Appl Biochem Biotechnol (2008) 151:610–617
DOI 10.1007/s12010-008-8268-2

Lactase Production by Solid-state Cultivation of *Kluyveromyces marxianus* CDBBL 278 on an Inert Support: Effect of Inoculum, Buffer, and Nitrogen Source

Luz Tovar-Castro · Mariano García-Garibay · Gerardo Saucedo-Castañeda

Received: 19 December 2007 / Accepted: 28 March 2008 /
Published online: 1 July 2008

Abstract A study was carried out to select the conditions for cultivation of *Kluyveromyces marxianus* CDBBL 278 in solid-state culture (SSC) using polyurethane foam (PUF) as an inert support. PUF was impregnated with culture media containing lactose (50 g/L) as the carbon and energy source. Evaluation of culture parameters during different growth phases was carried out by respirometry. The effect of inoculum level, buffer capacity of the medium, and nitrogen source upon the yield of biomass on lactose (*Y*x/s) and production of lactase and inulinase was investigated. The highest lactase titre was achieved with an inoculum level of 1×10^7 cells per gram of wet matter (gwm) and 20% of the total nitrogen source provided as urea. The best biomass yield (0.37) was obtained when less than 40% of the total nitrogen was provided as urea. Using potassium phosphate allowed 90% substrate consumption in 30 h. In the best conditions, intracellular lactase and extracellular inulinase activities of 1147.7 IU/g*X* and 241.6 IU/g*X* were obtained, respectively, with a lag phase of 13.8 h and a rate of respiratory activity (μ_{CO2}) of 0.23 ± 0.01 h^{-1}. To our knowledge, this is the first report on lactase production by *K. marxianus* CDBBL 278 in SSC. This study gives basic information about biomass yield and enzyme production using lactose as the sole carbon source in SSC on an inert support.

Keywords *Kluyveromyces marxianus* · Solid-state culture · Enzymatic activities

Introduction

The dairy industry generates a significant amount of by-products; whey in particular, can cause serious pollution problems [1] because of its high lactose content. The direct use of this sugar is difficult because of its low sweetness and its low solubility compared to sucrose [2]. Some microorganisms, such as yeasts of the genus *Kluyveromyces*, are able to

L. Tovar-Castro · M. García-Garibay · G. Saucedo-Castañeda (✉)
Departamento de Biotecnología, Universidad Autónoma Metropolitana Iztapalapa (UAMI),
San Rafael Atlixco, No. 186, Col. Vicentina, CP 09340 Delegación Iztapalapa, México
e-mail: saucedo@xanum.uam.mx

assimilate lactose [3, 4]. In particular, *Kluyveromyces marxianus*, previously known as *K. fragilis* and *Saccharomyces fragilis* [5], presents some interesting features such as a high capacity for converting substrate into biomass [6, 7], a large temperature range for growth, and a high capacity for producing enzymes such as lactase [5, 7], endo-polygalacturonase [8] and inulinase [9], as well as aromatic compounds such as 2-phenylethanol [10].

Many studies of *K. marxianus* have been carried out in liquid culture to investigate biomass or enzyme production [11–18]. However, although the potential applications are numerous, there have been few studies in solid-state culture (SSC), e.g., the production of inulinase [19], the production and optimisation of aroma compounds using factorial design and surface response methodology [20, 21], and the production of inulinase and its preliminary characterization using factorial design [22, 23]. It is worth noting that there are no reports of lactase production by *K. marxianus* CDBBL 278 in SSC.

The aim of this work was to investigate the effect of the inoculum level, the buffer capacity of the medium, and the nitrogen source upon biomass yield ($Y_{X/S}$) and enzymatic titres during SSC of *K. marxianus* CDBBL 278 on an inert support.

Materials and Methods

Microorganism

The yeast *K. marxianus* (CDBBL 278) was used in this study. It was maintained on dextrose-potato-agar (PDA), subcultured every 2 weeks and stored at 4 °C. The strain was preserved at −40 °C using 40% ethylene glycol as a cryoprotector.

Preparation of Inoculum

Inoculum was prepared in 25-mM potassium phosphate buffer, pH 5.5, containing (g/L): lactose 50, yeast extract 2.23, $(NH_4)_2SO_4$ 8.75, KH_2PO_4 2.72, and $MgSO_4{\cdot}7H_2O$ 0.518 [5, 16]. A loopful of cells from a stock culture was transferred into 60 mL of sterilized medium (121 °C for 15 min) in a 250-mL conical flask. The flasks were incubated at 30 °C for 24 h on a rotary shaker at 150 rpm.

Solid-state Culture

The SSC of *K. marxianus* was carried out in glass columns [20]. Polyurethane foam (PUF), with a particle size of 0.8–1.5 mm, was used as an inert support; it was impregnated with the same medium used for inoculum preparation. The corresponding lactose concentration in SSC was 0.18 g/g initial dry matter (gidm). The nitrogen source was modified as indicated below. Lactose was the only carbon source able to produce biomass and enzymes, as reported previously [3–7]. Each column was packed with approximately 20 g of wet matter (gwm), previously sterilized (121 °C for 15 min) and inoculated with a yeast suspension (1×0^7 cells/gwm). The packed material in each column corresponded to approximately 5 gidm.

The nitrogen source was provided as mixtures of ammonium sulfate (SA) and urea (U) in the following proportions (SA:U): 100:0; 80:20; 60:40; 40:60; 20:80, and 0:100. The media were isonitrogenated (N_T=1.85 g/L) and were prepared in 25-mM potassium phosphate buffer, pH 5.5. A moistened airflow of 0.5 mL of air per gwm was passed through the glass columns. The initial moisture and the water activity (A_w) were 75% and

0.997, respectively; columns were incubated at 30 °C. The cultures were performed in triplicate. Statistical analysis of results was carried out using the Duncan method with $\alpha<0.05$.

Sample Treatment

Four grams of fermented matter were weighed and 20 mL of 0.01% Tween 80 was added. This suspension was mixed thoroughly for 5 min with a magnetic agitator. Biomass was separated from the fermenting material by filtration using a stainless steel mesh sieve (No. 100, 0.1 mm diameter). The filtrate was centrifuged for 10 min at 10,902×*g*. The supernatant was used to determine the substrate and extracellular enzymatic activities, whereas the precipitate was dried at 60°C for 24 h to determine the biomass [24]. In separate samples, intracellular enzyme extract was obtained from the biomass precipitate after centrifugation, as indicated above. Biomass was disrupted by ultrasonic disruption (Mini-BeadBeater, Sartorius). Then, 1 mL of 25-mM potassium phosphate buffer, pH 5.5, was added and the extract centrifuged for 10 min at 20,128× *g*. The supernatant was used for intracellular enzymatic measurements.

Determination of Biomass and Lactose

CO_2 production was used as an indirect measure of *K. marxianus* growth [25]. CO_2 concentration in the dry air stream outlet from the fermentation column was monitored online using a gas chromatograph (Gow-Mac 580, Gow-Mac Instrumentation Co., Bethlehem, PA, USA), equipped with a thermal conductivity detector and an automatic injector; a gas separating concentric column (CTRI, Alltech) and a software integrator program (Chroma Biosystemes, France). Results were expressed in terms of carbon dioxide formation rate (CDFR, mgCO_2/h gidm) and total CO_2 formed (mgCO_2/h gidm). Lactose was determined by using a high-performance liquid chromatograph (HPLC) equipped with a refraction index detector and a Rezex 300-mm column (Phenomenex); 5 mN H_2SO_4 was used as the mobile phase at 0.5 mL/min, and column temperature was kept at 50 °C.

Enzymatic Determinations

The enzymatic activity of lactase EC 3.2.1.23 (β-galactosidase) was measured as follows: a volume of 0.2 mL of 34 mM *ortho*-nitrophenol beta-galactopiranoside (ONPG) was added to 0.1 mL of the intracellular enzymatic extract. Final volume was adjusted to 3 mL with 50-mM potassium phosphate buffer, pH 6.6. Magnesium and manganese salts were added [26]. The reaction mixture was incubated at 37 °C for 3 min. The reaction was monitored by reading the absorbance at 420 nm on a Shimadzu TCC-240 spectrophotometer. A standard plot was prepared with *o*-nitrophenol (ONP) solution in phosphate buffer. Adequate blanks were run simultaneously without the enzyme extract and substrate solutions. One International Unit (IU) of lactase activity was defined as the amount of enzyme that liberates 1 μmol of o-nitrophenol per minute under the conditions described.

Inulinase activity was measured as follows: 0.2 mL of 1% inulin (dissolved in 100-mM acetate buffer, pH 4.5) and 0.05 mL enzymatic extract were mixed and incubated at 37 °C for 15 min. After incubation, 0.25 mL of Somogyi reactive was added and the tubes were held in a boiling water bath for 10 min. Tubes were cooled in a glass water bath, then 0.25 mL of Nelson reactive was added, and samples were incubated for 30 min at room temperature; then 4 mL of water was added. The increase in absorbance at 580 nm, because

of the appearance of reducing groups [19, 27], was measured by means of a Shimadzu TCC-240 spectrophotometer. Standard plots were prepared with fructose solutions in 100 mM acetate buffer, pH 4.5. Blanks were run simultaneously without the enzyme extract and substrate solutions. An International Unit (IU) of inulinase activity was defined as the amount of enzyme that released 1 μmol of reducing sugars of fructose per minute under the assay conditions.

Determination of Moisture, pH, A_w, and Cell Concentration

The moisture content of the fermented solid was determined by gravimetry. The pH was determined using a previously calibrated potentiometer. An AQUA-LAB (CX-2) instrument was used to determine the water activity (A_W) from a portion of fermented wet matter. Yeast cell concentration was estimated by using a Neubauer hematocytometer.

Results and Discussion

Effect of Inoculum Level on the Solid-state Culture of *Kluyveromyces marxianus*

CO_2 formation is an indirect method for estimating biomass growth [25], allowing measurement of the carbon dioxide formation rate (CDFR, mg CO_2/h g idm) automatically online, without disturbing the culture. The integration of the CDFR vs time plot gives total CO_2 formed, which can be transformed into biomass by using a proper yield coefficient ($Y_{X/CO2}$). Analysis of these plots allows estimation of culture parameters during lag, exponential, and stationary phases of the SSC of this yeast.

Figure 1 shows the CDFR for the four levels of inoculation studied: 10^5, 10^6, 10^7, and 10^8 cells/gwm. Nearly 90% of the substrate was consumed in all cases; nevertheless, the lower the inoculum level the longer the culture time (Fig. 1). Table 1 shows lactase production and the total CO_2 formed for the four inoculum levels. Maximum lactase production was obtained with an inoculation of 10^7 cells/gwm. The enzyme activity (580.2 IU/g*X*) represents only 36% of that reported in the literature (1,590 IU/g*X*) for this strain in liquid culture [28]. The total CO_2 formed was not affected by the levels of inoculation, except for the lowest inoculum (10^5 cells/gwm), which could be explained by the development of contaminants because of the low initial concentration of cells.

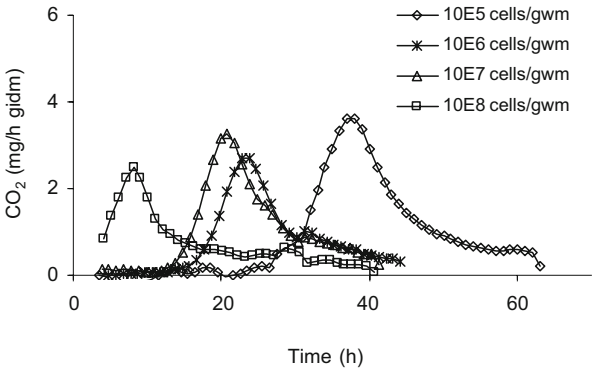

Fig. 1 Effect of inoculum level on CO_2 formation rate during solid-state cultures of *Kluyveromyces marxianus* CDBBL 278

Table 1 Total CO_2 formed and intracellular lactase activity of *Kluyveromyces marxianus* CDBBL 278 in solid-state culture at four levels of inoculation.

Level of inoculation (cell/gwm)	Total CO_2 (mg CO_2/gidm)	Enzymatic activity lactase (IU/g*X*)
1×10^5	55.0±0.01[a]	2.1±0.01[c]
1×10^6	34.4±0.01[b]	35.0±0.01[b]
1×10^7	34.6±0.04[b]	580.2±0.02[a]
1×10^8	34.5±0.03[b]	15.0±0.02[b]

Same letter indicates no significant differences ($\alpha<0.05$).

The lag phase increases as the inoculation level decreases (Table 2); a lag phase of almost 25 h was observed for the smallest inoculum level (10^5 cells/gwm). This occurs because the CO_2 concentration in the exhaust airflow depends on the microbial population; it decreased as the cell population decreased. In contrast, the lag phase for 10^7 and 10^8 cells/gwm was about 14.3 and 5.1 h (Table 2), respectively.

Table 2 shows the respiratory activity rate (μ_{CO2}) of *K. marxianus* for the four levels of inoculation; μ_{CO2} is considered an estimation of specific growth rate (μ). This was calculated by the slope in the linear zone of the natural logarithm of the total CO_2 versus time plot [25]. The maximum value of μ_{CO2} was reached with 10^8 cells/gwm and a significant difference was observed ($\alpha<0.05$) with respect to the other inoculum levels [29]. The inoculum level selected for further studies was 10^7 cells/gwm because of the high lactase titre obtained and because it will probably avoid problems of contamination.

Importance of Buffer Solution in the Solid Culture of *Kuyveromyces marxianus*

Figure 2 shows the CDFR for *K. marxianus* in the following conditions: without buffer, with 25-mM sodium acetate buffer and with 25-mM potassium phosphate buffer. Only in the latter case were the different growth phases clearly identified; negligible growth was observed in the other two conditions assayed. Under the study conditions, the pH of the media was not buffered at the end of the culture; no significant differences were observed in the final pH (2.7). This may be because this yeast produced organic acids and because ammonium sulfate was used as the nitrogen source [30].

Substrate consumption and the yield of biomass formed per lactose consumed ($Y_{X/S}$) were 96, 73, 39%, and 0.36, 0.24, and 0.25 for potassium phosphate, sodium acetate and without buffer, respectively.

Sodium acetate has been reported to have an inhibitory effect on the growth of this yeast. Sodium is an inhibitor of lactase production, which probably explains the negligible growth and, therefore, the absence of this enzyme under these conditions [31].

Table 2 Characteristic growth parameters of *Kluyveromyces marxianus* CDBBL 278 in solid-state culture at four levels of inoculation.

Level of inoculation (cell/gwm)	Lag phase (h)	μ_{CO2} (h^{-1})	R^2
1×10^5	32.5	0.26±0.013[a]	0.98
1×10^6	16.7	0.34±0.014[a]	0.98
1×10^7	14.3	0.32±0.025[a]	0.94
1×10^8	5.1	0.55±0.055[b]	0.94

Same letter indicates no significant differences ($\alpha<0.05$).

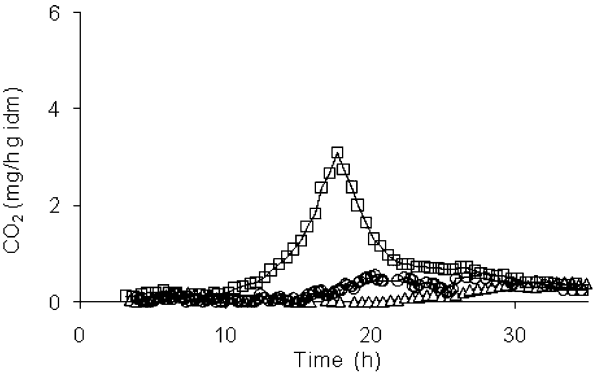

Fig. 2 Effect of the buffer capacity of the medium on CO_2 formation rate during the solid-state culture of *Kluyveromyces marxianus* CDBBL 278. Without buffer (*triangle*), with 25-mM sodium acetate buffer (*circle*) and with 25-mM potassium phosphate buffer (*square*)

Although the pH of the media was not buffered, a remarkable difference was observed when phosphate buffer was used: 90% of the lactose was consumed after 30 h incubation and the different growth stages were clearly identified during the SSC; a short lag phase (6 h) was observed, and the maximum CDFR was found after 18 h of incubation. These results suggest that regulation of pH could play an important role in culture performance.

Effect of Nitrogen Source on the Solid-state Culture of *Kluyveromyces marxianus*

Utilization of mixtures of urea and ammonium sulfate is a well-known strategy for pH control in SSC, but it also has an effect upon yeast growth. Thus, the effect of the nitrogen source on biomass yield and enzyme titres was studied by changing the ammonium sulfate (SA) and urea (U) ratio as indicated in the "Materials and Methods" section. Moisture content (71.4±1.5%) and A_w (0.994±0.002 units) remained practically constant during cultivation. For all the studied cases, the final pH of the culture was considerably acid and varied from 2.6 to 3.2, except in the case where only urea was used. Figure 3 shows the CDFR for *K. marxianus* in SSC using different mixtures of ammonium sulfate (SA) and urea (U) as the nitrogen source. Tables 3 and 4 show the effect of the nitrogen source upon the lag phase, growth rate, $Y_{X/S}$, substrate consumption, and enzyme titres during SSC of *K. marxianus*. The lag phases and the rates of respiratory activity (μ_{CO2}) were affected by the presence of urea as a nitrogen source. Small proportions of urea extended the lag phase of the culture (Table 3). Yeast growth was negligible when more than 40% of the nitrogen source was provided as urea. The best biomass yields and titres of lactase (1147.7 IU/g*X*)

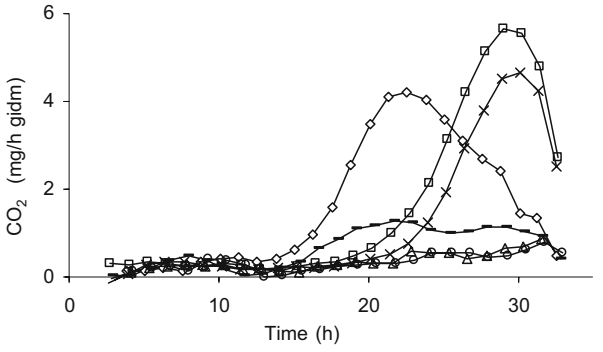

Fig. 3 Effect of nitrogen source on CO_2 formation rate during solid-state culture of *Kluyveromyces marxianus* CDBBL 278. SA:U ratio: 100:0 (*diamond*) ; 80:20 (*square*); 60:40 (*x*); 40:60 (*triangle*); 20:80 (*straight line*); and 100:0 (*circle*)

Table 3 Effect of the nitrogen source in the growth phases of *Kluyveromyces marxianus* CDBBL 278 in solid-state culture.

Nitrogen source (SA/U)	Lag phase (h)	μ_{CO2} (h^{-1})	R^2
100:0	13.8	0.23±0.008[a]	0.982
80:20	17.7	0.17±0.007[b]	0.964
60:40	17.8	0.18±0.006[b]	0.978

Same letter indicates no significant differences (α<0.05).

and inulinase activity (245.5 IU/g*X*) were observed when 20% of the nitrogen source was supplemented as urea (Table 4). Twice the lactase activity was obtained when 20% of the nitrogen source was provided as urea in comparison with no urea. The specific enzyme activity (IU/g*X*) represents 72% of the value reported in liquid culture for this strain. The best biomass yield (0.37) was attained when less than 40% of the total nitrogen was provided as urea, suggesting that the nutritional and culture conditions were adequate for this yeast. These results are consistent with those reported in the literature, indicating that cultures at pH 3 or lower can produce high titres of lactase [32, 33]. Further studies are needed to improve pH regulation in SSC of *K. marxianus*.

The inulinase activity titres were in the range of values reported in the literature [19, 22, 23]. On the other hand, the values obtained for lactase activity were still low with respect to those reported for liquid culture: 5910 [34], 5770 [26] and 2800 IU/g*X* [5] for *Kluyveromyces fragilis* NRLL-Y1109; nevertheless, the conversion of lactose to biomass represents an interesting feature for the treatment of dairy by-products.

Conclusions

Automated online monitoring of CO_2 concentration allowed the assessment of the different growth phases of *K. marxianus* CDBBL 278 and the best conditions for cultivation of this yeast in SSC. The highest lactase titre was achieved with an inoculum level of 1×10^7 cells per gwm and 20% of the total nitrogen source provided as urea. The higher biomass yield (0.37) was obtained when less than 40% of the total nitrogen was provided as urea. The use of potassium phosphate allowed 90% substrate consumption in 30 h. It is important to emphasize that this is the first report of lactase production in SSC of *K. marxianus*. The data contained in this work provide useful information on the potential for using dairy by-products for the production of biomass and different enzymes from *K. marxianus* in solid-state culture.

Table 4 Main culture variables during solid-state culture of *Kluyveromyces marxianus* CDBBL 278.

Nitrogen source (SA/U)	$Y_{X/S}$	Substrate consumption (%)	Lactase activity (IU/g*X*)	Extracellular inulinase activity (IU/g*X*)
100:0	0.35±0.004[a]	82.25±0.47	[a]659.7	203.1[b]
80:20	0.27±0.001[b]	85.61±0.02	[b]1147.7	241.6[a]
60:40	0.37±0.006[a]	77.82±0.15	[c]334.6	69.2[c]
40:60	0.18±0.01[c]	64.63±3.37	[d]	10.7[d]
20:80	0.19±0.001[c]	72.3±0.3	[d]	[e]
0:100	0.20±0.006[c]	62±1.92	[d]	[e]

Same letter indicates no significant differences (α<0.05).

Acknowledgments We are grateful to Dr. Alma Cruz Guerrero from UAMI for the donation of the yeast strain, and also to the finance granted by CONACYT (Mexico).

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