Provided for non-commercial research and education use.
Not for reproduction, distribution or commercial use.

This article was published in an Elsevier journal. The attached copy is furnished to the author for non-commercial research and education use, including for instruction at the author's institution, sharing with colleagues and providing to institution administration.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

http://www.elsevier.com/copyright

Author's personal copy

Available online at www.sciencedirect.com

ScienceDirect

Enzyme and Microbial Technology 42 (2008) 272–277

ENZYME AND MICROBIAL TECHNOLOGY

www.elsevier.com/locate/emt

Optimized amylolytic enzymes production in *Saccharomycopsis fibuligera* DSM-70554 An approach to efficient cassava starch utilization

C.F. González [a,1], J.I. Fariña [a,*], L.I.C. de Figueroa [a,b]

[a] *PROIMI-CONICET, Avenida Belgrano y Pje. Caseros, T4001MVB Tucumán, Argentina*

[b] *Microbiología Superior, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Tucumán T4000INI, Argentina*

Received 14 September 2007; accepted 8 October 2007

Abstract

Different nutritional and operative conditions for improving amylolytic enzyme production by the amylolytic yeast *Saccharomycopsis fibuligera* DSM-70554 were evaluated. Under selected conditions, cassava starch could be efficiently utilized. Culture conditions such as oxygen saturation (50% dissolved oxygen tension), temperature (34 °C) and pH (5.5) showed a significant influence on α-amylase and glucoamylase production. An optimized culture medium (OPT) formulated by the chemostat pulse-shift method, containing yeast extract as the most favorable organic nitrogen source, vitamins and certain salts ($MgCl_2$, $MnSO_4$, $CaCl_2$) allowed to achieve the highest amylolytic enzyme production, thus leading to an optimal liquefaction and saccharification of cassava starch. Tween 80 (0.2–1% w/v) demonstrated to be a favorable amendment for amylolytic enzyme production. Under the conditions herein described, a 97% degradation of cassava starch could be finally attained under batch culture mode. Maximal values of 633.3 $U\,L^{-1}\,h^{-1}$ of α-amylase and 72.1 $U\,L^{-1}\,h^{-1}$ of glucoamylase under optimized conditions represented ∼9 and ∼3-fold increments, respectively, as compared to preliminary fermentation assays under non-optimal conditions.

© 2007 Elsevier Inc. All rights reserved.

Keywords: *Saccharomycopsis fibuligera*; α-amylase; Glucoamylase; Fermentation; Cassava starch

1. Introduction

Cassava (*Manihot esculenta* Crantz) is a root crop of tropical American origin, and the fourth most important staple crop in the tropics. In the developing world it is surpassed only by maize, rice and sugarcane as a carbohydrates source; cassava's starchy roots produce more raw starch per unit of land than any other staple crop. Cassava is grown almost exclusively in arid and semiarid tropics, where it accounts for approximately 10 percent of the total caloric value of staple crops [1,2].

Cassava starch is composed of unbranched amylose (20 ± 5%) and branched amylopectin (80 ± 5%) both of which can be hydrolyzed either with acids or enzymatically (either with pure enzymes or amylase-producing microorganisms) to release glucose and maltooligosaccharides. Thereafter, both products can be easily transported across the cell membrane and metabolized by yeasts.

Starch hydrolyzing ability is reasonably widely distributed among genera and species of yeasts and that explains why several laboratories have been involved in recent years in the isolation, evaluation and/or the eventual construction of amylolytic yeast strains. The possibility to produce *in situ* amylolytic capability during fermentation stages would make unnecessary to purchase or produce amylolytic enzymes in a separate step [3,4] and is at this point where amylase-producing yeast research can do a significant contribution.

The yeast *Saccharomycopsis fibuligera* has been widely studied because of its high amylolytic activity in submerged cultures using starch as sole carbon source [5–7]. Making use of this enzymatic activity several works for ethanol [8–10], lactic acid [11] or biomass production by mixed cultures [12] were successfully carried out. However, despite the promising profile concerning *S. fibuligera* amylolytic activity, the physiology of amylolytic enzymes production when growing with cassava

* Corresponding author. Tel.: +54 381 4344888; fax: +54 381 4344887.
E-mail addresses: jifarina@yahoo.com, jifarina@primi.org.ar (J.I. Fariña).

[1] Current address: Microbiology and Cell Science Department, University of Florida P.O. Box 103610, Genetic Institute Building Room 306, 1376 Mowry Road, Gainesville, Florida 32610-3610, USA.

0141-0229/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2007.10.005

Author's personal copy

starch has been the subject of relatively few investigations [13].

On this context, the aim of this work was to determine the optimal nutritional and operative conditions for amylolytic enzymes production by *S. fibuligera* DSM-70554 when cultured with cassava starch as the sole carbon source and under different fermentation strategies, in order to improve cassava starch utilization.

2. Methods

2.1. Yeast strain and maintenance

S. fibuligera DSM-70554 (from DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) was maintained on YEPD slants containing, in $g\,L^{-1}$: glucose, 20; peptone, 20; yeast extract, 10; agar, 15; pH 4.5.

2.2. Culture media

- SM culture medium, in $g\,L^{-1}$: cassava starch, 5 or 10 (see Section 3); $(NH_4)_2SO_4$, 2; yeast extract, 1; KH_2PO_4, 2; $MgSO_4{\cdot}7H_2O$, 0.5; and pH 5.0.
- Optimized culture medium (OPT) for amylolytic enzymes production: as a consequence of the optimization protocols performed, it will be discussed and finally defined under Section 3.

2.3. Dissolved oxygen tension (DOT) effect

A 4-L LH-210 (Inceltech-Toulouse-France) fermentor with automatic control of dissolved oxygen, pH and temperature and operated under batch mode was used with a working volume of 2 L SM medium containing $5\,g\,L^{-1}$ cassava starch as sole carbon source. A loopful from a *S. fibuligera* starch culture (YEP-starch) was transferred to 100 mL SM medium in a 250 mL Erlenmeyer flask and incubated at 30 °C and 250 rpm for 16 h (final BDW $\sim$2.5–3 $g\,L^{-1}$). Fermentor was inoculated at $\sim$10% v/v and pH was maintained at 5.0 by the addition of either 0.5 N HCl or 0.5 N NaOH. Agitation was kept at 450 rpm and temperature at 30 °C. Dissolved oxygen was controlled at 10, 30 and 50% saturation by automatic air supply via a proportional integrative and derivative (PID) controller, with fluctuations lower than 5%. Results are the mean values of at least three independent assays.

2.4. pH and temperature optimization

A 2-L LH-502 (Inceltech-Toulouse-France) fermentor was operated in the temperature range of 30–37 °C, at 450 rpm and with a working volume of 500 mL SM medium. pH was varied between 4.0 and 6.5 by the addition of either 0.5 N HCl or 0.5 N NaOH. Fermentor was inoculated as above described (2.3.) and, at the exponential growth phase, medium inflow was started and the fermentor operated under continuous mode. The dilution rate used for these experiments was $0.18\,h^{-1}$, corresponding to a mean residence time of about 5.5 h. Steady state was assumed following at least three volume changes after the last change in flow rate, and given that biomass and starch concentrations remained constant. As reported, three to five volume exchanges are usually required after each change in dilution rate [14]. Culture medium was the same as that used for DOT optimization, but with $10\,g\,L^{-1}$ cassava starch as carbon source. Samples were withdrawn at each steady state.

2.5. Culture medium optimization

Assays were performed under the experimental conditions described for pH and temperature optimization (2.4.), starting with a medium containing $2\,g\,L^{-1}$ yeast extract and $10\,g\,L^{-1}$ cassava starch. Culture medium was optimized by the chemostat pulse-shift method described by Mateles and Battat [15], making possible the evaluation of a particular compound effect. Nutritional shifts including vitamins (biotin, calcium pantothenate, folic acid, inositol, niacin, *p*-aminobenzoic acid, pyridoxine hydrochloride, riboflavin and thiamine hydrochloride), $(NH_4)_2SO_4$, peptone, $MgCl_2$, $CaCl_2{\cdot}2H_2O$, trace elements G1 (H_3BO_3, $CuSO_4$, KI), trace elements G2 ($FeCl_3$, $MnSO_4{\cdot}7H_2O$, Na_2MoO_4, $ZnSO_4$), were separately injected into the growth vessel. After a single injection, cell concentration and amylolytic enzyme activities were analyzed. In the event that no significant change in cell concentration or amylolytic enzyme activities was observed, the incidence of that particular shift was neglected, and the next pulse proceeded after a new steady state was achieved.

2.6. Effect of Tween 80

Effect of different concentrations of Tween 80 (0.05, 0.2 and 1% w/v) was studied under batch mode by using the same equipment as described for DOT effect.

2.7. Analytical methods

Alpha-amylase activity was determined by measuring the reducing sugar groups enzymatically released from starch [16] with the 3,5-dinitrosalicylic acid (DNSA) colorimetric method [17]. Reaction mixture contained 100 μL supernatant obtained after culture broth centrifugation (5 min/5000 × *g*) and 400 μL 0.01 $g\,mL^{-1}$ soluble starch in 10 mM acetate buffer, pH 5.0. After incubating 15 min at 45 °C, during which enzymatic activity was linear, the reaction was stopped by adding 770 μL DNSA reagent. Released reducing sugars were determined at 590 nm. One unit of enzyme was defined as the amount of protein required to liberate reducing oligosaccharides equivalent to 1 μmol glucose $min^{-1}\,mL^{-1}$ of sample.

Glucoamylase activity was estimated according to Kim et al. [18]. A reaction mixture containing 200 μL 1.6% Lintner potato starch, 100 μL sodium acetate buffer (1 M, pH 5.0) and 700 μL culture supernatant was incubated at 55 °C for 30 min. Reaction was stopped by immersing the tube in a boiling water bath for 10 min and after cooling it, glucose was enzymatically measured with a glucose-oxidase kit (Glicemia enzimática, Wiener lab., Argentina). A heat-treated culture fluid (10 min/100 °C) was used as blank. One unit of enzyme was defined as the amount of protein required to release 1 μmol glucose $min^{-1}\,mL^{-1}$ of enzyme sample.

For starch determination 180 μL 1 N HCl were added to 180 μL supernatant. The mixture was boiled 45 min and then neutralized with 1 N NaOH [19]. Released reducing sugars were colorimetrically measured using the DNSA method [17].

Biomass dry weight (BDW) was determined by centrifugation of culture broth samples at 10,000 × *g* for 10 min, washing the pellet with distilled water and drying at 105 °C to constant weight.

3. Results and discussion

3.1. Effect of DOT level

The influence of dissolved oxygen tension (DOT) on the production of amylolytic enzymes during cassava starch fermentation by *S. fibuligera* was studied under batch culture mode at three different DOT levels (Table 1). At all the aeration conditions tested (10, 30, 50% DOT), growth profiles were quite similar and biomass reached a concentration around $2.5\,g\,L^{-1}$. This fact confirmed that, even at the lowest DOT tested, yeast growth did not become limited by oxygen supply. Free glucose in culture broth was detectable even at the exponential growth, denoting that starch hydrolysis rate surpassed sugar utilization rate (data not shown). Nevertheless, at 10% DOT, a certain delay in glucose accumulation suggested that starch hydrolysis may be oxygen-limited, though still enough to maintain normal growth.

While maximal biomass was similar at any of the aeration conditions tested, amylolytic activity was significantly higher

Author's personal copy

Table 1
Amylolytic activity in batch cultures at different DOT in SM medium with 5 g L^{-1} cassava starch

DOT (%)	Cultivation time (h)	Biomass (g L^{-1})	Yield[a] (g g^{-1})	α-amylase (U L^{-1})	Glucoamylase (U L^{-1})
10	24	2.30 ± 0.23	0.52 ± 0.15	310 ± 9	150 ± 24
	48	2.43 ± 0.18	0.53 ± 0.30	385 ± 22	280 ± 70
30	24	2.63 ± 0.05	0.51 ± 0.12	543 ± 18	170 ± 32
	48	2.70 ± 0.12	0.52 ± 0.23	464 ± 20	340 ± 50
50	24	2.40 ± 0.20	0.53 ± 0.21	1560 ± 20	570 ± 39
	48	2.36 ± 0.10	0.51 ± 0.18	974 ± 30	650 ± 10

[a] Yield (g g^{-1}): g_{BDW} g^{-1}consumed cassava starch.

at 50% DOT, indicating that enzyme production was markedly favored by aeration (Table 1). Even so, DOT levels above 50% could not be adopted because of the excessive foam arisen.

Alpha-amylase and glucoamylase production improved along with the increase in DOT level (Table 1). After 24 h of cultivation at 50% DOT (Fig. 1), the maximum α-amylase and glucoamylase titers could be achieved. However, α-amylase decreased at 48 h while glucoamylase remained constant (Fig. 1). This fact should be taken into account at the time of the process design, and it might be likely related to the physiology of cassava starch enzymatic attack.

3.2. Optimization of temperature and pH

Under the operative conditions tested, optimal pH for enzyme production was shown to be 5.5 with a specific α-amylase activity of 562 U g_{BDW}^{-1} (Fig. 2). Under steady-state conditions, glucoamylase activity was low and remained constant in a pH range from 4.0 to 6.5. Reasonably, highest α-amylase values correlated with lowest residual starch in culture broth (Fig. 2).

When growing in batch culture mode, *S. fibuligera* growth occurred at temperatures from 15 to 40 °C, with the optimum at about 30 °C and being suppressed at 40 °C (data not shown). Considering that vigorous growth took place at temperatures between 30 and 37 °C, amylolytic enzymes production was studied in continuous culture at temperatures within this range, and maintaining pH at 5.5 and DOT at 50%. Particularly, highest α-amylase production occurred at 34 °C (1000 U g_{BDW}^{-1}), decreasing by 66% at 37 °C (Fig. 3). These results are somewhat at variance with those of Ueda and Saha [20] who reported the highest amylolytic activity at 25 °C and pH 6.0, and with those of Futatsugi et al. [21] who established 30 °C and free pH as the optimal conditions. However, our results were similar to those obtained by Gogoi et al. [22], who

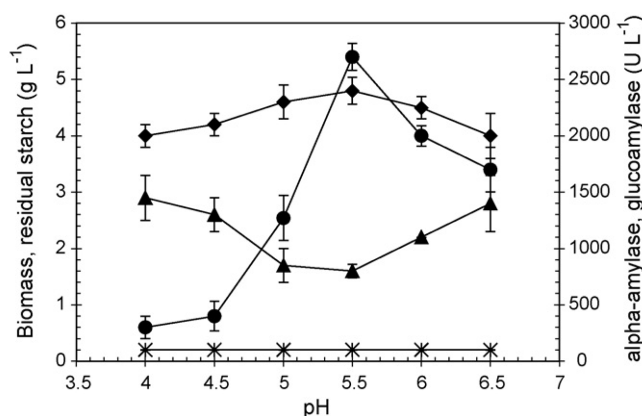

Fig. 2. Effect of pH on amylolytic enzymes production in SM medium with 10 g L^{-1} cassava starch as sole carbon source, at a dilution rate of 0.18 h^{-1}, 50% DOT and 30 °C. ♦ biomass, ▲ residual starch, ● α-amylase, ✳ glucoamylase.

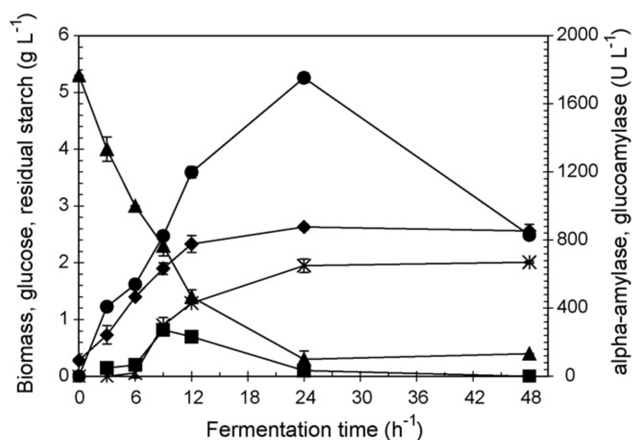

Fig. 1. Kinetic growth and amylolytic enzymes production in SM medium with 5 g L^{-1} cassava starch as sole carbon source, at 30 °C, 50% DOT and pH 5.0. ♦ biomass, ▲ residual starch, ● α-amylase, ✳ glucoamylase, ■ glucose.

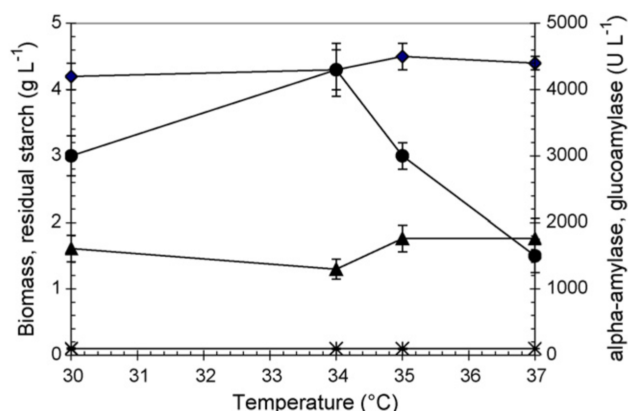

Fig. 3. Effect of temperature on amylolytic enzymes production in SM medium with 10 g L^{-1} cassava starch as sole carbon source, at a dilution rate of 0.18 h^{-1}, pH 5.5 and 50% DOT. ♦ biomass, ▲ residual starch, ● α-amylase, ✳ glucoamylase.

Author's personal copy

Table 2
Biomass and amylolytic enzyme activities under steady-state conditions after different nutritional shifts

Nutritional pulse-shift	Biomass ($g\,L^{-1}$)	α-amylase ($U\,g_{BDW}^{-1}$)	Glucoamylase ($U\,g_{BDW}^{-1}$)
Yeast extract, $2\,g\,L^{-1}$ [a]	3.70 ± 0.30	362 ± 29	21.6 ± 1.8
Vitamins [b,a]	4.60 ± 0.10	509 ± 11	15.2 ± 0.3
$(NH_4)_2\,SO_4$, $2\,g\,L^{-1}$	4.37 ± 0.20	437 ± 20	ND
Peptone, $1\,g\,L^{-1}$	4.80 ± 0.09	360 ± 7	ND
$MgCl_2$, $0.6\,g\,L^{-1}$ [a]	4.70 ± 0.10	740 ± 16	21.3 ± 0.4
$CaCl_2{\cdot}2H_2O$, $0.1\,g\,L^{-1}$ [a]	4.65 ± 0.12	766 ± 20	21.5 ± 0.6
G1	4.70 ± 0.20	766 ± 32	21.3 ± 0.9
G2	4.76 ± 0.26	794 ± 43	23.1 ± 1.3
$MnSO_4{\cdot}7H_2O$, $1\,mg\,L^{-1}$ [c,a]	4.74 ± 0.20	863 ± 36	38.0 ± 1.6

G1 = H_3BO_3 ($1000\,\mu g\,L^{-1}$), $CuSO_4$ ($80\,\mu g\,L^{-1}$), KI ($200\,\mu g\,L^{-1}$). G2 = $FeCl_3$ ($200\,\mu g\,L^{-1}$), $MnSO_4{\cdot}7H_2O$ ($400\,\mu g\,L^{-1}$), Na_2MoO_4 ($200\,\mu g\,L^{-1}$), $ZnSO_4$ ($400\,\mu g\,L^{-1}$). ND: not detectable.

[a] Compounds selected for the OPT medium.

[b] Vitamins: biotin ($2\,\mu g\,L^{-1}$), calcium pantothenate ($400\,\mu g\,L^{-1}$), folic acid ($2\,\mu g\,L^{-1}$), inositol ($2000\,\mu g\,L^{-1}$), niacin ($400\,\mu g\,L^{-1}$), p-aminobenzoic acid ($200\,\mu g\,L^{-1}$), pyridoxine hydrochloride ($400\,\mu g\,L^{-1}$), riboflavin ($200\,\mu g\,L^{-1}$), thiamine hydrochloride ($400\,\mu g\,L^{-1}$).

[c] $MnSO_4$ concentration was previously optimized in Erlenmeyer-flask scale.

reported best amylolytic enzymes production by *S. fibuligera* at 35 °C.

3.3. Culture medium optimization

S. fibuligera has been described as a yeast with restricted growth in synthetic medium [23] and unable to grow in vitamin-free medium [24]. Culture medium optimization started with a basic medium containing $2\,g\,L^{-1}$ yeast extract and $10\,g\,L^{-1}$ cassava starch. Yeast extract is perfectly known as a source of important growth factors for *S. fibuligera* and it has been already found an optimal organic nitrogen supplement for glucoamylase production by fungal cultures [25]. Nevertheless, as some vitamins might be denatured during autoclaving, then the significant increase in biomass production (24.3%, $P < 0.001$) and in specific α-amylase activity (40.6%, $P < 0.001$) after the vitamin pulse (Table 2) might be reasonable. Addition of nitrogen sources like ammonium or peptone had a negative effect, particularly on glucoamylase production ($P < 0.001$, for both N-sources), and in a lesser extent, on α-amylase ($P < 0.001$, for peptone). Similar results have been earlier reported by other authors [22,26]. $MgCl_2$ pulse-shift had no significant influence on biomass production ($P > 0.05$), whereas specific activity of amylases became significantly increased, especially for α-amylase (51.4% increase, $P < 0.001$). Trace elements (G1 and G2) and $CaCl_2{\cdot}2H_2O$ had no significant influence neither on the amylolytic enzymes production nor on biomass ($P > 0.05$). As a high sensitivity to an excess of $MnSO_4{\cdot}7H_2O$ had been previously noted for this strain in batch cultures (data not shown), the pulse shift assay was performed at $1\,mg\,L^{-1}$. That led to a moderately increased glucoamylase activity ($P < 0.001$). Therefore, the final composition of optimized culture medium (OPT) consisted in: yeast extract, $2\,g\,L^{-1}$; vitamins (composition and concentrations as described in Table 2); $MgCl_2$, $0.6\,g\,L^{-1}$; $MnSO_4{\cdot}7H_2O$, $1\,mg\,L^{-1}$; $CaCl_2{\cdot}2H_2O$, $0.1\,g\,L^{-1}$ and cassava starch, $10\,g\,L^{-1}$. Despite not a significant influence by $CaCl_2$ supplementation was noted, OPT medium finally included this salt because of its role as an important α-amylase stabilizer and cofactor [25].

3.4. Effect of dilution rate

Studies on the influence of dilution rate were performed with $5\,g\,L^{-1}$ cassava starch. Working at $10\,g\,L^{-1}$ cassava starch was not viable because the high biomass yields obtained at this starch concentration made difficult to operate the system at low dilution rates. All the other components were maintained in the same proportion to the carbon source according to preceding optimization data (see Section 3.3, OPT medium).

At dilution rates between 0.22 and $0.30\,h^{-1}$, maximum yield of biomass was $0.62\,g_{BDW}\,g^{-1}_{consumed\ starch}$ (Fig. 4). These results were in accordance with those reported elsewhere, e.g. $0.45\,g\,g^{-1}$ [27] or $0.84\,g\,g^{-1}$ [28] also for *S. fibuligera* and with starch as the sole carbon source.

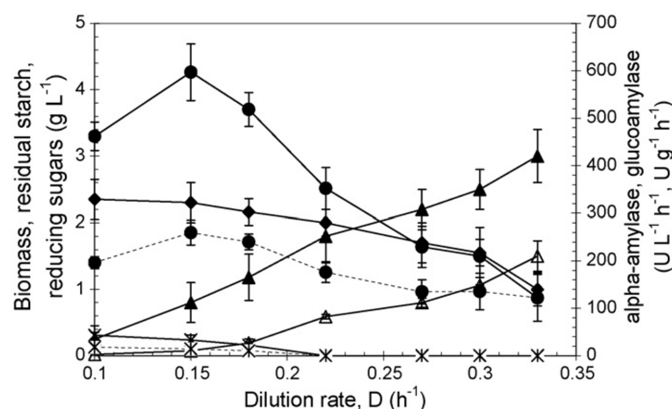

Fig. 4. Effect of dilution rate on amylolytic enzymes production, in OPT medium with $5\,g\,L^{-1}$ cassava starch as sole carbon source, at pH 5.5, 34 °C and 50% DOT. ♦ biomass, ▲ residual starch, △ reducing sugars, ● α-amylase (—, volumetric productivity, . . ., specific productivity), ✳ glucoamylase (—, volumetric productivity, . . ., specific productivity).

Author's personal copy

Table 3
Effect of Tween 80 on volumetric and specific amylolytic enzymes productivity

Culture media	α-amylase			Glucoamylase		
	($U\,g^{-1}$)	($U\,L^{-1}\,h^{-1}$)	($U\,g^{-1}\,h^{-1}$)	($U\,g^{-1}$)	($U\,L^{-1}\,h^{-1}$)	($U\,g^{-1}\,h^{-1}$)
OPT + 0.05% w/v Tween 80[a]	1161.3	516.7	82.0	161.3	41.7	6.7
OPT + 0.2% w/v Tween 80[a]	1416.7	633.3	100.7	208.3	52.1	8.7
OPT + 1% w/v Tween 80[a]	1292.3	616.7	94.1	266.2	72.1	11.1
OPT w/o Tween 80[a]	1033.3	383.3	67.8	183.3	45.8	7.6
DOT-culture conditions[b]	674.5	71.7	28.1	235.3	25.1	9.8

All presented values exhibited standard deviations below 10%, and are average of at least three replicates.
[a] These results were obtained after 24 h of incubation in batch cultures with Optimized culture medium (OPT, see Table 2) at pH 5.5, 50% DOT and 34 °C, with $10\,g\,L^{-1}$ cassava starch.
[b] Results corresponding to 24 h batch cultures performed under the same culture conditions as for DOT assays (see 2.3.), i.e. SM medium containing $5\,g\,L^{-1}$ cassava starch, incubated at 450 rpm, 30 °C and pH 5.0. Displayed values were obtained at 50% DOT.

Biomass productivity increased linearly with the increase in dilution rate, reaching a value of $0.186\,g_{BDW}\,g^{-1}_{consumed\,starch}h^{-1}$ at a dilution rate of $0.30\,h^{-1}$. This result was similar to that obtained by Lee and Simard [27]. The critical dilution rate calculated from washout assays [29] was $0.37\,h^{-1}$.

Maximal amylolytic enzyme values were found at $0.10\,h^{-1}$, reaching values of 430 and 4620 $U\,L^{-1}$ for glucoamylase and α-amylase, respectively. Glucoamylase activity was not detected at dilution rates higher than $0.18\,h^{-1}$ (Fig. 4). Amylolytic enzyme productivity decreased with the increase in dilution rate, showing maximum values of 597 $U\,L^{-1}\,h^{-1}$ and 259 $U\,g^{-1}_{BDW}\,h^{-1}$ for α-amylase, at a dilution rate of $0.15\,h^{-1}$ and, 43 $U\,L^{-1}\,h^{-1}$ and 18 $U\,g^{-1}_{BDW}\,h^{-1}$ for glucoamylase, at a dilution rate of $0.10\,h^{-1}$. The observed decrease when dilution rate was increased suggested that enzyme biosynthesis might be subjected to catabolic repression. This effect has been already observed in *S. fibuligera* growing with carbon sources such as glucose, maltose and starch [28].

3.5. Effect of Tween 80

The incidence of surfactants on microorganisms, as well as the mechanism by which they enhance enzyme production, are so far not well understood. References on this subject include for instance the finding of increased extracellular esterase activity in *Thermomonospora curvata* by Tween detergents [30] and the increase in α-amylase production by *Bacillus subtilis* when growing with surfactants [31]. Further positive effects of Tween on the extracellular enzyme production by different microorganisms have been already reported [32–35]. In the present case, the addition of Tween 80 to OPT medium significantly enhanced amylolytic enzyme production in *S. fibuligera* (Table 3).

4. Conclusions

The excreted amounts of α-amylase and glucoamylase by *S. fibuligera* DSM-70554 could be successfully manipulated by certain operative and nutritional conditions. Among the evaluated culture conditions, high oxygen saturation (50% DOT), temperature at 34 °C and pH 5.5 demonstrated to enhance enzyme production, particularly for α-amylase. Inorganic nitrogen (as ammonium) and peptone negatively affected enzyme biosynthesis, whilst yeast extract showed to be highly effective as an organic nitrogen source with no interference on the amylolytic enzymes production profile.

The addition of vitamins, Mg^{2+} and Mn^{2+} into the culture medium had a positive effect on enzyme production, and consequently, that allowed a good liquefaction and saccharification of cassava starch. On the other hand, the incorporation of surfactants such as Tween 80 appreciably improved enzyme production.

Under continuous culture conditions it was possible to achieve high amylolytic enzyme productivities (both volumetric and specific) up to a dilution rate of $0.10\,h^{-1}$. However beyond this point, they showed a marked decrease likely related to catabolic repression.

Maximal values of 633.3 $U\,L^{-1}\,h^{-1}$ of α-amylase and 72.1 $U\,L^{-1}\,h^{-1}$ of glucoamylase under optimized conditions (Table 3) represented ∼9 and ∼3-fold increments, respectively, when compared to the preliminary fermentation assays under non-optimal conditions.

Concluding, the use of the optimized culture medium (OPT) under the optimal operative conditions herein described, allowed *S. fibuligera* DSM-70554 to grow under batch culture mode leading to 97% degradation of cassava starch with a remaining 3% likely related to limit dextrins. Being cassava one of the most important staple crops in the tropics, and one of the most significant sources of carbohydrates in the developing world, the possibility to improve cassava starch utilization and conversion efficiencies by yeasts with biotechnological potential represents a great progress.

Acknowledgements

This work was partially supported by grants ERB-TS3*-CT94-0279 (Commission of the European Communities), 26/D 108 (CIUNT, Argentina) and PIP 0298/98 (CONICET, Argentina).

References

[1] Westby A. Cassava utilization, storage and small-scale processing. In: Hillocks RJ, Thresh JM, Bellotti AC, editors. Cassava: biology, production and utilization. Wallingford: CABI Publishing; 2002. p. 281–300.

Author's personal copy

[2] Petersen W, Umbeck P, Hokanson K, Halsey M. Biosafety considerations for selectable and scorable markers used in cassava (*Manihot esculenta* Crantz) biotechnology. Environ Biosafety Res 2005;4:89–102.
[3] Pandey A, Nigam P, Soccol CR, Soccol VT, Singh D, Mohan R. Advances in microbial amylases. Biotechnol Appl Biochem 2000;2:135–52.
[4] Aiyer PV. Amylases and their applications. African J Biotechnol 2005;4. pp. 1525–1295.
[5] González C. Estudio de levaduras amilolíticas de importancia industrial. Aspectos básicos y aplicados. Argentina: Universidad Nacional de Tucumán; Ph.D. Thesis; 2000.
[6] Hostinová E. Amylolytic enzymes produced by the yeast *Saccharomycopsis fibuligera*. Biologia Bratislava 2002;57:247–51.
[7] Eksteen JM, van Rensburg P, Cordero Otero RR, Pretorious IS. Starch fermentation by recombinant *Saccharomyces cerevisiae* strains expressing the α-amylase and glucoamylase genes from *Lipomyces kononenkoae* and *Saccharomycopsis fibuligera*. Biotechnol Bioeng 2003;84:639–46.
[8] Dostálek M, Häggtröm MH. Mixed cultures of *Saccharomycopsis fibuligera* and *Zymomonas mobilis* on starch. Use of oxygen as a regulator. Eur J Appl Microbiol Biotechnol 1983;17:269–74.
[9] Vijaya Sarathi RO, Bassappa SC. Direct fermentation of cassava starch to ethanol by mixed cultures of *Endomycopsis fibuligera* and *Zymomonas mobilis*; synergism and limitations. Biotechnol Lett 1996;18:1315–8.
[10] González C, Delgado O, Baigorí M, Abate C, de Figueroa LIC, Callieri DA. Ethanol production from native cassava starch by a mixed culture of *Endomycopsis fibuligera* and *Zymomonas mobilis*. Acta Biotechnol 1998;18:149–55.
[11] Häggström MH, Dostálek M. Regulation of mixed cultures of *Streptococcus lactis* and *Saccharomycopsis fibuliger*. Eur J Appl Microbiol Biotechnol 1981;22:216–9.
[12] Admassu W, Korus R, Heimsch R. Continuous fermentation of *Saccharomycopsis fibuligera* and *Candida utilis*. Biotechnol Bioeng 1984;27:1511–3.
[13] Figueroa LIC de, Rubinstein L, González C. Utilization of native cassava starch by yeasts. In: Spencer JFT, de Spencer ALR, editors. Methods in biotechnology, food microbiology protocols. Totowa, New Jersey: Humana Press; 2001. p. 307–17.
[14] Serra A, Strehaiano P, Taillandier P. Characterization of the metabolic shift of *Saccharomyces bayanus* var. *uvarum* by continuous aerobic culture. Appl Microbiol Biotechnol 2003;62:564–8.
[15] Mateles RI, Battat E. Continuous culture for media optimization. Appl Microbiol 1974;28:901–5.
[16] Laluce C, Mattoon JR. Developments of rapidly fermenting strains of *Saccharomyces diastaticus* for direct conversion of starch and dextrins to ethanol. Appl Environ Microbiol 1984;48:17–25.
[17] Miller GL. Use of dinitrosalycilic acid reagent for determination of reducing sugars. Anal Chem 1959;31:315–71.
[18] Kim K, Park Ch, Matton J. High-efficiency, one step starch utilization by transformed *Saccharomyces* cells which secrete both yeast glucoamylase and α-amylase. Appl Environ Microbiol 1988;54:966–71.
[19] Dubois M, Gilles KA, Hamilton JK, Roberts PA, Smith F. Colorimetric method for determination of sugars and related substances. Anal Chem 1956;28:350–6.
[20] Ueda S, Saha BCh. Behaviour of *Endomycopsis fibuligera* glucoamylase towards raw starch. Enzyme Microb Technol 1982;5:196–8.
[21] Futatsugi M, Ogawa T, Fukuda H. Scale up glucoamylase production by *Saccharomycopsis fibuligera*. J Ferment Bioeng 1993;76:419–22.
[22] Gogoi BK, Bezbaruah KR, Pillai KR, Baruah JN. Production, purification and characterization of an α-amylase produced by *Saccharomycopsis fibuligera*. J Appl Bacteriol 1987;63:373–9.
[23] Nwoguh CE, Berry DR. Effect of methionine on the growth and morphogenesis of *Saccharomycopsis fibuligera* in defined medium. Biotechnol Lett 1990;12:271–6.
[24] Kurtzman CP, Smith MTh. *Saccharomycopsis* Schiönning. In: Kurtzman CP, Fell JW, editors. The yeast, a taxonomic study. Fourth ed. Amsterdam, New York: Elsevier Science; 1998. p. 374–86.
[25] Anto H, Trivedi UB, Patel KC. Glucoamylase production by solid-state fermentation using rice flake manufacturing waste products as substrate. Biores Technol 2006;97:1161–6.
[26] Lachance MA, Blackwood AC. Etude de la croissance d'Endomycopsis fibuligera sur les résidus de pâtes alimentaires. Ottawa, Canada: Congress ACFAS; 1972.
[27] Lee BH, Simard RE. Growth of *Saccharomycopsis fibuligera* on enzyme hydrolysed potato starch. Develop Ind Microbiol 1983;25:459–66.
[28] Lemmel SA, Heimsch RC, Korus RA. Kinetics of growth and amylase production of *Saccharomycopsis fibuligera* on potato processing wastewater. Appl Environ Microbiol 1980;39:387–93.
[29] Pirt SJ. Principles of microbe and cell cultivation. New York: John Willey and Sons; 1975.
[30] Thies E, Jenkins T, Stutzenberger F. Effects of the detergent Tween 80 on *Thermomonospora curvata*. World J Microbiol Biotechnol 1994;10:657–63.
[31] Goes AP, Sheppard JD. Effect of surfactants on α-amylase production in a solid substrate fermentation process. J Chem Technol Biotechnol 1999;74:709–12.
[32] Kamande GM, Baah J, Cheng K-J, McAllister TA, Shelford JA. Effects of Tween 60 and Tween 80 on protease activity, thiol group reactivity, protein adsorption, and cellulose degradation by rumen microbial enzymes. J Dairy Sci 2000;83:536–42.
[33] Sun Y, Cheng J. Hydrolysis of lignocellulosic materials for ethanol production: a review. Biores Technol 2002;83:1–11.
[34] Zeng GM, Shi JG, Yuan XZ, Liu J, Zhang ZB, Huanga GH, et al. Effects of Tween 80 and rhamnolipid on the extracellular enzymes of *Penicillium simplicissimum* isolated from compost. Enzyme Microb Technol 2006;39:1451–6.
[35] Shi JG, Zeng GM, Yuan XZ, Dai F, Liu J, Wu XH. The stimulatory effects of surfactants on composting of waste rich in cellulose. World J Microbiol Biotechnol 2006;22:1121–7.