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GROWTH OF *CANDIDA UTILIS* IN SOLID STATE FERMENTATION

P. CHRISTEN,*‡ R. AURIA,* C. VEGA,† E. VILLEGAS† and S. REVAH†

**ORSTOM, Institut Français de la Recherche Scientifique pour le Développement en Coopération, Cicéron 609, Colonia Los Morales, CP 11530 México D.F., Mexico*
†Universidad Autónoma Metropolitana - Iztapalapa. IPH. Apdo. Postal 55-534. CP 09340, México D.F., Mexico

ABSTRACT

In this work, the growth of the yeast Candida utilis on different solid substrate (wheat bran) and supports (sugarcane bagasse and Amberlite resin) imbibed with a liquid culture medium was studied. Growth was followed by sugars consumption, carbon dioxide production rate (CDPR) and cell count. The results showed the ability of the yeast to grow on the three solid media with fairly good viability and total dextrose consumption in the case of sugarcane bagasse and Amberlite, and partial consumption of wheat bran sugars. After two or three days of culture, a five hundred fold increase in cell population was observed .

Key Words : Solid State Fermentation, Candida utilis, Cell Growth, Wheat Bran, Bagasse, Amberlite.

INTRODUCTION

The problem of contaminated dilute liquid ethanol streams may be solved by transforming the ethanol into more easily recoverable components[3]. One alternative could be to biologically convert the alcohol to either an ester, an aldehyde or other volatile compounds. Yeasts are involved in the production of flavoring compounds[8]: Kluyveromyces lactis has been reported to produce monoterpene alcohols[9]. Several authors have reported the biosynthesis of acetate esters by both whole-cell processes and enzymatic routes. For example, the production of intense fruity aromas due to the presence of isobutyl acetate and others ethylic esters by Dipodascus magnusii[11] has been studied. Specifically, it has been reported the conversion by Candida utilis of ethanol to ethyl acetate[2,3] and acetaldehyde[4] which have the potential use of "natural" flavor and fragrance compounds and C_2 to C_6 alcohols into their corresponding aldehydes by Pichia pastoris[27]. Lipases from molds and yeasts are known to synthetise flavor esters such as isoamyl or geranyl acetate propionate and butyrate[15], ethyl or hexyl propionate butyrate and hexanoate[17], and terpene alcohols esters[14]. Lipase from a Candida strain has been studied for the production of acetic and butyric esters from C_3 to C_5 alcohols[26]. Another enzyme : the alcohol acetyltransferase has been involved in flavor esters biosynthesis[1,29]. Various yeasts of the generi Saccharomyces and Hansenula produce ethyl acetate from dextrose and ethanol[30,24]. Nevertheless, Candida utilis is known to tranform ethanol to ethyl acetate in liquid fermentation with a higher yield and production rate than other known ester-producing yeasts[2,3].

‡ *corresponding author*

Yeasts - like fungi - are able to support fairly wide ranges of osmotic pressure and water activity. Growth and fermentative activity have been reported at aw's lower than 0.7[6]. It is one of the major reasons for regarding yeasts as microorganisms with promising potential in SSF culture conditions. SSF processes have gained renewed interest[19]and yeasts have been studied in the past ten years for various applications, such as protein enrichment of agricultural wastes (sweet potato[28], potato and cassava flour[21]); ethanol production from apple pomace[13], pinneaple waste[16], sweet sorghum[7] or rice and maize wastes[23]. Among the great diversity of yeasts studied, few papers report the use of Candida strains. Nevertheless, a study employing C. lipolytica on rice wastes medium and complemented with amylolytic enzyme showed the ability of the yeast to grow in solid state fermentation conditions and displayed the potential of respirometry to estimate microbial growth[22]. Another paper reported the use of a Candida utilis strain to ferment ryegrass straw to increase its in vitro digestibility, protein content and crude fat content[12]. The fermentation, achieved under semisolid conditions (70% moisture), showed the ability of the yeast to grow on pre-hydrolyzed straw and to support drastic conditions (temperature varying from 20°C to 40°C, and oxygen partial pressure close to zero)[12].

To solve the problem of gaseous ethanol containing streams, the biotransformation of ethanol in the gas phase into ethyl acetate is currently under study. As the first step, the feasability of growing Candida utilis is approached. The growth kinetics of the yeast in solid state fermentation using three different supports : natural without any culture medium (wheat bran), sugar cane bagasse and synthetic polymer (Amberlite), both complemented with an appropriate culture medium have been followed with cell count, dextrose consumption rate and respirometry.

MATERIALS AND METHODS

Microorganism and culture media.

Candida utilis ATCC 9950 (CDBB L245) was maintained on slants of potato dextrose agar medium (PDA) at 6°C. To prepare the inoculum, it was grown in 50 ml of dextrose (20 g/l) and malt extract (20 g/l) medium in 150 ml erlenmeyer flask with shaking at 200 rpm at 30°C. The yeast was then grown on the prepared support complemented with the minimal salts medium of Thomas and Dawson[25].

Substrate and supports preparation.

- Wheat bran was milled and sieved through -20+40 mesh screens to obtain particles of 0.42 to 0.82 mm in diameter. It was then autoclaved at 121°C for 15 min and its pH adjusted to 6.
- Sugarcane bagasse was washed with distilled water and prepared in the same way as the wheat bran.
- Amberlite (IRA 900) - an anionic ion exchange resin with a macroreticular structure - was purchased from Rhom and Hass and pretreated as described by Auria and al.[5].

Solid state culture conditions.

The initial conditions were :

. pH : 6 and Temperature : 30°C

. Moisture contents were chosen as the moisture saturation of each material: Wheat bran: 50% (w/w), Bagasse : 63% (w/w), Amberlite 58% (w/w).

. Yeast inoculum concentration : $1x10^7$ yeasts/g Initial Dry Matter (IDM).

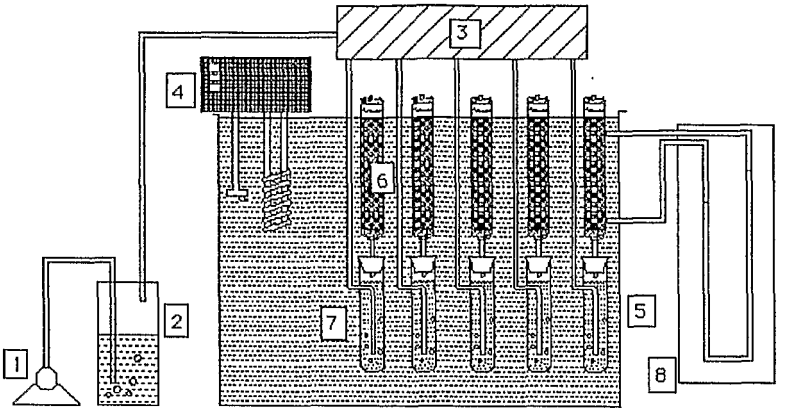
. Aeration rate : 0.1 l/h.g IDM

. Packing density : 0.3 g/cm^3 reactor except for Amberlite (0.6 g/cm^3).

. In the case of bagasse and Amberlite, the supports were complemented with the liquid culture medium to reach a dextrose concentration of 40 mg/g IDM. The wheat bran was complemented only with microelements used in the Thomas and Dawson's culture medium.

Fermentation system.

A schematic diagram of the experimental set up is shown in Figure 1.



1. Air supply, 2. Humidifier, 3. Air collector, 4. Temperature controller
5. Water bath, 6. Column fermentor, 7. Humidifiers, 8. Pressure drop gauge.

Figure 1 : Experimental set up for the Solid-State columns fermentors.

The columns were filled with aproximatively 12g of dry matter and fed with pre-humidified air. For each experimental sample, a column was removed from the water bath and total residual sugars, cell number, cell viability, pH and moisture content were determined. One column was maintained throughout the fermentation to assay exit gas composition.

Analytical procedures

Sugar determination : In the case of wheat bran, 2 g were heated for 2.5 h with 200 ml H_2O and 20 ml of HCl with reflux. The hydrolyzate was then refrigerated, neutralized with NaOH and discolored with the Carrez solution[20]. Sugar measurements were made with the phenol sulfuric method[10]. Reducing sugars in bagasse and Amberlite were measured by the DNS method[18] after washing 1 g of sample in 25 ml of water, homogenizing with Ultra turax, centrifugating and collecting the solids-free phase.

Cell number was determined by dispersing the samples in distilled water with 9 g/l NaCl, homogenizing with blender (1 g in 25 ml), adding methylene blue solution to determine the proportion of viable cells and counting in a Neubauer chamber. In liquid culture, it was found that 10^7 cells were equivalent to 0.092 mg of biomass (correlation coefficient = 0.993).

pH was determined by mixing 1 g of sample with 25 ml of distilled water and homogenizing for 5 mn.

Water content of the medium was determined by drying the sample in an oven at 95°C during 24 hours to a constant weight.

Carbon dioxide evolution in the exit gas of the column was measured with a gas chromatograph (Gow Mac, USA) equipped with thermal conductivity detector and a concentric column CTR1 (Alltech, USA). Carbon dioxide production rate values (CDPR) were then calculated with the following equation :

$$\mathbf{CDPR} = (\%\ CO_{2\ \text{produced}} \times F) / (100 \times W)$$

where F = Input air flow (ml/h) and W = Initial dry matter loaded in the column (g) This value is expressed as ml/g IDM . h. At the atmospheric pressure of Mexico (580 mm Hg), 1ml CO_2 corresponds to 0.0305 mmol CO_2.

RESULTS AND DISCUSSION

As a first part of this work, the ability of Candida utilis to grow on different complex carbon sources was studied. The yeast was cultured on Petri dishes with the carbon sources (20 g/l) mixed with agar-agar. Results are presented in Table1.

Table 1 : Growth tests of C. utilis on Petri dishes with different carbon sources (- none, + little, ++ good, +++ plentiful)

	24 h	48 h	120 h
Casein	-	-	-
Starch	+	+	++
Maltose	++	+++	+++
Cellulose	-	-	-
Pectin	++	++	++

The yeast displayed a significant growth when cultivated on maltose or pectin and surprisingly a regular growth on starch. This may be explained by a possible partial hydrolysis of the starch during the sterilization of the medium. From these data, it was also decided to test the growth ability of the yeast on heat treated wheat bran in SSF.

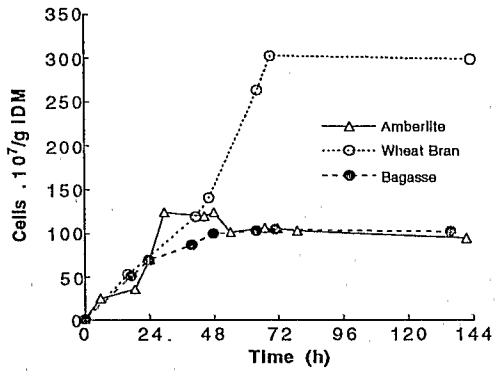


Figure 2 : Evolution of number of cells vs. time with 3 different supports.

Figure 2 shows that the time for reaching the maximum number of cells was about two days for the yeast grown on Amberlite and bagasse and three days on wheat bran. These maxima were 1.2 x10^9; 1.1 x10^9 and 3.0 x10^9 cells/g IDM which corresponds to 11.04; 9.66 and 27.6 mg/ g IDM of dry biomass respectively. No pseudo mycelium was observed. Wheat bran allowed the best growth because of its higher carbohydrates content (about 400 mg/g IDM) but the relatively lower growth rate in this case can be explained by the limited availability of assimilable sugars. Number of cells were higher than those obtained with C. utilis when grown during 3 days on pre-hydrolyzed ryegrass straw (1x10^9 from 1x10^8 initial cells/g IDM)[12].

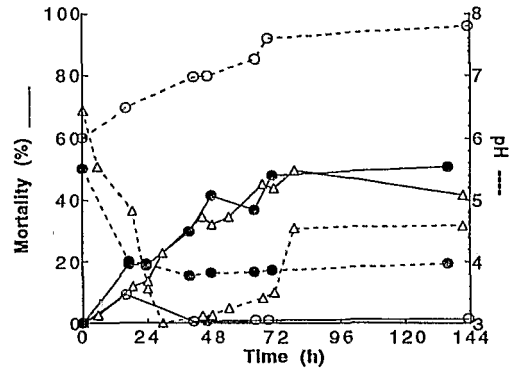


Figure 3 : Evolution of cells mortality and pH vs. time with three different supports. (Δ Amberlite, o Wheat bran, • Bagasse)

Mortality curves for Amberlite and bagasse were very similar, increasing slowly during the first 3 days to reach 50% while cell mortality remained close to zero for wheat bran throughout the fermentation (figure 3). This difference can be explained by the important drop in pH observed for both Amberlite and bagasse within the first 24 hours -probably due to the production of organic acids - and then remaining close to 4 for bagasse and 3 for Amberlite during two days. This showed the relatively poor buffering properties of these two supports. This can be explained also by the rapid dextrose consumption in both cases (figure 4). On the contrary, pH increased from 6 to nearly 8 for wheat bran probably because of the partial proteolysis of the bran. It may be observed in each case, that in spite of a previous buffering step of the solid substrates, pH regulation might become an important problem.

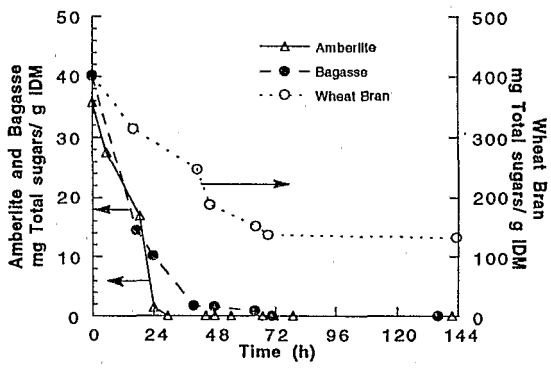


Figure 4 : Total sugar evolution vs. time for the three supports.

Figure 4 shows that for Amberlite and bagasse, all initial dextrose was consumed, in 24 and 48 hours, respectively, and for wheat bran residual sugars reached a final value of 130 mg/g IDM (67% of initial sugars consumed) after 72 hours of fermentation. These different sugar consumption rates may be explained by the different availability of carbohydrates in each substrate.

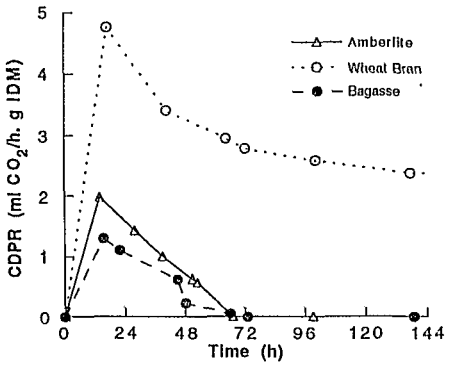


Figure 5: Evolution of carbon dioxide production rate vs. time for the 3 supports.

As may be expected, the highest carbon dioxide production rate (CDPR) - close to 5 ml/h . g IDM- was reached with wheat bran corresponding to the highest biomass production and highest sugar consumption (figure 5). CDPR maxima coincide in the three cases : around 20 hours, at the end of the exponential growth phase. Nevertheless, a major difference is observed between Amberlite and bagasse in one hand and wheat bran in the other hand : while in the two first cases, CDPR decreased to zero in the same manner as dextrose consumption, for wheat bran the value of CDPR remained constant at 2.5 ml/h . g IDM after 2 days of fermentation and the total sugars consumption stopped. It may be assumed that after consuming all the available sugars, the yeast began or continued to metabolize other kinds of substrates such as proteins or produced ethanol. This could explain the constant increase of pH.

In order to test the behavior of the yeast with higher dextrose concentrations, other fermentation runs with 135 and 200 mg/g IDM of dextrose on Amberlite were performed. Results are presented in Figure 6. For a dextrose concentration of 200 mg/g IDM, a much larger amount of biomass was produced (46.18 mg/g IDM) with nearly complete dextrose consumption and without any significant inhibition. Nevertheless, cell mortality was above 50% at the end of the fermentation probably due to the pH drop (2.58). Moreover, no net lysis was observed as may be evidenced by the stability of the cell count after the substrate was consumed. Table 2 summarizes the results of the growth of C. utilis.

Table 2 : Solid state fermentation results of Candida utilis grown on wheat bran, impregnated bagasse and Amberlite. * YX/S : Yield of substrate conversion into biomass.

Supports / Substrate	Amberlite	Amberlite	Amberlite	Sugarcane bagasse	Wheat bran
Initial sugars (mg/g IDM)	40	135	200	40	402
Residual sugars (mg/g IDM)	0	0	6.4	0	128
Final Biomass (mg/g IDM)	11.41	27.05	46.18	9.66	27.78
YX/S* (g/g)	0.285	0.200	0.238	0.241	0.131
Final pH	4.59	5.66	2.58	3.97	7.80
Initial moisture (%)	58	58	58	63	50
Final moisture (%)	55	61	68	68	57
CDPR max (ml/h.g IDM)	1.42	1.72	3.70	1.29	4.76

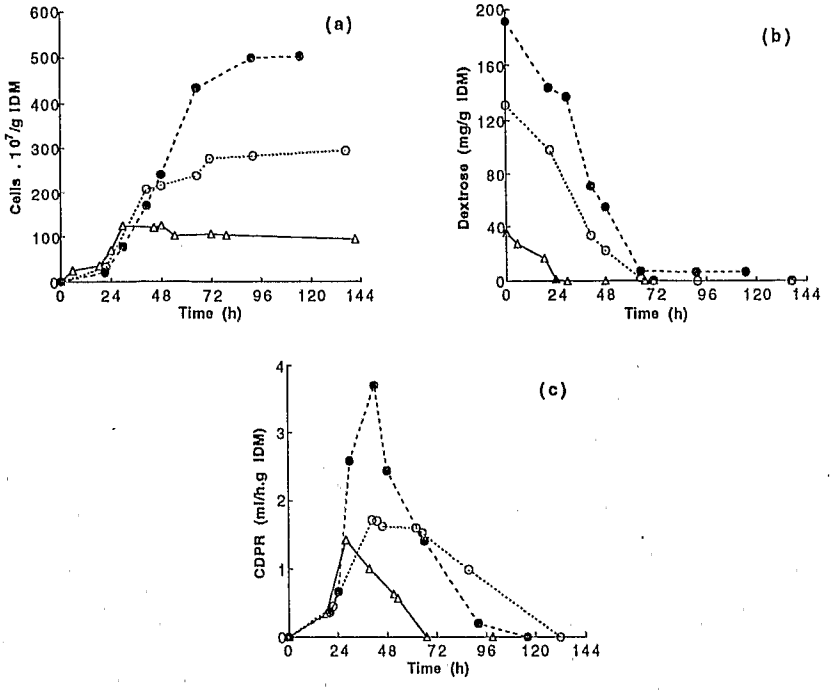


Figure 6 : Candida utilis growth on Amberlite with 3 different initial dextrose concentrations (Δ--Δ 40mg/g IDM, o...o135mg/g IDM, ●--● 200mg/g IDM). Cell growth (a); Dextrose consumption (b); Carbon dioxide production rate (c).

The yield values were found to be between 0.2 and 0.285 for Amberlite and cane bagasse while a lower value was found on wheat bran. Experiments performed in liquid fermentation with the dextrose- mineral medium showed similar yields. The values obtained are lower than those reported for the growth of C. utilis for biomass production but they reflect the effect of ethanol accumulation [25]. The maximum CDPR for Amberlite at 200 mg/g IDM is similar to those reported for A. niger on the same support[5].

CONCLUSION

The ability of Candida utilis to grow on solid substrate (wheat bran) or impregnated solid support (natural: sugarcane bagasse or synthetic: Amberlite) has been demonstrated. Using Amberlite with an initial concentration of 200 mg/g IDM, a 500 fold increase in biomass was observed. This biomass shows a good viability even after the substrate is utilized. Strong variations were found for the final pH and moisture content depending on the substrate. The use of the supported cells is now being studied for the transformation of ethanol to ethyl acetate.

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