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Article *in* World Journal of Microbiology and Biotechnology · January 2001

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World Journal of Microbiology & Biotechnology **17**: 767–771, 2001.
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Aroma compounds produced by *Kluyveromyces marxianus* in solid state fermentation on a packed bed column bioreactor

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Received 7 December 1999; accepted 7 September 2001

Keywords: Aroma compounds, cassava bagasse, *Kluyveromyces marxianus*, packed bed column bioreactors, solid state fermentation

Summary

Studies were carried out for the production of aroma compounds by *Kluyveromyces marxianus* grown on cassava bagasse in solid state fermentation using packed bed reactors, testing two different aeration rates. Respirometric analysis was used to follow the growth of the culture. Headspace analysis of the culture by gas chromatography showed the production of 11 compounds, out of which nine were identified. Ethyl acetate, ethanol and acetaldehyde were the major compounds produced. Lower aeration rate (0.06 l h^{-1} g^{-1} of initial dry matter) increased total volatile (TV) production and the rate of production was also increased at this aeration rate. Using an aeration rate of 0.06 l h^{-1} g^{-1} maximum TV concentrations were reached at 24 h and at 40 h with 0.12 l h^{-1} g^{-1}.

Introduction

In recent years, there has been a constant increase in efforts to utilize tropical agro-industrial residues such as coffee pulp and coffee husk, cassava bagasse and sugar cane bagasse. These residues are generated in large amounts during processing and their disposal rather causes serious environmental concern, as most of these residues at present do not find any application. One of the most important applications of these residues could be their utilization as carbon source for bioprocesses. Several processes have been reported that utilize these residues as raw material for the production of bulk chemicals and value-added fine products such as ethanol, single-cell protein (SCP), mushrooms, enzymes, organic acids, amino acids, biologically active secondary metabolites, etc. (Pandey & Soccol 1998).

Microorganisms play an important role in the generation of natural compounds, particularly in the field of food aromas. Several reports and reviews have been published on the production of volatile compounds (aroma compounds) by microorganisms (Janssens *et al.* 1988, 1992; Jiang 1995; Berger 1995; Christen *et al.* 1997; Bramorski *et al.* 1998; Soares *et al.* 2000). Although several bacteria, yeasts and fungi have been reported for the production of aroma compounds, a few species of yeasts and fungi have generally been preferred, and only a few of these find industrial application due to their generally regarded as safe (GRAS) status. Solid state fermentation (SSF) has been used for the production of aroma compounds by cultivating yeasts and fungi such as *Neurospora* sp. (Yamauchi *et al.* 1989; Pastore *et al.* 1994), *Zygosaccharomyces rouxii* (Sugawara *et al.* 1994), *Aspergillus* sp. (Humphrey *et al.* 1990; Ito *et al.* 1990), *Trichoderma viride* (Gervais & Sarette 1990), using pre-gelatinized rice, miso, cellulose fibres and agar, respectively. The production of intense fruity aromas by *Ceratocystis fimbriata* in SSF using wheat bran, cassava bagasse and sugar cane bagasse as substrates was explored by Christen *et al.* (1997). Bramorski *et al.* (1998) and Christen *et al.* (2000) reported the production of volatile compounds such as acetaldehyde and 3-methylbutanol by growing *Rhizopus oryzae* on tropical agro-industrial residues. Janssens *et al.* (1988) cultivated the yeast *Hansenula mrakii* in liquid fermentation for the synthesis of banana odour

compounds (isoamyl acetate). Two strains of the yeast *Kluyveromyces* sp. having the GRAS status, namely *K. lactis* and *K. marxianus* were used to produce aroma compounds such as monoterpene alcohols and isoamyl acetate (responsible for fruity aromas), in liquid fermentation (Fabre *et al.* 1995; Jiang 1995).

An adequate supply of O_2 has been termed an important parameter in SSF. It has been used to maintain aerobic conditions, eliminate CO_2 produced and control of temperature and moisture level of substrate (Pandey 1991). The analysis of headspace by gas chromatography (GC) of cultures could be used to quantify the oxygen uptake rate and carbon dioxide produced. These measures are often used for an indirect evaluation of biomass and are important to scale up SSF processes (Saucedo-Castañeda *et al.* 1994).

The aim of present work was to evaluate the production of aroma compounds in SSF by a GRAS strain of *K. marxianus* using cassava bagasse as substrate in a packed bed column bioreactor with forced aeration.

Materials and methods

Microorganism and inoculum

A strain of *K. marxianus* (ATCC 10022) was used in this study. It was maintained on yeast and malt extract agar (YMA) and stored at 4 °C. Inoculum was prepared by transferring a loopful of cells from a freshly grown culture to 100 ml of a liquid medium containing glucose and yeast extract (5 and 2%, respectively), held in 250 ml Erlenmeyer flask. The flask was incubated at 28 °C for 30 h on a rotary shaker (150 rev/min). Cells suspension contained 10^8 cells/ml, as counted using the Neubauer chamber.

Substrate preparation

Cassava bagasse was dried at 60 °C in an air oven for 24 h. The dried substrate was milled and sieved to obtain particles of 0.42–0.82 mm size. This was enriched with glucose (10%). After setting the initial pH and moisture at 5.0 and 60%, respectively, the substrate was autoclaved at 121 °C for 15 min and inoculated using 1×10^7 cells/g initial dry matter (IDM).

Fermentation conditions

SSF was carried out by packing pre-inoculated solid medium (20 g dry wt basis) into glass columns (4 cm diameter and 20 cm length). Figure 1 shows the schematic set-up of the fermentation system. The columns were put in a temperature-controlled (28 °C) water bath and connected with an air distributor. Saturated air was passed through the columns to prevent the drying of the substrate bed. Two different aeration rates, 0.06 and 0.12 l h^{-1} g^{-1} were used to study the influence of aeration rate on aroma production by the yeast culture.

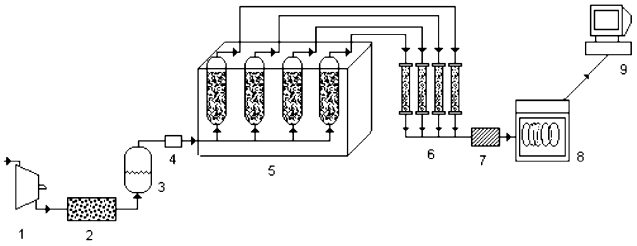

Figure 1. Schematic set-up of packed bed bioreactor system. (1) Air pump; (2) air filter; (3) air moisturizing unit; (4) air distributor; (5) bioreactor columns in water bath; (6) silica gel columns; (7) auto gas sampler; (8) gas chromatograph; (9) computer.

Two runs of each aeration rate were carried out and the experiments were done in duplicate.

Respirometric analysis

Respirometric analysis was followed by a GC with a thermal conductivity detector. Data were controlled by computer with the software CHROMA-Biosystèmes, in order to calculate carbon dioxide production rate (CDPR), oxygen uptake rate (OUR) and respirometric quotient (RQ), where:

$$\text{CDPR} = (\%\ \text{CO}_2\ \text{produced} \times F)/(100 \times W)$$
$$\text{in l h}^{-1}\text{g}^{-1}\ \text{IDM}$$

$$\text{OUR} = (\%\ \text{O}_2\ \text{consumed} \times F)/(100 \times W)$$
$$\text{in l h}^{-1}\,\text{g}^{-1}\ \text{IDM}$$

$$\text{RQ} = \text{CDPR}/\text{OUR}$$

where F is the air flow rate ($l\,h^{-1}$) and W the IDM load.

Analytical procedures

Aroma compounds produced in each column were measured by headspace analysis of the culture on an HP 5890 GC, equipped with a flame ionization detector at 250 °C. The operating conditions were: 30 m × 0.53 mm HP-INNOWAX polar capillary column, column temperature from 40 to 150 °C at a rate of 10 °C min^{-1}, injector temperature 250 °C. Total and individual volatiles were expressed as μmol/l of headspace, as ethanol equivalent. The compounds were identified by comparing their retention times with those of standard compounds. The final pH of the substrate was measured using a potentiometric method by homogenizing 1 g in 25 ml of deionized water. Reducing sugars were measured by the Somogyi-Nelson colorimetric method.

Results and discussion

Initial studies were carried out in 250 ml Erlenmeyer flasks using cassava bagasse as substrate. Two experi-

mental designs were done to optimize fermentation conditions such as temperature, glucose addition, inoculum size, initial pH and moisture of the medium. Using a 2^5 factorial design, addition of glucose and initial pH of the substrate was found statistically significant for aroma compound production. A second experimental design was performed to evaluate the effect of glucose addition and the initial pH of medium at two levels. According to the results, the best conditions to maximize volatile compound production were: pH 5.0, 60% of initial water content, 28 °C, inoculum size 1×10^7 cells/g IDM and 10% of glucose addition (Medeiros *et al.* 2000). The same conditions were applied to the experiments on packed bed column bioreactor. The results shown in the figures and tables are representative runs. The relative errors obtained were between 2 and 11%.

Production of total volatile compounds

As shown in Figure 2, a lower aeration rate (0.06 l h^{-1} g^{-1}) increased total volatiles (TV) production. TV produced with 0.12 l h^{-1} g^{-1} were less in quantity and the rate of production was also slower than with 0.06 l h^{-1} g^{-1}. Maximum TV concentrations were reached at 24 h with 0.06 l h^{-1} g^{-1} and at 40 h for 0.12 l h^{-1} g^{-1}. A total of 11 compounds were produced with cassava bagasse at both aeration rates, out of which ethyl acetate, ethanol and acetaldehyde were the major compounds (Figures 3 and 4). Higher aeration rate (0.12 l h^{-1} g^{-1}) resulted in decreased production of ethanol and acetaldehyde, showing that this affected their synthesis by the culture. The influence of oxygen on volatile compounds produced by *A. oryzae* has been discussed by Ito *et al.* (1990) who observed higher yields of alcohols and aldehydes by the lack of oxygen in the fermentation medium. Eight compounds including isoamyl alcohol, isoamyl acetate, ethyl propionate, propyl acetate, ethyl isobutyrate and butyl acetate were found in the headspace of the culture. Two compounds remained unidentified. All these compounds were produced in low concentrations (<1 μmol l^{-1} g^{-1} IDM). Table 1 shows the concentration of individual compounds produced at different aeration rates, as accumulated in the head-space at their maximum concentration. Table 2 shows the changes of pH and moisture in substrate at the end of fermentation. Evidently, at lower aeration rate, neither the pH nor the moisture contact changed significantly from their initial values of 5.0 and 60%, respectively. However at 0.12 l h^{-1} g^{-1} aeration rate, there was a fall in the pH of the substrate (3.9), although the moisture content only marginally increased (61.6%).

Fruity aromas produced by the yeast culture were attributed to the esters, as it is known that alcohols do

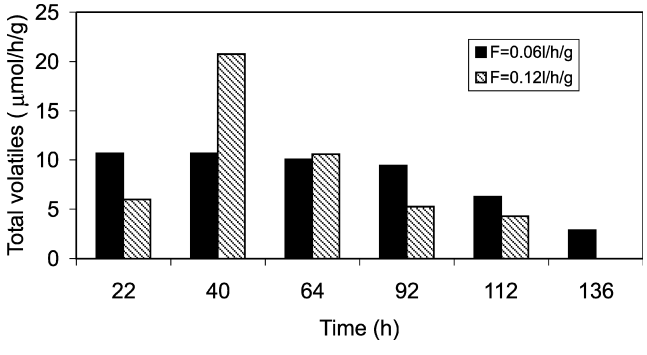

Figure 2. TV compounds produced in columns with cassava bagasse by *K. marxianus* with two aeration flow rates (*F*), at different times of fermentation.

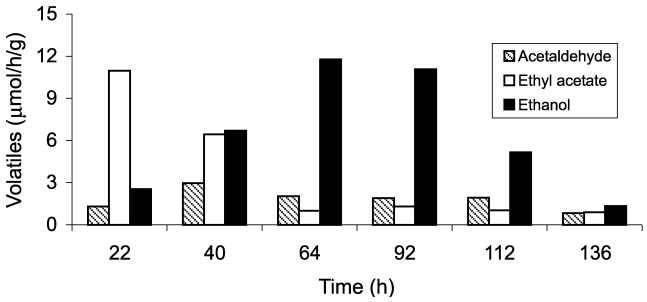

Figure 3. Major volatile compounds produced with cassava bagasse in columns fermentation with aeration rate 0.06 l h^{-1} g^{-1}, at different times of fermentation.

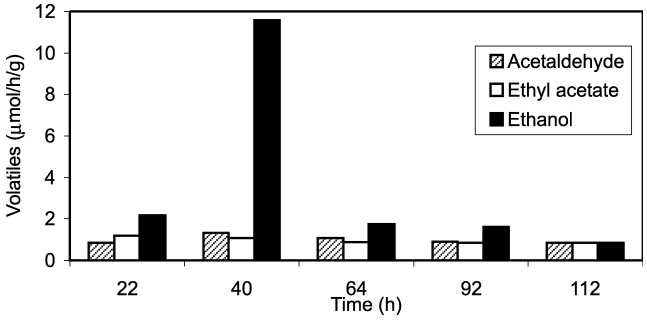

Figure 4. Major volatile compounds produced with cassava bagasse in columns fermentation with aeration rate 0.12 l h^{-1} g^{-1}, at different times of fermentation.

Table 1. Volatile compounds produced on cassava bagasse (pH 5.0 and 10% glucose) in packed bed bioreactor at maximum TV concentration at different aeration rates.

Volatile Compounds (μmol l^{-1} g^{-1})	Aeration rate (l h^{-1} g^{-1})	
	0.06	0.12
Acetaldehyde	0.54	0.55
Unidentified	0.97	0.45
Ethyl acetate	4.57	4.82
Ethanol	1.05	1.04
Propyl acetate	0.35	0.35
Butyl acetate	0.35	0.36
Ethyl propionate	0.36	0.35
Ethyl isobutyrate	0.35	0.35
Isoamylic alcohol	0.35	0.36
Isoamyl acetate	0.35	<1
Unidentified	<1	<1

Table 2. Final values of pH, water content and reducing sugars consumed for experiments on packed bed reactors at different aeration rates.

Determination	Low level of aeration	High level of aeration
Final pH	5.1	3.9
Reduced sugar consumed (g/g IDM)	0.32	0.31
Final water content (%)	60.8	61.6

not contribute so much in flavour, although together with other compounds affect the overall flavour quality. It is believed that the concentration of volatile compounds in the headspace of the flask culture is generally affected by several factors, chiefly with the nature and concentration of the fermentation medium and its vapour pressure. One reason could be that the concentration of the most volatile compound (acetaldehyde for example) measured in the headspace of the flask cultures was overestimated. The other one could be that the compounds with low vapour pressure such as isoamyl acetate have their concentration underestimated.

Respirometric analysis

The maximum respirometric activity, represented by CDPR and OUR (Figures 5 and 6), for both aeration rates, i.e. 0.06 and 0.12 l h^{-1} g^{-1}, was observed after 24 h of fermentation. CDPR was maximal at 31.5 and 38.9 h with 0.06 and 0.12 l h^{-1} g^{-1} aeration rates, respectively. Maximum TV concentration was detected at 24 h with 0.06 l h^{-1} g^{-1} and at 40 h with 0.12 l h^{-1} g^{-1} aeration rates. These results showed that production of volatile compounds was dependent on growth. The microbial culture used the carbon source and oxygen for growth, and maximum production of TV was just before or after maximal biomass growth. The respirometric quotient (Figures 5 and 6) with experiments using 0.12 l h^{-1} g^{-1} reached values greater than that obtained with 0.06 l h^{-1} g^{-1}. This was probably due to the lower OUR with 0.12 l h^{-1} g^{-1} aeration rate. The decrease in RQ showed that the oxygen and substrate were used by the yeast not

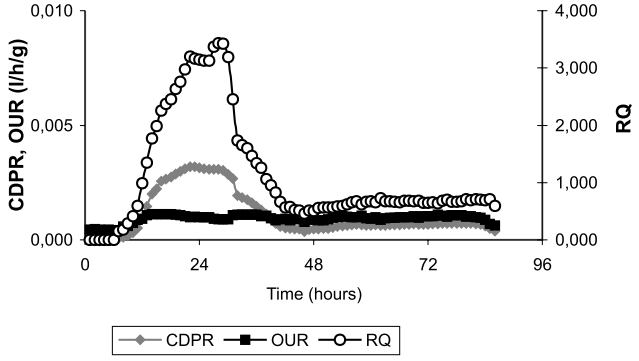

Figure 5. Carbon dioxide production rate and oxygen uptake rate by *K. marxianus* in cassava bagasse with aeration rate of 0.06 l h^{-1} g^{-1}.

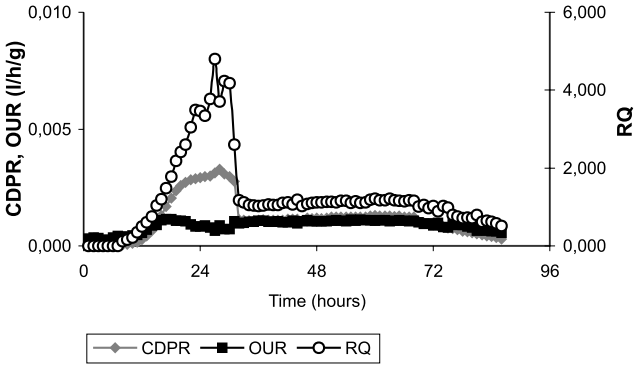

Figure 6. Carbon dioxide production rate and oxygen uptake rate by *K. marxianus* in cassava bagasse with aeration rate of 0.12 l h^{-1} g^{-1}.

only for biomass and CO_2 production, but for metabolite production (volatile compounds). After 60 h of fermentation, CO_2 production was constant. Bramorski *et al.* (1998a) and Meza *et al.* (1998) using CO_2 production as a growth indicator, also found correlation between the growth of *C. fimbriata* on different media and production of the volatile compounds, showing that the maximum volatile production always occurred a few hours before or after the maximum respirometric activity.

Conclusions

The results show the feasibility of packed bed column bioreactors in SSF to produce fruity aroma materials by a GRAS strain of *K. marxianus*. Cassava bagasse was found to be a suitable substrate for aroma production by the yeast culture. Eleven compounds were separated by GC headspace analysis of the culture. Interestingly, the predominant compounds were ethyl acetate, ethanol and acetaldehyde. The fruity aroma was attributed to the productions of esters. The influence of the aeration rate on both volatile production and carbon dioxide was demonstrated. A correlation was observed between growth and volatile compound production.

Acknowledgements

One of the authors (CRS) thanks CNPq for a scholarship under Scientific Productivity Scheme.

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