Appl Biochem Biotechnol (2010) 162:2058–2066
DOI 10.1007/s12010-010-8981-5

Study of Starch Degradation by Yeasts During Fermentation for Using in Animal Feed

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Received: 16 November 2009 / Accepted: 26 April 2010 /
Published online: 9 May 2010

Abstract Sixteen amylolytic yeasts, drawn from culture collections or isolated from different environments, were used in the present study. Experiments were performed to evaluate the starch degradation due to exocellular activity. The culture media of growth was optimized to find the maximum activity, and some strains produced an extracellular amylolytic enzyme when cultured in presence of starch in the medium. On the other hand, one yeast showed activity in cell extract when cultured under varying conditions in a bench bioreactor; this strain would thus be suitable for use as an animal feed supplement, both as a source of proteins and nucleic acids and in terms of its amylolytic activity.

Keywords Starch degradation · Yeast fermentation · Bioreactor scale-up · Amylolytic activity · Animal feed

Introduction

The EU ban on antibiotic animal-growth promoters from the start of 2006 prompted a marked increase in the demand for alternative feed supplements. General considerations regarding sustainability require that the demand of a constantly growing population for food products of animal origin be met by enhancing animal productivity rather than by increasing agricultural yield per unit of surface area.

Research is currently addressing the potential use of prebiotics, dicarboxylic acids, plant enzymes, derivatives, and microbial biomass, and particularly the effect of these feed additives on rumen fermentation. A number of studies have also focused on the use and limitations of these additives in field conditions and on compliance with EU legislation [1].

The potential use of exogenous enzymes to enhance the digestibility of plant substrates has also been studied, with particular reference to fibrolytic and amylolytic enzymes. Fibrolytic enzymes—primarily cellulases and xylanases—increase the fermentation of

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crops such as corn silage, alfalfa hay, and yucca and are secreted by anaerobic bacteria typically found in the rumen [2, 3]. A number of microorganisms able to break down lignocellulosic material in agricultural by-products are being studied as producers of hydrolytic enzymes. Although lignocellulose has a certain value as a cattle feed, its bioavailability requires the breakdown of lignin by means of pretreatment with depolymerizing enzymes, ligninases, xylanases, and pectinases [4].

Little interest has been shown in amylolytic activity, even though starch is a major component of high-yield diets in cattle. Certain yeast and mould strains isolated from various sources, though aerobic, offer considerable potential, since their secreted enzymes are partially adsorbed into the cell wall, thus providing a biomass rich in proteins, nucleotides, and vitamins, as well as a cell extract with hydrolytic activity [5–9]. Random hydrolysis of starch by alpha-amylases produces low-molecular-weight oligosaccharides and, eventually, maltotriose and maltose from amylose and limit dextrins, and maltose and glucose from amylopectin. Addition of alpha-amylase to the diet alters the rumen fermentation profile, increasing blood butyrate levels both in transition dairy cows and in calves, with beneficial epithelial effects. Increased plasma beta-hydroxybutyrate and nonesterified fatty acids pre- but not postpartum and a tendency for increased plasma glucose postpartum demonstrate shifting reliance from lipid- to carbohydrate-based metabolism postpartum in cows fed with alpha-amylase [10]. The administration of amylases promotes the growth of beneficial rumen bacteria, which in the presence of starch grow very slowly, thus modifying ruminal starch digestion and increasing productivity. Beneficial effects include weight gain, improved finishing, increased protein yield, and reduced milk fat proportion [11].

Addition of bacterial alpha-amylases to a lamb diet high in grains with low digestion rates has been shown to improve partial efficiency by increasing ruminal starch digestion [12].

Amylolytic activity is largely governed by the half-life of rumen enzymes, which may be inactivated by rumen proteases. Increased resistance to degradation requires further exploration, via a number of approaches including immobilization [13] and the use of cell extracts rich in cell wall proteins with amylolytic activity.

The present study sought to evaluate the starch-degradation capacity of several amylolytic microorganisms with exocellular activity, grown on an optimized culture medium. Cell extract activity was also analyzed with a view to its possible use as an animal feed additive providing biomass and enzyme activity.

Materials and Methods

Amylase Zymogram

The microorganisms listed in Table 1 were screened on Vogel minimal salt agar medium with added 1% soluble starch. Plates were inoculated with 5 μL of a preculture and incubated for 3 days at 30 °C and then stained with 50% Lugol's solution (*v*/*v*).

Optimization of the Culture Medium

(a) Yeasts were grown in two different media: on Vogel minimal salt medium with 1% (*w*/*v*) soluble starch on another medium with added 0.2% (*w*/*v*) yeast extract and 0.1% peptone (*w*/*v*)

Table 1 Microorganisms used for the study.

Specie	Collection
Saccharomyces cerevisiae	UCLM 383
Pichia farinosa	CECT 1447
Debaryomyces hansenii	UCLM ns1
Pichia anomala	CECT 10320
Pichia jadinii	CECT 1060
Saccharomyces cerevisiae	UCLM S377
Candida molischiana	ATCC 2516
Saccharomyces cerevisiae	UCLM S325
Saccharomyces cerevisiae	CBS 7958
Sympodiomyces attinorum	CBS 9734
Lipomyces tetrasporus	CBS 5910
Debaryomyces occidentalis	CBS 819
Pichia burtonii	CLIB 35
Saccharomycopsis capsularis	CLIB 37
Saccharomyces cerevisiae	CECT 1389
Bacillus cereus	CECT 495

UCLM Universidad de Castilla La Mancha, *CECT* Colección Española de Cultivos Tipo, *CLIB* International Center for Microbial Resources, *CBS* Centraalbureau voor Schimmelcultures, *ATCC* American Type Culture Collection

(b) In the light of the results obtained, a second assay was performed to optimize the starch and peptone concentrations, using a medium containing 0.2% yeast extract, 0.1–2% peptone (0.1%, 0.5%, 1%, and 2%), and 2%, 6%, or 8% starch. Two hundred fifty-milliliter flasks containing 100 mL of medium were inoculated with 10^6 cells/mL of a preculture of CLIB 37.

Study of Starch Degradation

Once optimized, the culture medium, those strains displaying the largest hydrolysis zones on minimal Vogel agar with added soluble starch (1%) was measured.

Yeasts for testing were precultured in a broth containing 0.2% yeast extract, 0.1% peptone, and 1% soluble starch as inductor. One hundred-milliliter flasks containing 50 mL of the optimized medium were inoculated with 10^6 cells/mL and incubated in a thermostat-controlled shaker bath at (150 rpm) at 30 °C for 96 h.

Samples were taken at different incubation times (0, 24, 48, 72, and 96 h) in order to measure starch degradation and perform a viable count on solid medium.

Aerated Fermentation in a Birreactor (Scale Up)

Strain yielding the best results (*Sympodiomyces attinorum*) in the preceding assay was grown in a 3-L BioBundle bioreactor (Applikon^r), equipped with pH, temperature, and dissolved oxygen sensors, and a mechanical shaking system. A total of four fermentations were carried out; the variables analyzed in each fermentation are shown in Table 2. Of optimized broth, 800 mL was inoculated with 10^7 cells/mL, and starch degradation was measured at different intervals (1, 8, 21, 26, 31, 45, and 50 h).

Table 2 Aerated fermentation in a bioreactor (scale up) of *Sympodiomyces attinorum*.

Fermentation	Temperature (°C)	pH[a]	Air flow (l/lh)	Agitation (rpm)
1°	30	–	6.4	300
2°	30	–	17.1	300
3°	30	5.5	17.1	300
4°	34	–	6.4	300

[a] Control by phosphoric acid (8% *v*/*v*) and NH_3 (3%)

Amylolytic Activity in Cell Extract

Biomass from second and third fermentations was recovered from 8 mL of each culture. Cell extracts were obtained following the crude-extract protocol [14]. Cell extract was added to 4 mL of optimized starch medium, and hydrolysis was checked at regular intervals for 50 h.

Statistical Assays

All experiments were done in triplicate, and their standard deviation was calculated and represented in figures. ANOVA was applied with the package; SPSS version 15.0 was used for all statistical procedures.

Results and Discussion

The yeasts and bacteria listed in Table 1 were screened on starch zymograms to check for extracellular amylases. A *Bacillus cereus* (CECT 495) strain with demonstrated amylolytic activity was used as reference strain.

After Lugol staining, all except seven of the 16 strains tested (*Debaryomyces hansenii* (UCLM ns 1), *Saccharomyces cerevisiae* (UCLM S377 and S325), *Pichia anomala* (CECT 10320), *Pichia farinosa* (CECT 1447), *Pichia jadinii* (CECT 1060), and *Lipomyces tetrasporus* (CBS 5910)) displayed a hydrolysis halo when grown on minimal Vogel starch agar medium.

Optimization of the Culture Medium

(a) Although Vogel's medium is suitable for moulds, it is not always ideal for growing yeast cultures, perhaps due to its reduced capacity for biosynthesis. For comparative purposes, therefore, growth and amylolytic activity were also measured on another enriched medium (peptone plus yeast extract), designated “modified medium”; both media contained 1% starch. Since peptone and yeast extract stimulated starch hydrolysis (data not shown), Vogel's medium was omitted in later assays.

(b) Having established that the substrate should contain a nitrogen source (peptone) and a source of minerals and vitamins (yeast extract), it was then necessary to identify the optimum starch concentration to stimulate enzyme synthesis. For this purpose, various peptone/starch formulations were tested using CLIB 37 strain (*Saccharomycopsis capsularis*), which had displayed an intermediate digestion halo on Vogel's starch.

In the first experiment [7], the peptone concentration was kept constant at 0.1%, and 4%, 6%, and 8% starch concentrations were tested. Figure 1a shows the evolution of starch hydrolysis: it is showed that from 24 to 72 h, it indicated that the best results were obtained with the 6% starch-containing medium; for economic reasons, it was deemed preferable to determine the minimum concentration ensuring maximum activity. Lower (4%) and intermediate (5%) concentrations were therefore tested; the best results were found for 5% and 6% concentrations; the 5% medium was thus selected as being the most economical (Fig. 1b).

Having optimized the starch concentration (5%), peptone levels ranging from 0.1% to 2% were tested; the results are shown in Fig. 2. Increasing the peptone concentration did not appreciably enhance amylolytic activity: after 72 h, the 2% peptone medium had only degraded 40% of starch. It also can be corroborated because at 48 h, the starch degradation in the lower peptone concentration medium (0.1%) showed the highest hydrolysis.

As expected, culture-medium pH values varied with peptone, reaching values of around 4 with a peptone concentration of 0.1% and approaching neutral at the highest peptone concentration. However, since hydrolysis results confirmed that amylase secretion was not affected by slightly acid pH values, peptone content was kept at only 0.1% for economic reasons.

Study of Starch Degradation

Having optimized the culture medium (5% starch, 0.2% yeast extract, and 0.1% peptone), starch degradation as a function of time was measured in those yeast strains from Table 1

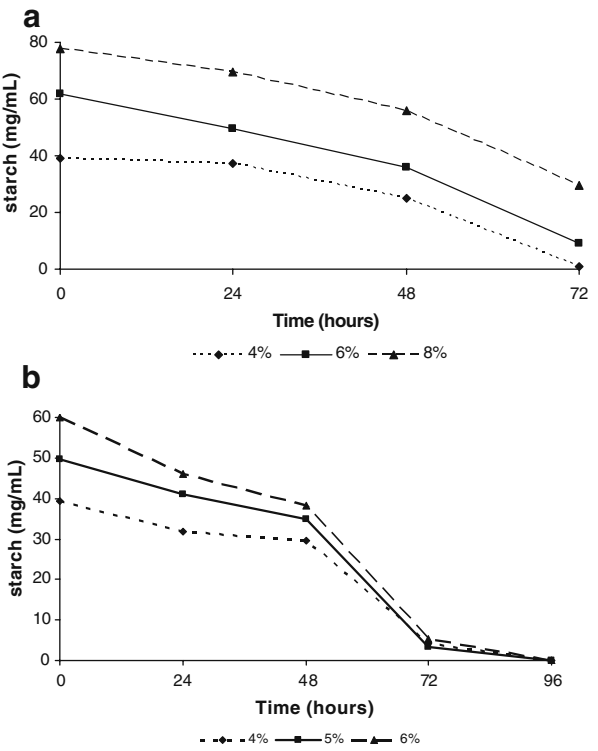

Fig. 1 Starch degradation by CLIB 37 strain in a medium with different starch concentrations ((**a**) 4%, 6%, and 8%; (**b**) 4%, 5%, and 6%)

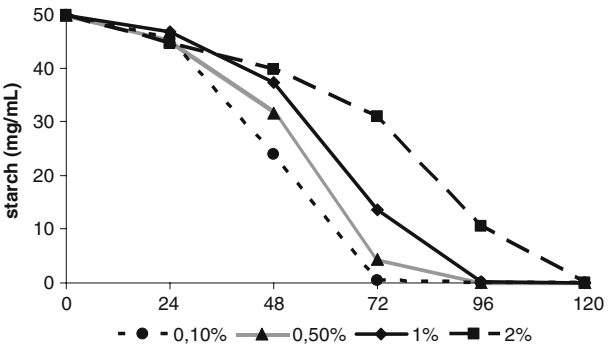

Fig. 2 Starch hydrolysis by CLIB 37 strain in a medium with 5% of starch concentration and different peptone concentrations (0.1%, 0.5%, 1%, and 2%)

which had displayed activity in qualitative assays: *Pichia burtonii* (CLIB 35), *S. capsularis* (CLIB 37), *S. cerevisiae* (CBS 7958), *S. attinorum* (CBS 9734), *Debaryomyces occidentalis* (CBS 819), *S. cerevisiae* (CECT 1389), *S. cerevisiae* (UCLM 383), and *Candida molischiana* (ATCC 2516). A *B. cereus* (CECT 495) strain with demonstrated amylolytic activity was used as reference strain. Results are shown in Fig. 3.

Of the strains displaying slow starch degradation (Fig. 3a), i.e., showing little or no activity, the worst were *C. molischiana* and *P. burtonii*, which, during 100 h of growth, had failed to degrade any starch. It also can be observed in ANOVA results; at 48 h of growth, the starch concentration was significantly different to all the others, but there was no difference between them. Two other strains, *S. cerevisiae* (CBS 7958) and *D. occidentalis*, though degrading almost 50% of the starch in 48 h (significantly different values to the other yeasts), cease to act thereafter, perhaps due to inhibition caused by substrate saturation. Finally, the behavior of *S. capsularis* was different (ANOVA), and it displayed

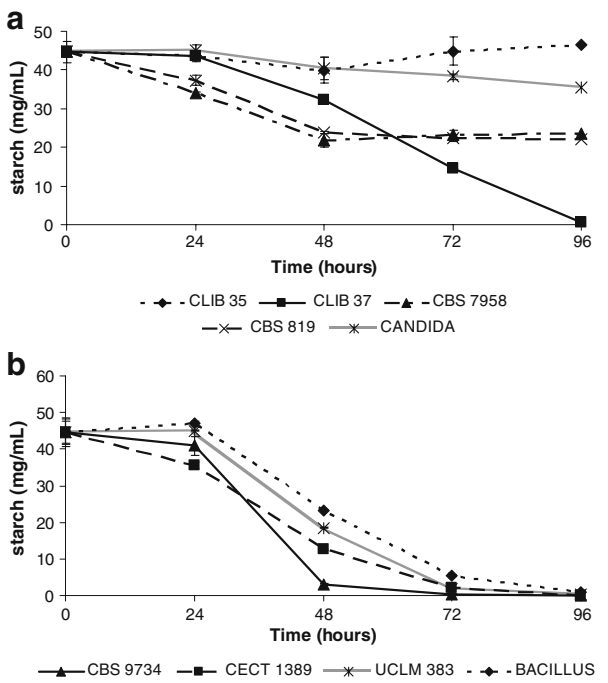

Fig. 3 **a**, **b** Starch degradation by some yeasts strains and *Bacillus cereus*

more regular hydrolytic activity, with a latency phase of almost 20 h, but consuming almost all the starch in 96 h. Viable cell counts confirmed activity data; strains with weak hydrolytic capacity also displayed little population growth. None of these strains exceeded 10^8 cells/mL.

Figure 3b shows amylolytic activity for *B. cereus* and for the some of the strains yielding the best results. All strains acted much more quickly than those indicated earlier. At 48 h of growth, all of them showed significantly differences in starch degradation. *S. attinorum* proved exceptionally swift, hydrolyzing almost all the starch in the medium after only 48 h; by comparison, *B. cereus* performed less well, taking almost 100 h to completely consume the substrate. The two *S. cerevisiae* (UCLM 383 and CECT 1389) strains also performed reasonably well. Viable cell counts for the two best strains, *S. attinorum* and *S. cerevisiae* (UCLM 383), at 48 h were 1.3×10^8 and 05×10^8 cell/mL, respectively, and at 96 h, the counts reached 7.7×10^8 and 9.2×10^8 cell/mL.

Although four of the strains showed in Fig. 3b performed well, *S. attinorum* was selected because it provided the best results at 48 h and also because it is an aerobic strain and therefore better suited for use in an aerated bioreactor; though showing good hydrolytic capacity, some *Saccharomyces* strains display the Crabtree effect, leading to the production of small but undesirable amounts of ethanol.

Aerated Fermentation in a Bioreactor (Scale Up)

S. attinorum was used in four "batch" fermentations performed in an aerated bioreactor fitted with a shaking system, varying the rate of airflow, pH, and process temperature (Table 2). Starch degradation for each experiment is shown in Fig. 4. Comparing with previous results, it is observed how during the scale up in all fermentations, the starch degradation is quicker (at 30 h, almost 100% of starch is already consumed). The most favorable conditions were achieved either with controlled pH (third fermentation) or at a higher temperature (34 °C, fourth fermentation); after 20 h, almost all the starch had been consumed; nevertheless, in the other two assays, the residual starch concentration was around the half at the same time. These data suggest that the airflow concentration does not affect.

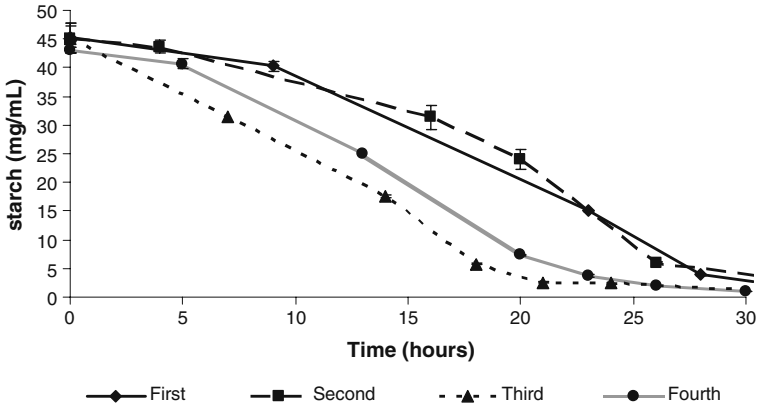

Fig. 4 Starch hydrolysis by *Sympodiomyces attinorum* in a bioreactor with agitation under different variables

Table 3 Yield of four fermentations at 32 h (Y=biomass obtained/consumed starch×100) expressed as biomass obtained respect to starch degradation.

Fermentation	mg dry matter/mL	mg/mL consumed starch	Y×100
1°	3.17	44.61	7.11
2°	4.92	39.10	12.57
3°	6.46	44.76	14.42
4°	3.69	42.22	8.75

However, in terms of yield (Y=biomass obtained/substrate consumed×100), the best results were achieved in the second and third fermentations either because a large amount of biomass was produced (third) or because the starch consume was lower (second). These results are showed in Table 3.

Amylolytic Activity in Cell Extract

Biomass from second and third fermentations was recovered from 8 mL of each culture, to ascertain whether cell extract was suitable both as a source of protein and nucletoides and if they were able to degrade starch. The cellular extract was incubated with 4 mL of optimized medium (5% starch, 0.1% peptone) at 28 °C during 50 h. As Fig. 5 shows, cell extract was able to degrade starch, displaying a performance similar to that of culture supernatant: after 24 h, i.e., roughly the duration of rumen digestion, almost 90% of the starch in the medium had been consumed.

In all cases, the standard deviation was lower than 10%, as it is observed in the figures.

Conclusions

The work development presents some important conclusions about the starch degradation by microorganisms and their use in animal feed. *S. attinorum* and two *S. cerevisiae* displayed the best behavior during starch degradation, even more than the positive control (*B. cereus*). Due to Crabtree effect that *S. cerevisiae* presents, *S. attinorum* (an aerobic strain) was chosen for further studies. In the scale up of the process, the pH and temperature

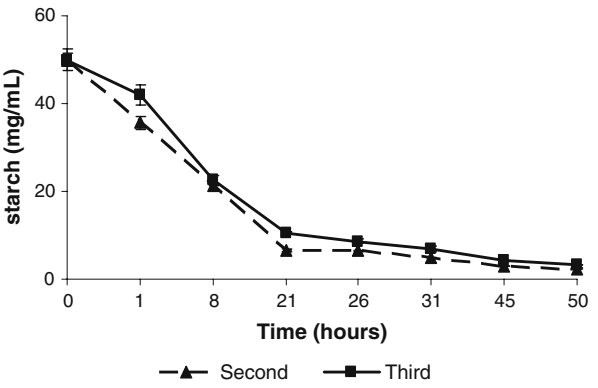

Fig. 5 Starch degradation by *Sympodiomyces attinorum* extract

control affected the activity, but regarding to the yield, the most important variable was the pH. The airflow seemed not to affect the amylolytic capacity.

The main conclusion of the work is that both the cell extract and the culture supernatant *S. attinorum* would be useful as animal feed additives; they could also be used together as a feed supplement as a fermented liquid enriched with cell extract.

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