Growth of *Candida utilis* on Amberlite with Glucose and Ethanol as Sole Carbon Sources

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The results of Candida utilis *growth on an anionic resin (Amberlite) at high glucose concentration and using ethanol as the sole carbon sources are presented. The yeast consumed 240 mg glucose (g initial dry matter)$^{-1}$ (IDM) reaching a final population of 5.6x10^9 cells (g IDM)$^{-1}$ (initial inoculum size: 1x10^7 cells (g IDM)$^{-1}$. It was also shown that respirometry was a reliable on-line method for monitoring growth. The respiratory quotient (RQ) showed the changes in the metabolism of the yeast during glucose consumption, from a fermentative to an oxidative route. When* C. utilis *was grown on gaseous ethanol enriched air, a final population of 3.25x10^9 cells (g IDM)$^{-1}$ was attained. The importance of mineral salts concentration in the nutritive medium was clearly demonstrated. A two fold increase in the population was obtained when the mineral medium was not limiting. Small amounts of acetaldehyde and ethyl acetate were detected at the outlet of the reactor (1.88 µl l^{-1} and 0.87 µl l^{-1}, respectively). Ethanol accumulated in the reactor up to 120 mg (g IDM)$^{-1}$ (probably an inhibitory level for this yeast). RQ remained constant at around 0.6 during the fermentation.*

Solid state fermentation (SSF) is an old technique that has been recently revaluated and modernized to enhance protein content of agro-industrial wastes (1,2,3) or to produce enzymes (4), secondary metabolites (5), spores of fungi (6) or cheese or fruity flavors (7,8). Some reports deal more specifically with yeasts employed in SSF systems (9,10,11) and recently, the ability of a *Candida utilis* strain to grow on various substrate/supports was demonstrated (12).

Furthermore, yeasts from the genius *Candida* are known to be able to convert ethanol to ethyl acetate (13,14) or acetaldehyde (15). Both components present an economical interest in the food additives industry (16).

In this work, the effect of inoculum concentration and high glucose concentrations on growth of *C. utilis* on a synthetic support (Amberlite) under SSF conditions was evaluated in terms of kinetic and respirometric parameters. The ability of this yeast to grow and produce ethyl acetate and acetaldehyde when fed with air enriched with gaseous ethanol was also studied.

MATERIAL AND METHODS

Microorganism

The yeast *Candida utilis* ATCC 9950 (CDBB L245) was used in all experiments. It was periodically transfered on potato dextrose agar slants and stored at 4°C.

Culture media

The inoculum was grown on a liquid medium composed with glucose (20 g/l) and malt extract (20 g/l) in 150 ml Erlenmeyer flasks agitated at 200 rpm at 30°C for 20h. The yeast was then grown on Amberlite IRA-900 (Rohm & Haas), an anionic resin, prepared according to Auria *et al.*(17) and imbibed with the minimal salts medium described by Thomas and Dawson (18). This nutritive medium was used with different glucose concentrations from 40 to 240 mg/g Initial Dry Matter (IDM). For the case of cultures on ethanol, glucose (40 mg/g IDM) was used in the beginning of the fermentation and then gaseous ethanol was fed.

Solid State Fermentation procedure

Cultures were performed in the set up represented in Figure 1.

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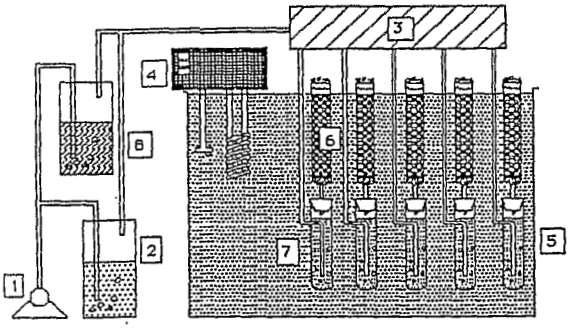

1. Air supply, 2. Humidifier, 3. Air Collector, 4. Temperature controller
5. Water bath, 6. Column fermentor, 7. Humidifiers, 8. Ethanol supply

Figure 1. Experimental set up for SSF.

In the experiments run with ethanol, the alcohol was fed to the columns by bubbling air in pure solution as it was done for water in air.
The initial culture conditions were: pH, 6; Temperature, 30°C; Moisture content, 58% (w/w); yeast inoculum concentration, $1x10^7$ cells/g IDM except when mentioned; aeration rate, 0.1 or 0.05 l/h.g IDM, ethanol rate feed, 5 µl/h.g IDM and packing density, 0.6 g/ml.

Analytical methods

Biomass was determined by direct cell count and viability with methylene blue coloration as described previously (12). In liquid culture, it was found that 10^7 cells correspond to 0.092mg (12). pH was measured with a Conductronic pH meter and Aw with an Aqualab CX-2 apparatus (Decagon, USA). With the same sample, glucose was determined by the dinitrosalicylic acid method (19) and residual ethanol was measured by gas chromatography. Headspace analysis of the exit air was achieved for acetaldehyde, ethanol and ethyl acetate determination. Gas chromatography analysis was made with a Hewlett-Packard chromatograph equipped with a flame ionization detector. Nitrogen was used as carrier gas at a 4 ml/mn rate. Split ratio was 1:50. Temperature were: injector and detector, 180°C; oven, 40°C. Separation was achieved with a Megabore HP-1 column (Length, 5m; Inner diameter, 0.53 mm). Concentrations were reported as µl liquid/l gas.
Respirometry (O2 and CO2 measurements) was realized with a Gow-Mac chromatograph equipped with a thermal conductivity detector and a concentric column CTR-1 (Alltech, USA). Helium was used as carrier gas (flow rate, 60 ml/mn). Carbon dioxide production rate (CDPR), oxygen uptake rate (OUR) and respiratory quotient (RQ) were calculated as follows :
CDPR = (%CO2 produced x F) / (100 x W)
OUR = (%O2 consumed x F) / (100 x W)
RQ = CDPR/OUR.

RESULTS AND DISCUSSION

Glucose as sole carbon source

.Influence of inoculum size

These experiments were run with an initial glucose concentration of 135 mg/g IDM and inoculum concentration of 1.4, 2.2 and $3.6x10^7$ cells/ml respectively.

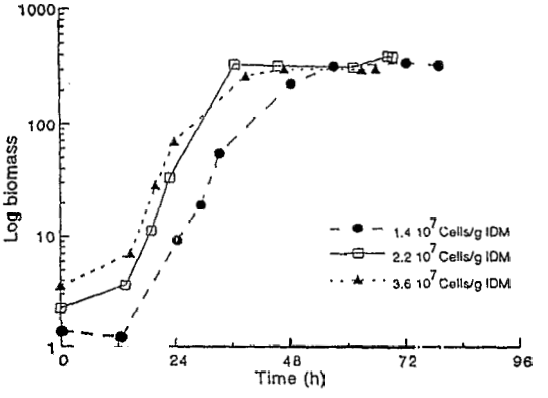

Figure 2. Logarithmic evolution of biomass vs. time. Influence of inoculum size.

In Figure 2, it can be seen that inoculum size did not have a strong influence on growth rate. There is not a significant influence on the final biomass (between 300 and $350x10^7$ cells/g IDM) and all the glucose was exhausted after 50 hours. The same conclusions were obtained in submerged cultures.

.Influence of initial glucose concentration

Three different glucose concentrations were studied: 40, 135 and 240 mg/g IDM. It appeared that the phase lag and the fermentation time were longer, and the maximum biomass was reached later for higher glucose concentrations (Cf Figure 3). These maxima were related to the initial glucose concentration (more than $550x10^7$ cells/g IDM

for 240 mg/g IDM).

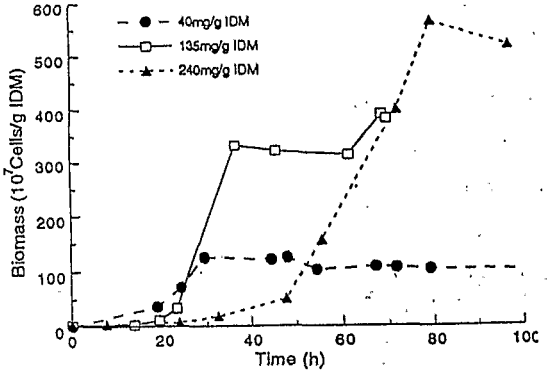

Figure 3. Evolution of biomass vs. time. Influence of initial glucose concentration.

Glucose was totally exhausted in all cases at different time courses (Figure 4). It can be seen that glucose concentration in SSF system has a strong influence on Aw (for the same initial moisture content). The relatively low value of Aw at the beginning of the fermentation for 240 mg/g IDM can explain the largest lag phase. When glucose was exhausted, Aw reached a value of 0.99 in both cases.

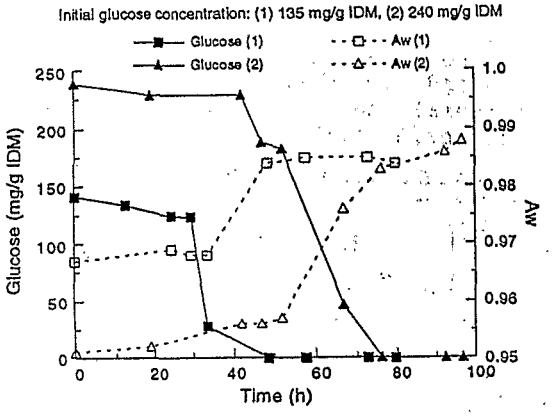

Figure 4. Evolution of glucose concentration and Aw vs. time. Influence of initial glucose concentration.

In Figure 5, it can be observed that despite the previous neutralization of the support (to pH 6), a dramatic drop in pH was observed (around 3.2 for low glucose concentration and 2.4 for high concentrations). This severe drop can be explained by the excretion of organic acids (e.g. acetic) to the medium. These final values were reached faster for lower glucose concentrations which was related to growth and ammonium sulfate consumption and proton excretion. Mortality among the yeast cells was low except for the final time for the higher glucose concentration. This behavior was related to low pH (2.3) and lack of carbon sources.

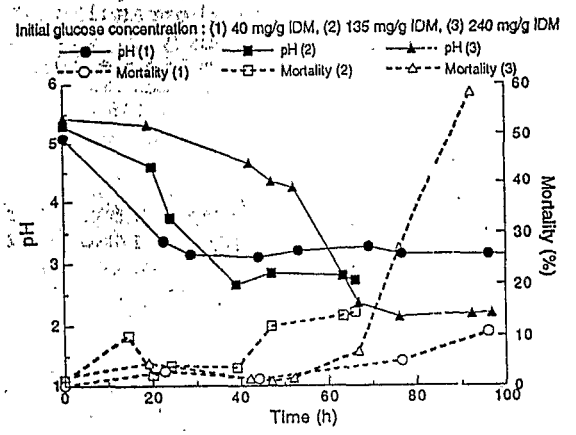

Figure 5. Evolution of pH and mortality vs. time. Influence of initial glucose concentration.

Figure 6 shows the CDPR evolution for different initial sugar concentrations. These curves present a maximum peak corresponding to the maximum volumetric growth rate. These maxima were reached in 28h, 34h and 55h respectively and are proportional to the initial glucose concentration.

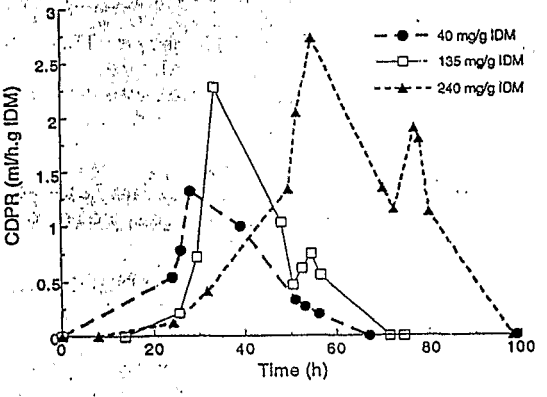

Figure 6. Evolution of CDPR vs. time. Influence of initial glucose concentration.

In the cases of 135 and 240 mg/g IDM, a second peak smaller and later was observed. It appeared when glucose was exhausted and was probably due to a diauxic effect from other substrates released previously in the medium (e.g. ethanol or organic acids). The maxima values of CDPR reached are comparable with those obtained by *Auria et al.* (17) for *Aspergillus niger* grown on the same support.

Table 1. Summing up of the results of the glucose fermentation by *C. utilis* grown on Amberlite. (* Cf Figure 10)

Initial glucose (mg/g)	40	135	240
X max	11.41	27.05	52.07
Fermentation time (h)	29.5	47	80.5
CDPR max	1.42	2.28	2.73
Δ R.Q.*	1.1/1.3	1.1/1.3	1.0/2.5
μ max	n.c.	0.55	0.58
Yx/s	0.283	0.201	0.225
Rx max	0.74	0.83	1.97

From table 1, it can be concluded that biomass production, in the range of the glucose concentrations studied, was proportional to the initial concentrations. The substrate conversion yields into biomass kept within a range of 0.2 to 0.28 with highest value for lowest concentration. There is no significant influence of glucose concentration on growth rate. On the contrary, the volumetric productivity (Rx) is greatly increased when substrate concentration is higher. There is also a clear influence of glucose level on the orientation of the metabolism. The respiratory quotient reached values superior to 2 (fermentative route) for 240 mg/g IDM, when these values kept near above 1 at lower concentrations (oxidative pathway). *C. utilis*, when grown on Amberlite, displayed a good tolerance toward high glucose levels, low pH value and seemed to be able to assimilate ethanol - or other metabolites released in the medium.

Ethanol as consecutive carbon source

.Influence of mineral medium concentration

Two experiments were made: one with mineral medium concentration corresponding to 40 mg/g IDM of glucose, the other one multiplied by a factor of 3.5 In both cases, the fermentation was initiated with glucose (40 mg/g IDM) and ethanol vapor phase fed when glucose was totally exhausted (after 24 hours). Growth evolution is shown in Figure 7.

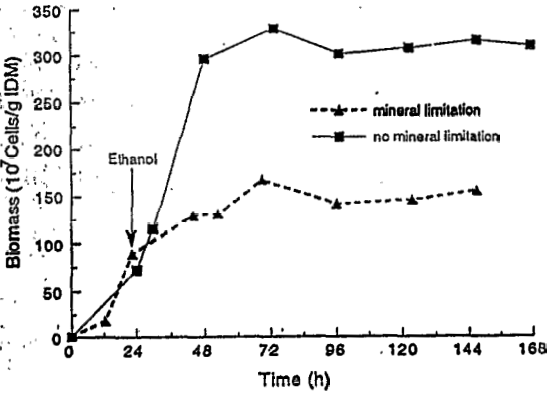

Figure 7. Evolution of biomass vs. time. Influence of mineral medium concentration. Growth on ethanol.

The interest of using the higher mineral salts concentration was clearly demonstrated since a final population of 330×10^7 cells/g IDM was reached against 150×10^7 cells/g IDM for the lower concentration. The maxima were reached in the same time (about 75h). The fact that the increase in biomass was not proportional to the mineral medium concentration implies that there is another limitation. Only the second experience (with the highest mineral salt concentration) is described hereafter.

.Growth and substrates consumption

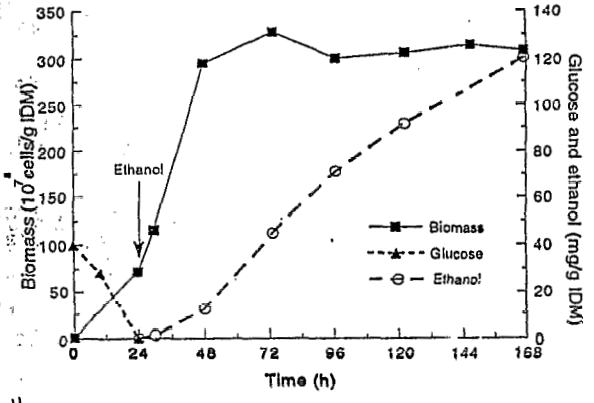

Figure 8. Evolution of biomass and substrates vs. time. Growth on ethanol.

From figure 8, it can be observed that the maximum population is reached after 75 hours when ethanol concentration in the medium was about 40 mg/g IDM and mortality yield was 16%. It can be seen that ethanol in the column increased constantly and with a higher velocity after 24 hours of culture on this substrate, which probably meant that the concentration reached was limitating but not lethal for the yeast (about 20 mg/g IDM). Meanwhile, the mortality yield increased up to 80% at the end of the fermentation. The yeast displayed a better tolerance toward ethanol than observed previously (13), which can be due to a protective effect of the support. The pH dropped to 2.5 in the first 30 hours and then kept constant around this value. Water activity was constant above 0.98 all along the experiment. It can be concluded from these data that SSF is an adequate system for the growth of *C. utilis* on gaseous substrate.

.Ethanol, ethyl acetate and acetaldehyde evolution in the exit gas

According to Armstrong *et al.* (13), ethyl acetate production from ethanol for *C. utilis* follows three steps:

1.Ethanol + O2 ----> Acetaldehyde
2.Acetaldehyde + O2 -----> Acetic acid
3.Ethanol+Acetic ac. --->Ethyl acetate + H20

These reactions are catalyzed by alcohol dehydrogenases (steps 1 and 2) and by an esterase (step 3).
The evolution of ethanol, acetaldehyde and ethyl acetate concentrations in the exit gas is plotted in figure 9.

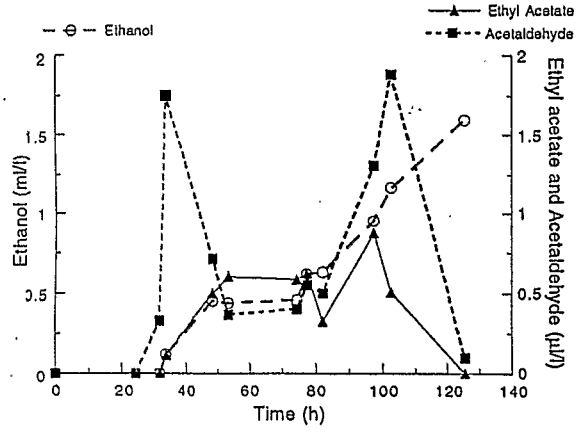

Figure 9. Headspace of solid state culture of *C. utilis* grown on ethanol.

The curves shown in figure 9 can be divided in three regions. From 24 to 55 hours, exit ethanol concentration increased as well as ethyl acetate and acetaldehyde, this one presented a maximum around 36 hours. From 55 to 75 hours, a steady state was set and the three concentrations remained constant. From 75 hours, when growth stopped, ethanol concentration increased again in relation to the concentration of the liquid phase, reflecting the fact that it was not consumed. No evidence was found that non-growing *C. utilis* cells produced ethyl acetate or acetaldehyde.
Most of the time, acetaldehyde levels were above those of ethyl acetate. Levels of both compounds were low probably because ethanol concentration was too high, pH low and hence inhibited redox enzyme system of the yeast. It should be observed that neither acetaldehyde nor ethyl acetate were detected in the solid state medium. By integration of the ethyl acetate and acetaldehyde curves, it was calculated that 23 mg and 38 mg were produced respectively.
Finally, a comparison of the respiratory coefficients in three cases is presented in figure 10.

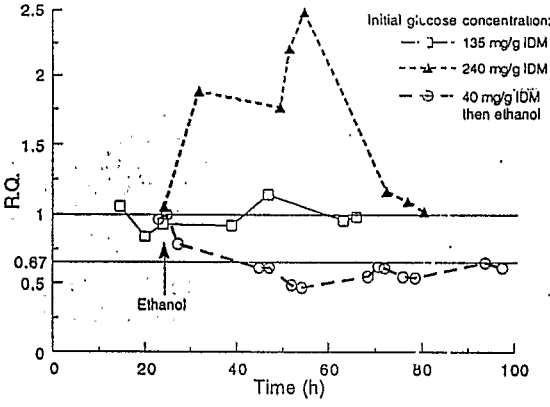

Figure 10. Evolution of the respiratory quotient vs. time on different glucose concentrations and on ethanol.

The evolution in each case is very significative:

. for 135 mg glucose/g IDM, RQ kept constant around a value of 1, characteristic of the oxidative metabolism of glucose, where :

$Glucose + 6O_2 \rightarrow 6CO_2 + 6H_2O + 28\ ATP$
(theoretical RQ = 1)

. for 240 mg glucose/g IDM, RQ varied between 1 and 2.5. This is characteristic of a partial orientation of the metabolism toward a fermentative route *sensu stricto*, where :

$Glucose \rightarrow 2CO_2 + 2\ Ethanol + 2\ ATP$
(theoretical RQ $\rightarrow \infty$)

. for growth on ethanol, RQ was constant around 0.6. This represents the oxidative metabolism of ethanol where:

$Ethanol + 3O_2 \rightarrow 2CO_2 + 3H_2O + 11ATP$
(theoretical RQ = 0.67)

Although *C. utilis* showed its capacity to grow on ethanol as sole carbon source, the overall results of the fermentation are lower than those obtained on glucose (μmax, Rx max) basically for the reason given above (cf Table 2).

Table 2. Kinetic parameters of the fermentation of ethanol by *C. utilis* on Amberlite.

X max	30.17
CDPR max	2.15
Δ R.Q.	0.5/0.72
μ max	0.13
Rx max	0.41

CONCLUSION

In this work, the influence of glucose concentration on growth was shown. It was demonstrated that total biomass, lag phase duration and yield of substrate conversion into biomass were dependent on initial glucose concentration as well as the type of metabolism (oxidative or oxidative/fermentative) as shown by the respiratory quotients study. Also, even at the higher glucose concentration, the relatively low water activity observed did not avoid the growth. The main limitation is finally due to the strong pH drop and the accumulation of ethanol in the water phase.

A SSF system was successfully designed to study the growth of the yeast with gaseous ethanol. It was shown that mineral salts concentration could be a limiting factor for growth and that *C. utilis* was able to use ethanol for its growth and to produce other metabolites. Moreover, the SSF system provided the advantage to increase the tolerance of the yeast toward ethanol. Although the conditions were not optimized, low excretion of ethyl acetate and acetaldehyde were obtained in the outlet gas phase.

R. Marcos was a post-graduate student from ENSBANA (Dijon-FRANCE). This work was achieved under research agreement between UAM (MEXICO) and ORSTOM (FRANCE).

NOMENCLATURE

Aw	Water activity (-)
CDPR	Carbon dioxide production rate (ml/h.g IDM)
F	Inlet aeration rate (ml/h)
OUR	Oxygen uptake rate (ml/h.g IDM)
R.Q.	Respiratory quotient (-)
Rx	Volumetric growth rate (mg biomass/g.h)
W	Initial dry matter weight (g)
X	Biomass (mg/g IDM)
Yx/s	Biomass yield on substrate (g biomass/g substrate)
μ	Specific growth rate (h^{-1})

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