobilized *R. nigricans* cells, the concentrations of fumaric acid were about 4 times

higher than in the cultures containing free cells. The culture of *R. oryzae* was grown in a form of rigid sphere. This is the reason why no crucial differences in fumaric acid concentrations were recorded for the immobilized cells cultures in comparison to the cultures containing free cells.

Adsorption of *Rhizopus nigricans* biomass in polyurethane foam contributed not only to the higher levels of fumaric acid concentrations in the culture filtrates, but also enabled fast and sterile change of the production media, and this improvement has a great significance when the multiple changes of the broth occur. Despite that, this carrier has many advantages; it is cheap, easily accessible, durable and has no influence on cells.

## CONCLUSIONS

1. *R. nigricans* immobilized on polyurethane foam produced 1.8%-2.5% of fumaric acid in the presence of pure glucose as a source of carbon, *i.e*. 2-6-fold higher concentrations than the non-immobilized biomass.

2. *R. oryzae* biomass produced 2.2-2.9% of fumaric acid and the application of polyurethane foam had no effect on the productivity of fumaric acid.

3. Apple juice can be successfully utilized during fumaric acid production by *R. nigricans* and *R. oryzae*, because the yield of the acid was about 2.8% after 6 days of cultivation, and after the change of the production medium, the fumaric acid concentration was 3.2%, *i.e*. 28% more than in cultivation containing pure glucose.

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## BIOSYNTEZA KWASU FUMAROWEGO PRZEZ GRZYBY Z RODZAJU *RHIZOPUS* Z WYKORZYSTANIEM SOKU JABŁKOWEGO

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Celem pracy była biosynteza kwasu fumarowego przez grzybnie *Rhizopus nigricans* 3/2 i *R. oryzae* 3/6 DSM 905 oraz wykorzystanie jako podłoża produkcyjnego soku jabłkowego zawierającego 5% cukrów redukujących i 5%-owy roztwór glukozy.

Hodowle prowadzono z grzybnią unieruchomioną na piance poliuretanowej jak też z grzybnią wolną, w kolbach stożkowych na wstrząsarce rotacyjnej w temp. 28°C jak również w fermentorze Gallenkamp o poj. 7,5 L stosując trzy teflonowe dyski pokryte pianką poliuretanową. Brak cukru w podłożu był sygnałem do wymiany pożywki i tę czynność powtarzano trzykrotnie.

Wyniki badań wykazały, iż unieruchomiona grzybnia *R. nigricans* produkowała od 1,8% do 2,5% kwasu fumarowego w obecności czystej glukozy tj. od 2–6-ciu razy więcej niż grzybnia wolna. Natomiast w hodowlach grzybni *R. oryzae* oznaczono od 2,2% do 2,9% kwasu fumarowego i nie stwierdzono różnicy w ilości wytwarzanego kwasu przez grzybnię wolną i immobilizowaną ponieważ grzybnia wolna sama ulegała immobilizacji (rys. 1 i 3). Sok jabłkowy może być z powodzeniem stosowany jako podłoże hodowlane do biosyntezy kwasu fumarowego przez badane grzyby gdyż uzyskano średnio 2,8% kwasu po 6 dobach hodowli, a po wymianie pożywki stężenie kwasu w podłożu wynosiło ok. 3,2%, tj. o ok. 28% więcej niż w hodowlach zawierających czystą glukozę (rys. 2 i 4).