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# Cultivation of *Candida langeronii* in sugar cane bagasse hemicellulosic hydrolyzate for the production of single cell protein

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**Summary**

Sugar cane bagasse hemicellulosic fraction was hydrolysed by treatment with 70 mg of sulphuric acid per gram of dry mass at 125 °C for 2 h. The hydrolysate was used as the substrate to grow *Candida langeronii* RLJ Y-019 at 42 °C; initial pH 6.0; stirring at 700 rev/min and aeration at 1.0 and 2.0 v/v/min. The utilization of D-xylose, L-arabinose, and acetic acid were delayed due to the presence of D-glucose, but after D-glucose depletion the other carbon sources were utilized. The kinetic parameters calculated for both cultivations at 1.0 and 2.0 v/v/min included: maximum specific growth rate ($\mu_{max}$) of 0.29 ± 0.01 $h^{-1}$ and 0.43 ± 0.016 $h^{-1}$, yields ($Y_{x/s}$) of 0.36 ± 0.012 and 0.40 ± 0.012 $g_x/g_s$ and productivity ($Q_x$) of 0.81 ± 0.016 and 0.97 ± 0.012 $g_x$/l/h, respectively, and compared favourably with published results obtained with *Candida utilis* and *Geotrichum candidum*. *Candida langeronii* 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. The biomass contained 48.2, 1.4, 5.8 and 23.4% of total protein, DNA, RNA and carbohydrate, respectively and contained essential amino acids for animal feed.

## Introduction

Bagasse, the residue left after extraction of sucrose from sugar cane, contains ca. 30–35% hemicelluloses (Roberto *et al*. 1994), which has the advantage of being one of the few lignocellulosic wastes which become available in large localized quantities in the course of normal agricultural practice. Mild acid treatment yields a mixture of monosaccharides, mainly pentoses, with D-xylose as the main component (Du Toit *et al*. 1984). Besides sugar, the hydrolysate contains acetic acid, furfural, hydroxymethyl-furfural and soluble lignin.

For biomass production, it is advantageous to use a yeast capable of growth on L-arabinose as well as on acetic acid. As compared to other microorganisms, the yeasts are more suitable for usage as a source of food (Bhattacharjee 1970). They contain more nitrogen than fungi and algae and more ash than bacteria (Kihlberg 1972). *Candida utilis* is frequently used for biomass production, because of its ability to utilize a variety of carbon sources rapidly and with high protein yield. However, the inability of *C. utilis* to utilize L-arabinose has prompted a search for alternative organisms. Apart from the assimilation of L-arabinose, the selection criteria included the ability to grow in the absence of vitamins or other growth factors and at temperatures above 40 °C. The objective of this study was to select a yeast with a better potential than *C. utilis* for biomass production and in particular from the pentose sugars occurring in sugar cane bagasse hemicellulose hydrolysate.

## Materials and Methods

*Isolation procedures*

Isolations were made by inoculating static test tubes with the respective samples under selective conditions in 10 ml of M-1 medium (Table 1) containing trace element 1 ml/l. The trace element solution contained (g/l): $CuSO_4 \cdot 5H_2O$, 2.5; $FeCl_3 \cdot 6H_2O$, 2.7; $MnSO_4 \cdot H_2O$, 1.69; $Na_2MoO_4 \cdot 2H_2O$, 2.42; $ZnSO_4 \cdot 7H_2O$, 2.87; $CoCl_2 \cdot 6H_2O$, 2.38 and $H_2SO_4$ (conc.), 3 drops. The isolations were conducted at pH 4.5 and 45 °C. The carbon source (10 g/l) was autoclaved separately. Samples from tubes showing growth were purified by streaking out on agar plates of the same medium. Isolates were maintained on agar slants of M-3 medium (Table 1) at 4 °C.

*Evaluation procedures*

Selected isolates were evaluated in 250-ml shake flasks equipped with side arms to facilitate turbidity measurements, contained 50 ml of M-2 medium (Table 1), and trace element 1 ml/l. The carbon source was 10 g/l except in the case of acetic acid, which was added at 3.0 g/l. The experiments were conducted at 42 °C and

*Table 1.* Defined medium compositions.

| Components | Concentration (g/l) | | | | |
|---|---|---|---|---|---|
| | M-1 | M-2 | M-3 | M-4 | M-5 |
| D-Xylose | – | – | 30.0 | 30.0 | – |
| D-Glucose | – | – | 10.0 | – | – |
| L-Arabinose | – | – | 5.0 | – | – |
| Peptone | – | – | 5.0 | 2.0 | – |
| Yeast extract | – | – | 5.0 | 2.0 | – |
| Sodium acetate | 15.0 | 15.0 | – | – | – |
| Citric acid | 0.2 | 0.2 | – | – | – |
| $(NH_4)_2HPO_4$ | – | – | – | 2.0 | – |
| $(NH_4)_2SO_4$ | 2.0 | 2.0 | – | – | 4.0 |
| $KH_2PO_4$ | 1.0 | 1.0 | – | 1.0 | 8.0 |
| $MgSO_4 \cdot 7H_2O$ | 0.2 | 0.5 | – | 0.5 | 0.25 |
| $CaCl_2 \cdot 2H_2O$ | 0.1 | – | – | – | 0.02 |
| NaCl | 0.1 | – | – | – | 0.08 |
| $MnSO_4 \cdot 4H_2O$ | – | – | – | – | 0.01 |
| $FeSO_4 \cdot 7H_2O$ | – | – | – | – | 0.005 |

M-1, Isolation medium; M-2, Evaluation medium; M-3, Maintenance medium; M-4, Inoculum preparation medium; M-5, Hydrolysate medium.

pH 6.0, unless stated differently in the text, on a Climo shaker at 200 rev/min. Each 250-ml shake flask received 1 ml inoculum, which was prepared in 150-ml shake flasks containing 15 ml of the same medium. The carbon source and cultivation conditions were same as that used in the 250-ml shake flask.

*Identification of isolates*

The isolate producing maximum concentration of biomass was identified according to the conventional systems for yeast identification (Barnett *et al.* 1983).

*Raw material (Substrate)*

Mill run bagasse contains 45–52% moisture, was air dried and milled to pass through a 0.75-mm screen before use. The composition of dry bagasse is presented in Table 2.

*Dilute acid hydrolysis of sugar cane bagasse*

Acid hydrolysis was performed at 125 °C for 2 h with an initial liquid to solid ratio of 5:1. The final concentration of sulphuric acid in the suspension was 70 mg/g of dry bagasse. Solid residue, after hydrolysis was separated by filtration and the hydrolysate was neutralized.

*Neutralization and pretreatment of hemicellulose hydrolysate*

Hydrolysate pH was adjusted to 10 with $Ca(OH)_2$, the resulting precipitate removed by centrifugation, and then reacidified to pH 6, followed by further centrifugation. The hemicellulose hydrolysate composition is summarized in Table 3.

*Table 2.* Major components of dry sugar cane bagasse*.

| Component | Percent |
|---|---|
| Cellulose | 38 |
| Hemicellulose | 33 |
| Lignin | 22 |
| Ash | 3 |

* Trickett and Neytzell-de wilder 1982.

*Table 3.* The average composition of sugar cane bagasse hemicellulose hydrolysate.

| Component | Concentration (g/l) |
|---|---|
| D-Xylose | 47.2 ± 0.82 |
| D-Glucose | 4.5 ± 0.17 |
| L-Arabinose | 6.2 ± 0.29 |
| Acetic acid | 10.7 ± 0.68 |
| Furfural | 0.83 ± 0.04 |
| Hydroxymethyl-furfural | 0.07 ± 0.02 |
| Ash | 1.0 ± 0.12 |

* Each values corresponds to the mean of three experiments ± SD.

*Microorganism*

The isolate, which was capable of producing maximum concentration of biomass from bagasse hemicellulose hydrolysate, was identified as *Candida langeronii* and maintained on agar slants, M-3 medium (Table 1), at 4 °C.

*Inoculum preparation*

The inoculum was prepared by transferring a loopful of cells in 50 ml, of M-4 medium (Table 1), and grown at 42 °C for 24 h on a Climo shaker at 200 rev/min. The cells were harvested aseptically, and used as inoculum.

*Hydrolysate medium*

Each litre of treated bagasse hemicellulose hydrolysate was supplemented with M-5 medium (Table 1), pH adjusted to 6.0.

*Equipment and cultivation conditions*

Batch cultivations were performed in a 5-L Microferm fermentor (NBS Co., Inc., NJ, USA) (2-L working volume) equipped with instrument and controllers for the measured parameters such as agitation, temperature, pH and dissolved oxygen and fitted with a reflux cooler in the gas exhaust to minimize evaporation. An antifoam, 1 ml (FG-10, Dow Corning Corporation, USA) was added. Reactor contents were stirred at 700 rev/min to ensure homogeneous mixing and air flow rates were set at (1.0 and 2.0 v/v/min). Temperature and pH were maintained at 42 ± 0.2 °C and 6.0 ± 0.2. Inoculum concentration was 5 g/l. Dissolved oxygen tension was never less than 40% of saturation. Aliquots of 5 ml were taken at different times to determine the concentrations of biomass, sugars, D-xylitol and acetic acid in the broth. All the experiments were made in triplicate.

*Analytical methods*

Cell density was determined as described (Bjorling & Lindman (1989). Total nitrogen, lipids, Lowry protein, and nucleic acid were determined by the standard methods (Herbert *et al.* 1971), using bovine serum

albumin fraction V (Sigma) as protein standard. Amino acid content was determined as described (Simpson *et al.* 1976). The carbohydrates and total reducing sugars were estimated by the anthrone and Somogyi–Nelson methods (Umbreit *et al.* 1972). Sugars and D-xylitol concentrations were determined by high-performance liquid chromatography and acetic acid by gas chromatography as described (van Zyl *et al.* 1988). The concentrations of furfural and hydroxymethyl-furfural were determined as described (Pessoa *et al.* 1996). Dissolved oxygen was measured with a standard galvanic type oxygen electrode.

*Calculation of the kinetic parameters*

Maximum specific growth rate, yields and productivities were calculated as described by Pirt (1975).

*Chemicals*

D-Xylose, D-glucose, L-arabinose and bovine serum albumin (Sigma), peptone and yeast extract (Difco), and all other chemical used were of analytical grade.

## Results and Discussion

*Isolation*

A total of 64 samples, including soil from various environments, and infected plant material (leaves, roots and flowers) were investigated. A total of seven isolates capable of utilizing D-xylose or both D-xylose and L-arabinose in vitamin-free medium at 42 °C were isolated.

*Preliminary evaluation*

Only three isolates were capable of vigorous growth on both D-xylose and L-arabinose in a vitamin-free medium at 42 °C. Four other isolates utilized D-xylose at 42 °C, but their specific growth rates were appreciably lower than the latter isolates (data not shown). These three isolates meeting the selection criteria were isolated from the soil samples collected from the area where bagasse, pressmud and other sugar mill processing wastes were dumped for the last two decades.

*Microorganism*

The isolate selected for biomass production was evaluated in terms of protein yield and other growth characteristics. This isolate showed the following advantages over an industrial reference strain, *C. utilis*. It uses L-arabinose and grows at temperatures as high as 45 °C in contract to *C. utilis* which cannot utilize L-arabinose and grows at 35 °C. This can substantially reduce cooling costs and assist in controlling contamination. It showed a much higher tolerance to furfural, are inevitable by-product of bagasse hydrolysis.

*Acid hydrolysis of the hemicellulose component of sugar cane bagasse*

The dry bagasse was acid hydrolysed at 125 °C for 2 h. Further increase in cooking time is counteracted by the increased breakdown of D-xylose to furfural. Acid concentrations higher than 70 mg/g of dry bagasse appear to have a limited beneficial effect on the production of fermentables. It is desirable to keep the acid concentration as low as possible so as to minimize the cost of chemicals and reduce equipment corrosion. Hydrolysate contained 47.2 ± 0.82 g/l D-xylose, the major component of hemicellulose sugars. Besides sugar, the hydrolysate contained acetic acid, furfural and hydroxymethyl-furfural. These compounds act as inhibitors of microbial growth (Tran & Chambers 1985; Pfeifer *et al.* 1996).

*Overliming*

Substantial precipitation of calcium sulphate (gypsum) occurs when $Ca(OH)_2$ is used to neutralize hydrolysates. The resulting precipitate is removed to improve hydrolysate fermentability. It is likely that some inhibitory components are precipitated by divalent calcium ions, or bind to or otherwise associate with precipitated solid gypsum (van Zyl *et al.* 1988). The mechanism of action of overliming remains unclear. Overliming resulted in the loss of D-glucose (9%), D-xylose (3%), L-arabinose (8%) and acetic acid (27%). To reduce these losses, the shift to higher pH during the liming process must be kept to a minimum (Fein *et al.* 1984). However, many conflicting reports exist concerning the extent to which inhibitory components are removed by overliming. For example, Strickland & Beck (1984) observed that overliming treatment reduced the concentration of furfural and possibly of metal ions, whereas van Zyl *et al.* (1988) and Amartey & Jeffries (1996) reported 17 and 43% decrease in acetic acid concentrations following neutralization of bagasse and corn-cob acid hydrolysed hemicellulose hydrolysates with lime, respectively.

## Bioreactor cultivation

Two batch cultivations were performed with *Candida langeronii* RLJ Y-019 (Figures 1 and 2) at different aeration rates (1.0 and 2.0 v/v/min). Initial pH (6.0) promoted cell growth impeding the permeation of dissociated acetic acid through the cell membrane (Prior 1984). Besides, the inhibitory effects of the furfural and hydroxymethyl-furfural were minimized at pH 6.0, as also observed by Pessoa *et al.* (1996). Furfural and hydroxymethyl-furfural in concentrations of 0.83 ± 0.04 and 0.07 ± 0.02 g/l, respectively did not inhibit the cell metabolism. Probably no negative influence on cell respiration and on oxidative phosphorylation occurred. According to the literature (Delgene *et al.*

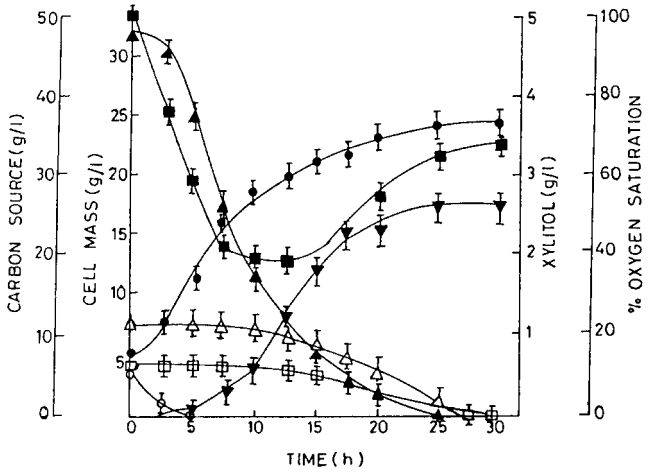


*Figure 1. Candida langeronii* growth in sugar cane bagasse hemicellulose acid hydrolysate. Cell mass (●), concentrations of D-xylose (▲), D-glucose (○), acetic acid (△), L-arabinose (□), D-xylitol (▼), and percent oxygen saturation (■). Growth conditions: aeration, 1.0 v/v/min; agitation, 700 rev/min; temperature, 42 °C, and pH, 6.0.

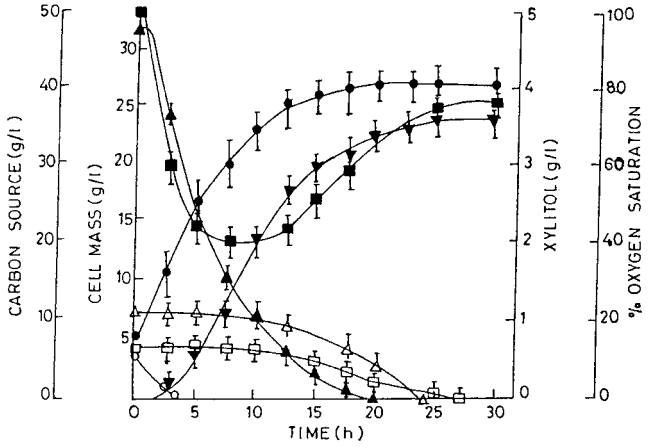


*Figure 2. Candida langeronii* growth in sugar cane bagasse hemicellulose acid hydrolysate. Cell mass (●), concentrations of D-xylose (▲), D-glucose (○), acetic acid (△), L-arabinose (□), D-xylitol (▼), and percent oxygen saturation (■). Growth conditions: aeration, 2.0 v/v/min; agitation, 700 rev/min; temperature, 42 °C, and pH, 6.0.

1988; Silva *et al.* 1995) furfural can be assimilated by yeasts or fungi.

Figures 1 and 2 show that D-glucose was utilized first (within 5 h after inoculation) and D-xylose utilization commenced shortly before the depletion of D-glucose. Acetic acid and L-arabinose were assimilated toward the end of the exponential phase. The delay in the assimilation of L-arabinose and acetic acid following D-glucose depletion in hemicellulose hydrolysate was probably due the presence of D-xylose. The ability of this strain to utilize L-arabinose gives an additional advantage over *C. utilis* and *Geotrichum candidum* (Holder *et al.* 1989). L-Arabinose can constitute up to 10.7% of the total hemicellulose sugars present in acid hydrolysate (Table 3).

A biomass with a concentrations of 24.3 ± 0.30 and 26.67 ± 0.36 g/l and yields of 0.36 ± 0.012 and 0.40 ± 0.012 $g_x/g_s$ were obtained when cultivated at aeration rates of 1.0 and 2.0 v/v/min, respectively. Holder *et al.* (1989) found a yield of 0.27 and 0.33 $g_x/g_s$, when *C. utilis* and *G. candidum* were grown in bagasse hemicellulose hydrolysate medium, respectively, whereas Meyer *et al.* (1992) observed yield of 0.28 $g_x/g_s$ when *C. utilis* was grown in simulated bagasse hemicellulose hydrolysate. The kinetic parameters are summarized in Table 4.

Under aerobic conditions, many yeasts have a potential for producing polyhydric alcohols as by-products of the D-xylose metabolism. D-Xylitol, the most common of these alcohols, was detected in the medium. The higher aeration rate (2.0 v/v/min) enhanced D-xylitol production as also observed by Pfeifer *et al.* (1996).

Figures 1 and 2 show that there was no inhibition of growth and substrate utilization by the yeast when grown in hydrolysate medium. This is in contrast with the findings of Watson *et al.* (1984). This shows that the isolate used in the present study may be resistant to these inhibitors.

When microorganisms are grown in a medium containing multiple carbon sources, a diauxic growth and sequential substrate utilization are frequently observed (Epps & Gale 1942; Siro & Lovgren 1979). Although no diauxic growth was observed in the present investigation, sequential utilization did occur. The utilization of D-glucose followed by D-xylose is preferred. The differential utilization of monosaccharides is due to metabolic differences, mutual competition for a transporter and for the capture of the induction enzymes (Gong 1983).

The high protein content 48.2% makes this isolate more attractive for single cell protein production. Biomass contained 1.4, 5.8 and 23.4% of DNA, RNA and carbohydrate, respectively and compares well to the values reported for *C. utilis* grown in synthetic medium indicating the importance of this yeast for single cell protein production (Alroy & Tannenbaum 1973).

The protein produced by *Candida langeronii* in the bagasse hemicellulose hydrolysate contains most of the amino acids essential for animal feed and its quality is comparable to soy bean protein (Table 5). Its high lysine and threonine content suggest that this yeast protein

*Table 4.* Kinetic parameters of *Candida langeronnii* RLJY-019 grown in sugar cane bagasse hemicellulose hydrolysate medium.

| Parameters | Bagasse hemicellulose hydrolysate Aeration rates (v/v/min) | |
|---|---|---|
| | 1.0 | 2.0 |
| $X_{max}$ (g/l) | 24.30 ± 0.30 | 26.67 ± 0.36 |
| $Y_{x/s}$ ($g_x/g_s$) | 0.36 ± 0.012 | 0.40 ± 0.012 |
| $Q_x$ ($g_x$/l/h) | 0.81 ± 0.016 | 0.97 ± 0.012 |
| $\mu_{max}$ ($h^{-1}$) | 0.29 ± 0.01 | 0.43 ± 0.016 |
| $X_{max\ xi}$ (g/l) | 2.45 ± 0.03 | 3.75 ± 0.164 |
| $Q_{xi}$ ($g_{xi}$/l/h) | 0.08 ± 0.01 | 0.14 ± 0.016 |
| $Y_{xi/s}$ ($g_{xi}/g_s$) | 0.038 ± 0.001 | 0.053 ± 0.005 |

* Each values corresponds to the mean of three experiments ± SD.

*Table 5.* Amino acid composition of protein produced by *Candida langeronii* RLJ Y-019 and other protein sources.

| Amino acid | Concentration (as % of total protein) | | | | | | |
|---|---|---|---|---|---|---|---|
| | *C. langeronii* RAJ Y-019[a] | *C. tropicalis* IZ1824[b] | *C. utilis* ATCC 9255[c] | FAO[d] | Soy bean[e] | Whole wheat[f] | Ruminats feed |
| Lysine | 7.80 | 7.22 | 6.80 | 6.60 | 6.60 | 1.90 | 3.20 |
| Histidine | 2.45 | 2.43 | 1.70 | – | – | 2.20 | – |
| Arginine | 4.66 | 4.63 | 6.50 | – | – | 4.20 | – |
| Threonine | 5.10 | 4.98 | 2.50 | 2.80 | 4.30 | 2.70 | 1.97 |
| Cystine | 0.70 | – | 0.70 | 2.00 | 1.60 | 1.90 | 2.74 |
| Valine | 4.50 | 4.76 | 4.80 | 4.20 | 5.00 | 4.10 | 2.70 |
| Methionine | 0.75 | 1.60 | 0.80 | 2.20 | 1.30 | 1.50 | 0.72 |
| Isolucine | 4.10 | 3.99 | 3.90 | 4.20 | 4.90 | 4.20 | 2.57 |
| Leucine | 6.50 | 6.45 | 6.10 | 4.80 | 8.00 | 7.00 | 3.80 |
| Tryptophan | – | – | – | 1.40 | 1.40 | 0.30 | 0.80 |
| Phenylalanine | 3.5 | 3.55 | 3.50 | 2.80 | – | 5.50 | 2.20 |

[a] This work; [b] Pessoa *et al.* 1996; [c] Prior *et al.* 1981; [d] Araujo and D'souza 1986; [e] Lo and Moreau 1986; [f] Gnevol 1957.

should be utilized as a feed supplement, specially in diets based on cereals (Kihlberg 1972).

## Conclusions

The data presented in the present article demonstrate that sugar cane hemicellulosic bagasse can be easily hydrolysed and the resulting sugar solution can be used as a cultivation medium for the production of single cell protein.

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