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Cultivation of *Candida blankii* in simulated bagasse hemicellulose hydrolysate

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SUMMARY

All four *Candida blankii* isolates evaluated for growth in simulated bagasse hemicellulose hydrolysate utilized the sugars and acetic acid completely. The utilization of D-xylose, L-arabinose and acetic acid were delayed by the presence of D-glucose, but after glucose depletion the other carbon sources were utilized simultaneously. The maximum specific growth rate of 0.36 h^{-1} and cell yield of 0.47 g cells/g carbon source assimilated compared favorably with published results obtained with *C. utilis*. *C. blankii* appeared superior to *C. utilis* for biomass production from hemicellulose hydrolysate in that it utilized L-arabinose and was capable of growth at higher temperatures.

INTRODUCTION

The hemicellulose fraction of sugar cane bagasse is easily hydrolyzed and extracted by mild acid treatment, yielding D-xylose, D-glucose and L-arabinose as the major sugars [5] as well as acetic acid originating from the acetyl groups of the xylan. For biomass production, it is advantageous to use a yeast capable of growth on L-arabinose as well as on acetic acid.

It is known that D-glucose often inhibits the utilization of other carbohydrates [13] due to catabolite repression. Heredia and Ratledge [9] examined the utilization of glucose and xylose by ten yeast species and only *Candida curvata* D was capable of simultaneous sugar assimilation. Hsiao et al. [12] found that the yeasts *Candida utilis*, *Rhodotorula toruloides*, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* utilized D-xylose only after depletion of D-glucose during growth on a mixture of these sugars. Futhermore, D-glucose strongly inhibited utilization of both D-xylose and D-xylitol by *C. utilis* and *R. toruloides*. The presence of D-glucose in bagasse hemicellulose hydrolysate may therefore cause diauxic growth and thereby prolong the cultivation time in batch cultures.

The growth of four *C. blankii* isolates for SCP production in a simulated sugar cane bagasse hemicellulose hydrolysate was evaluated.

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MATERIALS AND METHODS

Microorganisms

The *C. blankii* isolates UOVS-63.1, UOVS-64.1 and UOVS-64.2 were all isolated from soil obtained from the Senekal district [14]. Isolate CSIR-ESP94, also a soil isolate, was obtained from J.P. van der Walt of the CSIR, Pretoria.

Reactor cultivation

Batch cultivations were conducted in a Multigen F-2000 (New Brunswick Scientific Co.) bioreactor fitted with a reflux cooler on the gas exhaust to minimise evaporation. The 2-l glass vessel contained 1 l medium consisting of (g/l) citric acid 0.25; $(NH_4)_2SO_4$ 17; KH_2PO_4 3; $MgSO_4 \cdot 7H_2O$ 0.8; $CaCl_2 \cdot 2H_2O$ 0.05; antifoam 1 ml (Foam-Knox, Chemserve, Johannesburg); trace elements solution 1 ml, [4] with a mixture of 7.2 g/l D-xylose, 0.5 g/l D-glucose, 0.8 g/l L-arabinose and 1.5 g/l acetic acid, representing the ratios occurring in bagasse hemicellulose hydrolysate [20], added to the medium so that the total carbon source amounted to 10 g/l. The dissolved oxygen tension (DOT) was maintained at 20% air saturation using a polarographic oxygen electrode (WTW, Weilheim, F.R.G.) linked to a dissolved oxygen controller [3]. The agitation speed varied between 200 and 600 rpm during the cultivation while the aeration rate was maintained at 1.0 l/min. The cultivations were conducted at 38 °C and the pH was automatically controlled at pH 5.5 with 5 N HCl and 5 N NaOH. A 20-ml volume of the inoculum was used to inoculate the reactor.

Utilization of sugar mixtures

The growth and utilization of different mixtures of carbon sources by *C. blankii* UOVS-64.2 were determined in 1-1 Erlenmeyer shake flasks containing 100 ml medium at pH 5.5 and 38 °C on a rotary shaker at 180 rpm. The mineral medium was the same as that used during bioreactor cultivation, except that the KH_2PO_4 concentration was increased to 10 g/l for additional buffering capacity and the carbon sources were supplied at approximately the same concentration range, as indicated in Fig. 2. Each shake flask received 2 ml inoculum.

Inoculum

The yeasts were inoculated from 24-h-old glucose-peptone-yeast extract agar slants into 500-ml Erlenmeyer shake flasks containing 50 ml medium (pH 5.5) with 10 g/l D-xylose and incubated for 24 h at 30 °C on a rotary shaker at 160 rpm. The medium was the same as that used in the 1-1 shake flasks as described above.

Analytical procedures

Sugars were determined by high performance liquid chromatography and acetic acid by gas chromatography as described by Van Zyl et al. [20]. Growth was monitored with a Klett-Summerson colorimeter at 640 nm. Dry cell mass was determined gravimetrically by drying centrifuged and washed samples to constant mass at 105 °C. The protein content of the cells was determined by the biuret method [8] using bovine serum albumin, fraction V (Sigma Chemical Co., St. Louis) as protein standard.

RESULTS

Reactor cultivation

Figure 1 depicts the time course of growth and substrate utilization of isolate UOVS-64.2 in simulated hemicellulose hydrolysate. The other *C. blankii* isolates gave very similar results (data not shown). Although no diauxic growth was observed with any of the yeasts examined, D-glucose was utilized first and D-xylose utilization started on depletion of D-glucose (Fig. 1). Acetic acid and L-arabinose were assimilated only towards the end of the cultivation.

There was very little difference between the yield coefficients, growth rates and protein contents of the *C. blankii* isolates, except in the case of isolate CSIR-ESP94 which exhibited a slightly lower growth rate than the other isolates (Table 1). This isolate consistently gave μ_{max} values on D-xylose lower than the other isolates [14].

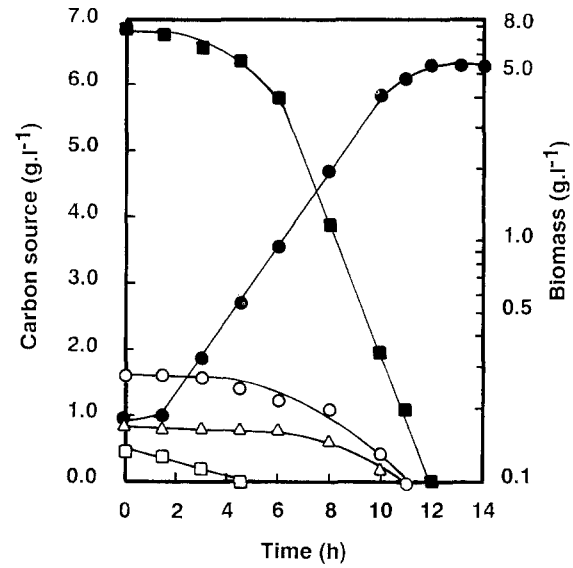

Fig. 1. Biomass production and carbon source utilization by *Candida blankii* during cultivation in a simulated bagasse hemicellulose hydrolysate at 38 °C and pH 5.5. Symbols: (●) biomass; (□) D-glucose; (■) D-xylose; (△) L-arabinose and (○) acetic acid.

TABLE 1

Comparative evaluation of *Candida blankii* isolates grown in 10 g/l simulated bagasse hemicellulose hydrolysate at 38 °C and pH 5.5 in a bioreactor in terms of specific growth rate (μ_{max}), cell yield ($Y_{x/s}$), protein yield ($Y_{p/s}$) and protein content (% protein). The cultivation conditions of *C. utilis* and *Geotrichum candidum* differed from those of the other cultures (see references). The yield coefficients are expressed in terms of carbon substrate assimilated.

Yeasts	μ_{max} (h^{-1})	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (g/g)	Protein (%)
Candida blankii				
UOVS-63.1	0.37	0.46	0.22	48.8
UOVS-64.1	0.38	0.46	0.22	47.4
UOVS-64.2	0.36	0.47	0.21	47.0
CSIR-ESP94	0.31	0.48	0.23	48.4
Candida utilis				
ATCC 9256[a]	0.15	0.25	nd	nd
ATCC 9950[b]	nd	0.28	0.11	39.3
ATCC 9256[c]	0.30	0.38	nd	nd
Geotrichum candidum				
AN1[a]	0.20	0.39	nd	nd

[a] In a mixture of 40 g/l xylose and 15 g/l sodium acetate [10].
[b] In bagasse hemicellulose hydrolysate [21].
[c] In spent sulphite liquor permeate [19].
nd: not determined.

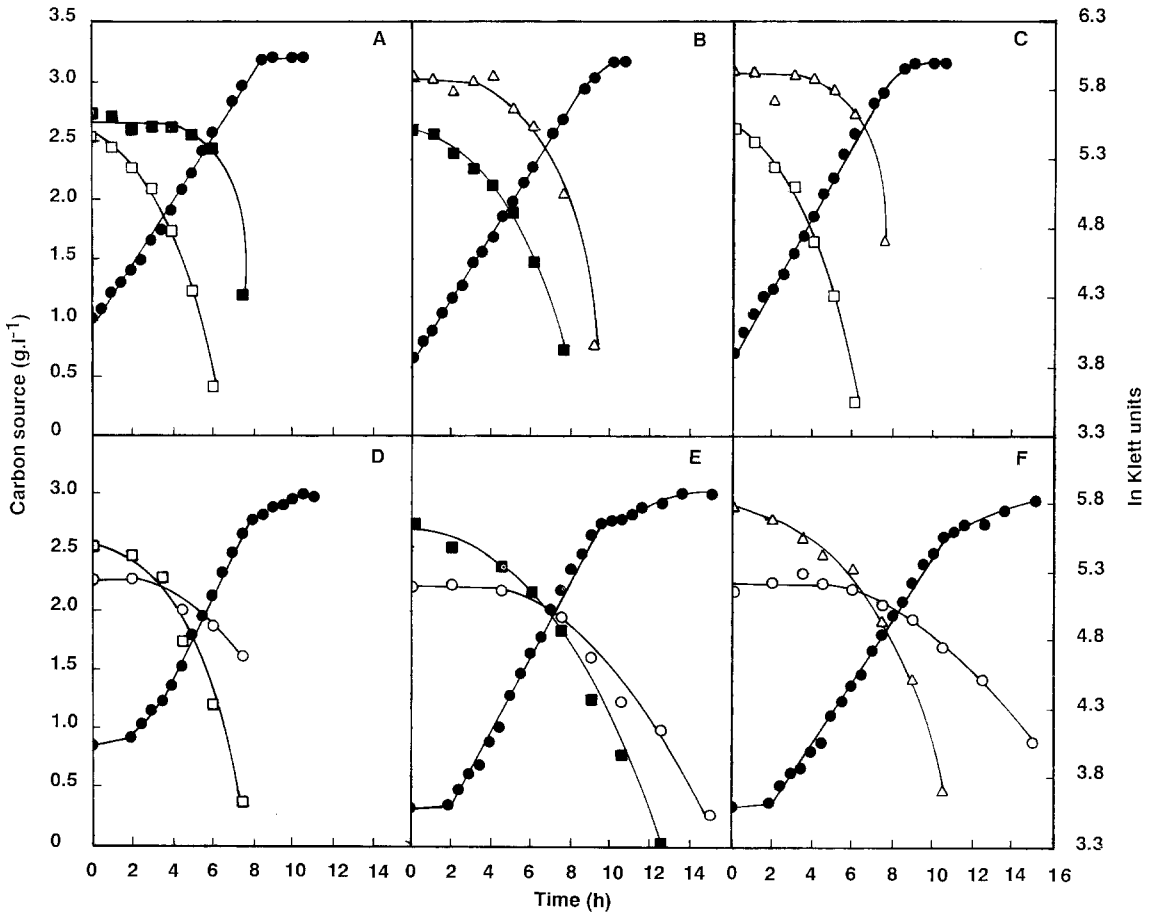

Fig. 2. Utilization of different carbon source mixtures by *Candida blankii* in shake flasks at pH 5.5 and 38 °C. Symbols: (●) turbidity in Klett units; (□) D-glucose; (■) D-xylose; (△) L-arabinose and (○) acetic acid.

Utilization of sugar mixtures

Utilization of D-xylose and L-arabinose were inhibited by D-glucose (Fig. 2A, C), but utilization of the second carbon source commenced before glucose depletion. In contrast with the results obtained with the arabinose and acetic acid mixture (Fig. 2F), D-xylose also inhibited the utilization of L-arabinose (Fig. 2B), although L-arabinose utilization started before D-xylose depletion. A lag phase in growth was observed only in the presence of acetic acid (Fig. 2D, E, F). Although delayed initially, acetic acid utilization started prior to depletion of the carbohydrate. Repression did not appear to be involved in the delay of substrate utilization in any of the mixtures, as indicated by the absence of a diauxic growth lag.

DISCUSSION

Diauxic growth and sequential substrate utilization are frequently observed when microorganisms are exposed to multiple carbon substrates [6, 7, 18]. D-Xylose utilization by yeasts has also been reported to be inhibited by D-glucose [2]. Although no diauxie was observed with any of the four yeasts examined, sequential substrate utilization did occur. Substrates utilized sequentially in batch culture may be utilized simultaneously in carbon limited continuous culture at low dilution rates where the residual glucose concentration is low [17], indicating that a threshold glucose concentration has to be exceeded before catabolite repression is manifest. This may explain the utilization of D-xylose in the presence of low concentrations of D-glucose reported here.

Similar to the inhibition of the utilization of D-xylose and hexose sugars by D-glucose in *Candida shehatae* and *Pichia stipitis* [2] and *C. utilis* and *Rhodotorula toruloides* [12], D-glucose inhibited the utilization of D-xylose and L-arabinose in *C. blankii* (Fig. 2A, C). In *Pichia stipitis* D-glucose also inhibited D-xylose utilization, while L-arabinose was utilized concurrently with the residual xylose when gown aerobically on a mixture of these sugars [1]. This pattern of substrate utilization was also observed

in the simulated hydrolysate (Fig. 1). The delay in the utilization of L-arabinose and acetic acid following glucose depletion in the simulated hydrolysate was probably due to the presence of D-xylose, as indicated by Figs. 2B and E.

The lag phase in growth observed in the presence of acetic acid (Fig. 2) suggested that acetic acid was inhibitory to the cells. The undissociated form of acetic acid enters the cell by diffusion, where it dissociates, inducing acidification of the cytoplasma [15], resulting in enhancement of the membrane-bound ATPase activity and harmful dissipation of ATP [16]. The delay in acetic acid utilization during cultivation in the simulated hydrolysate was in contrast with the findings of Holder et al. [11] who found that *Candida utilis* and *Geotrichum candidum* utilized acetic acid and glucose simultaneously without lag when these yeasts were grown in bagasse hemicellulose hydrolysate medium.

The cell yield ($Y_{x/s}$), protein yield ($Y_{p/s}$) and protein content of the different *C. blankii* isolates were similar (Table 1). *C. blankii* CSIR-ESP94 exhibited a slightly lower maximum specific growth rate than the other isolates. The high protein content, which varied between 47 and 48%, makes these *C. blankii* isolates attractive for SCP production. Although the culture conditions for the cultivation of *C. utilis* and *G. candidum* differed from those used for the *C. blankii* isolates, it is evident that the *C. blankii* isolates outperformed both these organisms on bagasse hydrolysate (Table 1). The results obtained with *C. utilis* on spent sulphite liquor permeate, which is similar in composition to bagasse hemicellulose hydrolysate [19], also compared unfavourably with the *C. blankii* isolates. Unfortunately both Holder et al. [11] and Streit et al. [19] did not determine the protein yield and protein content of the yeasts examined, which are important parameters in investigations on single cell protein production.

The ability of these isolates to utilize L-arabinose gives them an advantage over *C. utilis* and *G. candidum*, because these yeasts failed to utilize the L-arabinose present in bagasse hemicellulose hydrolysate [11]. L-Arabinose can constitute up to 9.3% of the total hemicellulose sugars of acid-hydrolysed sugar cane bagasse [20]. A further advantage is that these *C. blankii* isolates grew well at a relatively high temperature of 38 °C.

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