ISSN 1330-9862
(FTB-1700)

original scientific paper

Use of Potato Nitrogen Concentrate in the Production of α-Amylase by *Aspergillus oryzae*

Marie-Astrid Dolnik[1], Eric Thaller[1], Ferdinand Karner[1], Werner A. Hampel[1], Ulrich Stifter[2], Eduard Taufratzhofer[2], Marnik-Michel Wastyn[2] and Bernhard F. Adamitsch[1]*

[1]Institute of Chemical Engineering, Section of Industrial Microbiology and Bioengineering, Vienna University of Technology, Getreidemarkt 9/166, A-1060 Vienna, Austria

[2]Zuckerforschung Tulln GmbH, Reitherstraße 21-23, A-3430 Tulln, Austria

Received: March 30, 2006
Accepted: May 23, 2006

Summary

The influence of various nitrogen sources and media supplements on α-amylase (EC 3.2.1.1) formation by *Aspergillus oryzae* ATCC 1011 was investigated in shake flask experiments and batch fermentations. Both inorganic and organic nitrogen-containing supplements have been applied, while corn starch and ammonium sulphate were used as the major source of carbon and nitrogen, respectively. Shake flask experiments revealed that potato nitrogen concentrate (PNC) is almost equivalent to corn steep liquor (CSL) in supporting amylase formation. A pretreatment step consisting of clarification of the turbid material did not show any significant effect. The replacement of the inorganic nitrogen source by sodium nitrate led to lower enzyme yields. Other complex supplements may reduce the enzyme level formed, *e.g.* casein hydrolysate, or increase the amylase titre slightly, *e.g.* yeast extract or malt extract. Cultivations in instrumented bench top reactors on media supplemented with PNC led to higher cell growth rates and yields of α-amylase in comparison with the medium without any supplement. Replacement of PNC by CSL revealed a slightly increased enzyme level, which is in the range of 9–17 % after 100 h of cultivation. Only minor differences were revealed in the growth kinetics and enzyme formation when PNC was used as the sole nitrogen source, replacing a mixture of soybean meal, yeast extract, malt extract and casein hydrolysate in bioreactor cultivations with lactose as the carbon source. However, metabolic differences as seen from the course of dissolved oxygen tension (DOT), α-amino nitrogen concentration and the amount of acid needed to maintain a constant pH were observed.

Key words: α-amylase, *Aspergillus oryzae*, potato nitrogen concentrate (PNC)

Introduction

Some products of agricultural industrial processing such as molasses, corn steep liquor (CSL), oilseed cake, *etc.* are used as cheap and effective growth supporting components in media for industrial microbial fermentation processes. Potato nitrogen concentrate (PNC), a product of starch production from potatoes, is a highly viscous liquid of dark colour with a high content of nitrogen. After the removal of starch and fibrous material from the pulp, the residual liquid is treated by heat to coagulate protein and the supernatant liquid concentrated to approximately 70 % of dry matter to give the so-called PNC. The typical composition of PNC is given in Table 1. As a source of nitrogen together with soluble polysaccharides, it seems to be predestined as a valuable

*Corresponding author; Phone: ++43 01 58 801 17 222; E-mail: astriddolnik@hotmail.com

Table 1. Typical composition of potato nitrogen concentrate as provided by Agrana Zucker und Stärke AG, Gmünd, Austria

Component	Composition
Dry matter	60–70 %
Insoluble material	3–3.5 %
Total nitrogen	17–27 g/L
α-Amino nitrogen	10–11 g/L
Protein (Bradford, as BSA)	~3.2 g/L
Total carbohydrates	220–240 g/L
Reducing sugars (as glucose)	10–11 g/L

medium component for the formation of amylolytic enzymes by filamentous fungi.

Aspergillus oryzae has been used in food industry and enzyme production for more than a hundred years. Traditional applications such as the production of α-amylase are still topics of interest. For obtaining high productivity, the influence of various culture conditions and the effect of several medium components on growth and enzyme formation using different organisms and strains have been studied in detail by several authors (*1–6*). For industrial production of amylases by fungal cells, media containing lactose and various complex standard constituents like soybean meal, yeast extract, malt extract or protein hydrolysates have been proposed by several authors (*7,8*). Although glucose is a carbon source widely used for growth, some authors (*4,5*) proposed to include cheaper polysaccharides, most having α-1,4-linked glucose units, like starch or starch hydrolysates such as maltodextrin or Nutriose®. In addition, various inorganic and organic substances like ammonium, nitrates, urea or L-asparagine were tested as potent nitrogen sources, but nevertheless some reports were contradictory to the others (*1–3*). The addition of complex materials such as CSL, yeast extract, malt extract, *etc*. as media supplements, which serve as a source of nitrogen or assist in supporting growth or product formation, was reported elsewhere (*9*). Even the effects of detergents such as Tween 80 or Span 40 on amylase formation by filamentous fungi were tested (*10*).

The experiments reported here focus both on the comparison of different conventional medium supplements with PNC, and the use of PNC as the sole nitrogen source regarding the influence on α-amylase formation by *A. oryzae*. Experiments were carried out by cultivation in shake flasks as well as in bench top bioreactors run in parallels.

Material and Methods

Microorganism

The wild type strain *A. oryzae* ATCC 1011 was used. The organism was subcultured monthly on potato dextrose agar slants and stored at 4 °C.

Substrates and chemicals

Maisita® (corn starch), PNC and CSL were products of Agrana Zucker und Stärke AG, Gmünd, Austria. PNC and CSL contained 28 and 41 g/L of α-amino nitrogen, respectively, calculated as glycine. The syrups were diluted before use with an equal amount of distilled water forming a turbid solution (PNC_{turbid} and CSL_{turbid}, respectively). As a pretreatment step, the precipitate was removed by centrifugation (3200 × *g*; 30 min), resulting in a clear substrate (PNC_{clear} and CSL_{clear}, respectively). Soybean meal (0.8 % α-amino nitrogen) was purchased from Vollkraft, Grimmenstein, Austria. Yeast extract (4.1 % α-amino nitrogen), malt extract (0.2 % α-amino nitrogen) and casein hydrolysate (6.1 % α-amino nitrogen) were of bacteriological grade and obtained from Merck KGaA, Darmstadt, Germany. All other chemicals were of analytical grade.

Shake flask cultivations

The preculture contained 245 mL of basal medium (*11*) of the following composition (unless stated otherwise, the amounts are in g/L): corn starch 40.0, $(NH_4)_2SO_4$ 16.0, KH_2PO_4 4.0, citric acid·H_2O 10.9, acetic acid (≥99.5 %) 6.9 mL/L, $MgSO_4{\cdot}7H_2O$ 0.6, $FeCl_3{\cdot}6H_2O$ 0.1, and it was supplemented with 2.1 g/L of CSL_{turbid}. Using 1-litre Erlenmeyer flasks, the medium was sterilized in an autoclave at pH=6.8 (120 °C, 20 min). Cultivations were performed at 28 °C on an orbital shaker (200 rpm, 2.5 cm orbit). Precultures for shake flask and bioreactor experiments were prepared by inoculation with 5 mL of spore suspension (spores of one agar slant harvested with 1.1 % KCl; spore count approx. 10^7 mL^{-1}).

In the shake flask experiments for studying the effect of different nutrients on amylase formation, CSL in the above described medium was replaced either by PNC (3.0 g/L), yeast extract (2.1 g/L), malt extract (39.2 g/L) or casein hydrolysate (1.3 g/L). In the experiments testing the inorganic nitrogen source, $(NH_4)_2SO_4$ was replaced either by $NaNO_3$ (21.0 g/L) or NH_4Cl (12.9 g/L). The amount of replaced nutrient was calculated based on its α-amino nitrogen content for complex components and molecular nitrogen content for inorganic salts. A volume of 5 mL of a 50-hour-old preculture was used as inoculum. Experiments were carried out in 1-litre Erlenmeyer flasks containing 250 mL of medium and were all done in duplicates. Samples were taken aseptically after about 50 h and at the end of the cultivation.

Bioreactor cultivations

For bioreactor cultivations where PNC was applied as a medium supplement, either CSL_{turbid} (2.1 g/L), PNC_{turbid} (3.0 g/L) or no complex supplement was added to the basal medium. Moreover, 0.5 g/L of Tween 80 were added to reduce wall growth. A volume of 100 mL of a 50-hour-old preculture was used as inoculum.

For cultivations with PNC as the sole nitrogen source the medium had the following composition (in g/L): lactose 19.0, $MgSO_4{\cdot}7H_2O$ 0.16, $Na_2HPO_4{\cdot}2H_2O$ 1.78, KH_2PO_4 0.54 and PNC_{clear} 84.0 mL/L. Vitamins (biotin 0.02, choline chloride 69.0, niacin 3.8, calcium D-pantothenate 0.9, pyridoxine hydrochloride 0.4, riboflavin 0.3 and thiamine hydrochloride 0.5, all in mg/L) were added aseptically after sterilisation. The reference medium (*8*) had the following composition (in g/L): lactose 19.0,

soybean meal 7.4, yeast extract 6.0, malt extract 3.04, casein hydrolysate 2.6, $MgSO_4 \cdot 7H_2O$ 0.16, $Na_2HPO_4 \cdot 2H_2O$ 1.78 and KH_2PO_4 0.54. Reactors were inoculated with 100 mL of a 50-hour-old shake flask preculture grown on reference medium.

The cultivations were carried out as batch-fermentations (1.1 L of initial volume) in bench-top bioreactors (1.7 L of total volume, 2-stage 4-blade Rushton-type turbine and baffles, Applikon, Schiedam, the Netherlands) for a period of about 100 h at an initial pH of 6.5 to 6.8, 28 °C and constant aeration at 0.8 vvm compressed air. Oxygen saturation was monitored online, pH=6.5 was held constant by controlled addition of 2 M sodium hydroxide solution and 1 M sulphuric acid, samples were taken every 6 to 14 h and foaming was controlled manually by aseptic addition of antifoam reagent and by initial addition of 3 drops of antifoam agent (Glanapon DG 160, Busetti, Linz, Austria).

Determination of biomass dry mass

Samples taken from the bioreactor were intensively mixed with 5 PTFE-coated iron balls for 1 min (Vortex-Genie 2, VWR Scientific Industries, New York, USA). A volume of 10 mL of suspension was filtered through a tared glass fibre filter (BMC, Stölzle-Oberglas, Köflach, Austria) and the filtrate was recollected in a test tube. Subsequently, the filter was washed with distilled water and dried in the microwave oven until constant mass (3 × 3 min, 500 W; cooled off in between). It must be stated that biomass dry mass was distorted by initially insoluble medium components when soybean meal was used.

α-Amylase assay

The enzyme activity was determined in the clear filtrate of the sample suspension. The assay is based on the determination of reducing sugars using 3,5-dinitrosalicylic acid (DNS) as described by Miller (*12*). The method was modified for the present needs: 0.2 mL of adequately diluted sample were mixed with 0.8 mL of substrate solution (200 mg of soluble starch dissolved in 100 mL of buffer – 0.15 M KH_2PO_4 and 0.5 mM $CaCl_2$, pH=6.0) and incubated for 15 min at 37 °C. The reaction was stopped by adding 1 mL of stop solution (13.5 g of crystalline phenol in 350 mL of NaOH (10 %) mixed with 510 g of KNa tartrate in 800 mL of distilled water). After the addition of 1 mL of DNS solution (1.5 %), the samples were heated in boiling water for 5 min and then allowed to cool down in running tap water. For the blank the stop solution was added prior to the substrate solution. After the addition of 9 mL of distilled water, the absorption of the properly mixed solution was measured at 540 nm. The reducing sugars formed by enzymatic activity were calculated by subtracting the reducing sugar contents of the sample and the blank, using a standard curve (0.1–1.0 mg/L of glucose). All measurements were performed in duplicates.

Total carbohydrates assay

The method of Dubois *et al.* (*13*) was used with lactose for calibration.

α-Amino nitrogen assay

The assay is based on the method described by Moore and Stein (*14*). The reagent was prepared by dissolving 1 g of ninhydrin in 30 mL of ethylenglycol monomethylether and 10 mL of 4 M acetate buffer, pH=5.5. After adding 0.15 g of hydrindantin, the solution was stored in darkness over night. A volume of 0.5 mL of adequately diluted sample was mixed with 0.5 mL of ninhydrin reagent, incubated in boiling water for 15 min and then allowed to cool in cold water for 15 min. To 0.8 mL of this solution, 4 mL of ethanol (50 %) were added and the absorbance was measured at 570 nm. For calibration, a standard curve was prepared using glycine (0.05–0.5 mg/L).

Results

α-Amylase formation on different complex medium supplements

The experiments aimed to compare the effect of PNC with other common complex medium supplements, such as CSL, yeast extract, malt extract and casein hydrolysate on fungal growth and amylase formation. The amount of the added supplement was calculated on the basis of its α-amino nitrogen content, which was equivalent to 3.0 g/L of PNC. A second point of interest with regards to industrial application was to investigate the necessity of clarifying CSL and PNC as a pretreatment step. The results (Fig. 1) are the calculated averages of duplicates from two independent shake flask experiments. There were no significant differences in enzyme activity between the particular media after 46 h of cultivation. The α-amylase activity obtained levels between 275 and 308 μkat/L. After 94 h of cultivation the medium supplemented with casein hydrolysate contained the lowest enzyme titre. In contrast, the addition of PNC, yeast extract or malt extract resulted in highly productive media. Moreover, the application of a pretreatment step to PNC or CSL, which forms a clear solution by removing the precipitated substances, rendered slightly

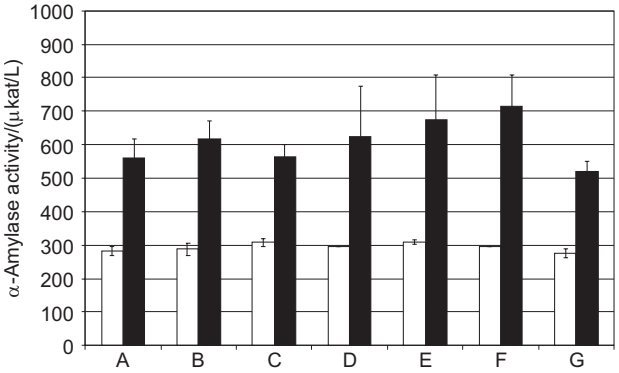

Fig. 1. Effects of various complex nitrogen-containing supplements on α-amylase formation by *Aspergillus oryzae* ATCC 1011 in shake flask experiments. □ extracellular α-amylase activity after 46 h of cultivation, ■ extracellular α-amylase activity after 94 h of cultivation; error bars indicate standard deviation; A= PNC_{turbid}, B=PNC_{clear}, C=CSL_{turbid}, D=CSL_{clear}, E=yeast extract, F=malt extract, G=casein hydrolysate

increased enzyme activity levels compared to the untreated material.

Results of the determination of biomass concentration are given in Fig. 2. After 46 h of cultivation there were some differences in the levels of the formed biomass, indicating differences in the growth rate; the media supplemented with malt extract and casein hydrolysate lagged significantly behind the rest. After 94 h of cultivation the increase in biomass concentration in the different experimental runs was rather small (0.85–2.01 g/L) compared to the amount of biomass formed within the first 46 h; thus indicating the existence of limiting growth conditions in the later period. Nevertheless, the differences in biomass concentration were too small to enable a proper assessment and classification of the effect of supplements on fungal growth. Using PNC or CSL as a supplement, a pretreatment step resulting in a clarified preparation did not significantly influence the growth rate of the organism.

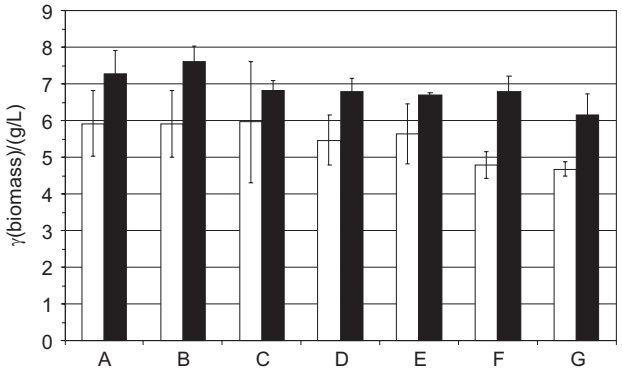

Fig. 2. Effects of various complex nitrogen-containing supplements on biomass formation by *Aspergillus oryzae* ATCC 1011 in shake flask experiments. □ concentration of dry biomass after 46 h of cultivation, ■ concentration of dry biomass after 94 h of cultivation; error bars indicate standard deviation; A=PNC_{turbid}, B=PNC_{clear}, C=CSL_{turbid}, D=CSL_{clear}, E=yeast extract, F=malt extract, G=casein hydrolysate

α-Amylase production in media with different inorganic nitrogen sources

These series of tests were performed to have a look at the effect of different inorganic nitrogen sources on amylase formation by *A. oryzae*. The ammonium sulphate in the basic medium was replaced by sodium nitrate or ammonium chloride and enzyme activity was estimated after 46 and 94 h of cultivation. Results are shown in Table 2 and indicate that greater part of the enzyme is formed during the later phase of culture development. Whereas at 46 h of cultivation ammonium chloride as nitrogen source leads obviously to a quicker enzyme formation and a higher titre, the level of amylase formed at the end of cultivation is almost equivalent to that found with ammonium sulphate as nitrogen source. With sodium nitrate as the sole inorganic nitrogen source, the lowest amylase titre can be detected in the medium after both 46 and 94 h of cultivation. Obviously, it needs more energy for its assimilation to fungal biomass, reducing in this way the amount of amylase formed.

Table 2. Effects of various inorganic nitrogen sources on the formation of α-amylase by *Aspergillus oryzae* ATCC 1011

Nitrogen source	α-Amylase activity/(μkat/L)	
	Cultivation time/h	
	47	94
Ammonium sulphate	176	838
Sodium nitrate	172	475
Ammonium chloride	246	770

α-Amylase formation with complex supplements

To estimate the effect of PNC on the amylase formation by *A. oryzae*, experiments were performed in an instrumented bench top bioreactor with media containing either PNC as complex supplement or no complex nitrogen source. Samples for amylase determination were withdrawn at intervals. As seen from Fig. 3, there was an almost uniform progression of amylase formation for the first 49 h on both media. After a period of comparable enzyme formation in both media, differences appeared after 49 h of cultivation. α-Amylase activity formed in the PNC-supplemented medium increased significantly faster compared to the medium with exclusively inorganic nitrogen. After 99 h of cultivation the final α-amylase titre produced on the PNC-containing medium exceeded that obtained on the non-supplemented medium significantly (1350 μkat/L with PNC *vs.* 880 μkat/L without PNC). Due to wall growth in the bioreactor, the estimation of fungal biomass from the withdrawn samples was not representative.

In several industrial microbial cultivations CSL is commonly used as a supplement supporting growth or product formation. In order to evaluate the effect of PNC on amylase formation by *A. oryzae*, it was compared to the influence of the standard additive CSL. Regarding the level of α-amylase activity, the two media showed a very similar course until 50 h of cultivation (500 μkat/L

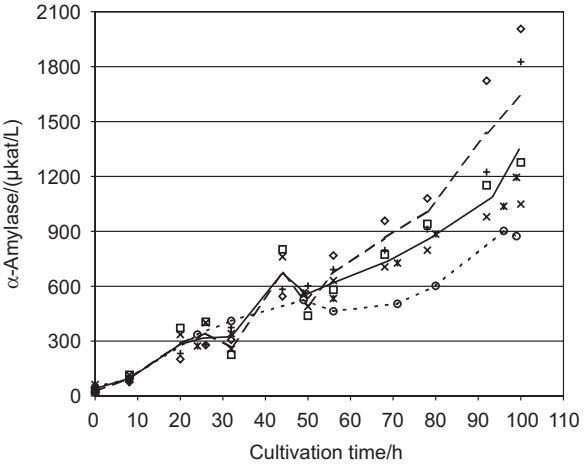

Fig. 3. Cultivation of *Aspergillus oryzae* ATCC 1011 in bioreactor on media containing starch and ammonium sulphate: extracellular α-amylase activity in the medium supplemented with CSL (□, ◇; – – –), with PNC (+, x, *; —) and without PNC (o; ···); results are calculated averages of duplicates; lines are estimated averages of independent runs

on CSL *vs.* 550 μkat/L on PNC). Thereafter the medium with CSL addition showed slightly increased yields of α-amylase activity, leading to significantly higher enzyme titre at 100 h of cultivation (1640 μkat/L on CSL *vs.* 1350 μkat/L on PNC).

Formation of α-amylase with PNC as the sole nitrogen source

Further cultivations in bioreactors were done to elucidate the differences in the kinetics of enzyme formation and fungal growth in media containing PNC and a mixture of complex constituents. Fig. 4 shows the time course of the measured biomass concentration, the amylase activity and the α-amino nitrogen level for cultivations done in triplicates. Cell growth was almost exponential for about 15 h in both media with several succeeding linear phases decreasing in slope; no significant lag phase was observed although both cultivations were inoculated with cells grown on reference medium. The differences in biomass concentration in the initial phase of the cultivations were due to insoluble medium components in soybean meal. Biomass concentrations could not be compared in the initial phase of the cultivations. Only minor differences were found for the biomass concentration at the end of the cultivations. The concentration of total carbohydrates measured in the medium decreased from 22.9 to 6.7 g/L and from 27.3 to 9.4 g/L for the reference cultivation and the cultivation with PNC, respectively. Regarding the α-amylase activity in the two media, a very analogous course was detected and only minor differences were observed until the end of cultivation. An almost linear increase was observed for the first 70 h. The α-amylase activity attained levels from 6800 to 10 000 μkat/L at the end of the cultivation. These values are up to 6-times higher compared to media with starch as the carbon source, indicating an inducing effect of lactose, which is quite frequently mentioned in literature (*15*,*16*).

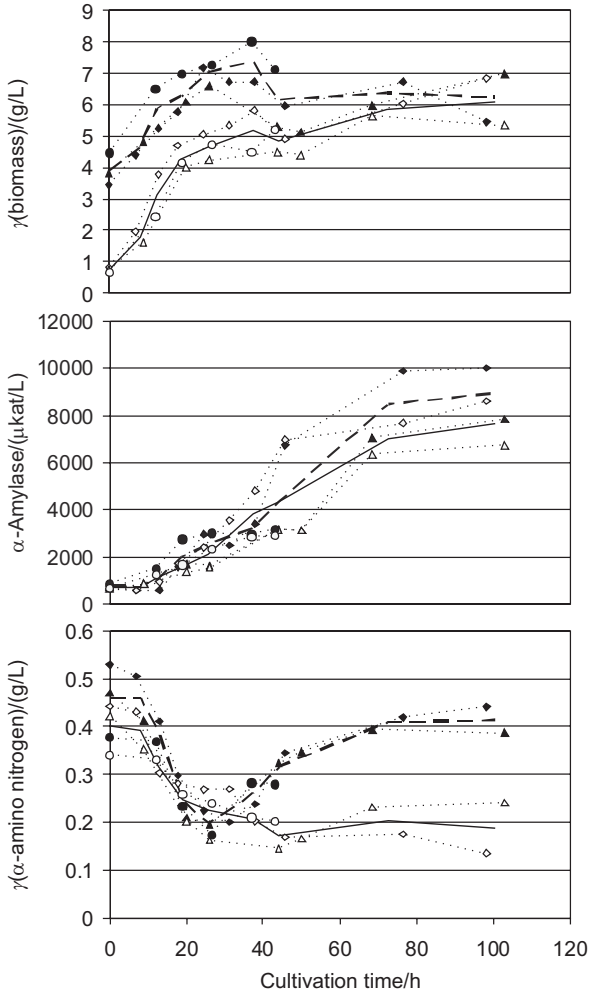

Fig. 4. Course of biomass, α-amylase activity and α-amino nitrogen in three parallel bioreactor cultivations (♦ first run, ▲ second run, ● third run) [filled symbols correspond to reference medium, empty symbols to PNC medium]. The dashed bold line represents the approximated expected course for reference medium and the continuous bold line the approximated expected course for PNC medium

Simultaneous to fungal growth, the α-amino nitrogen concentration was decreasing in the first part of the cultivation. After 40 h of cultivation the α-amino nitrogen level stayed almost constant in the PNC medium, whereas it increased starting at 25 h and attained a constant level after 80 h for the reference medium. This effect may be caused by the release of proteolytic enzymes, which solubilized the insoluble proteins of soybean meal, thus resulting in an increase of the α-amino nitrogen level and a decrease in the insoluble material as estimated by the determination of biomass.

Some insights into the metabolic activity of the fungal cells were given by the dissolved oxygen tension (DOT). The plots in Fig. 5 are averages of three independent cultivations as referred above. Only in media containing PNC as a nitrogen source the rapid decrease of DOT is suddenly interrupted, forming a peak at 15 h of cultivation, thus indicating a change in fungal metabolism. Limitation in oxygen supply occurred 20 h after inoculation and was maintained for further 20 h; thereafter the level of dissolved oxygen increased slowly, sometimes fluctuating with a sudden return close to saturation level at almost 75 h of cultivation. This indicated the utilization of substrates (they were difficult to meta-

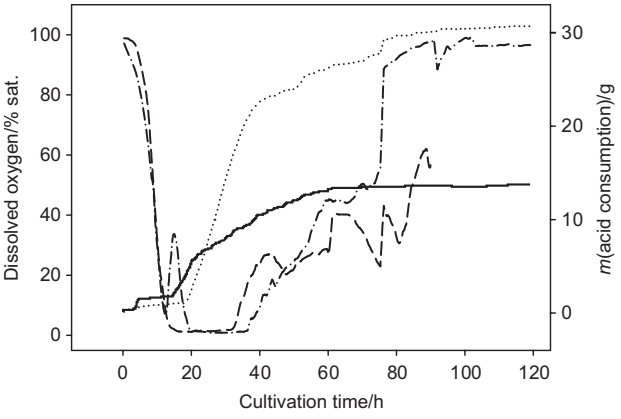

Fig. 5. Course of dissolved oxygen concentration (– – – reference medium, – · – · – medium with PNC) and the amount of added sulphuric acid (— reference medium, · · · medium with PNC) for maintaining a constant pH (average of three independent cultivations)

bolize by the fungal cells) and their depletion from the medium. Moreover, it coincided with the termination of amylase synthesis. Some additional information was obtained from the amount of neutralizing agent needed to maintain a constant pH in the culture fluid. Addition of mineral acid started with the onset of oxygen limitation and ended with substrate exhaustion. Obviously, it was the result of the dissimilation of physiologically alkaline compounds such as organic acids, amino acids or proteins by the fungus. The effect is more intensive in media containing PNC as nitrogen source than in the reference medium.

Discussion

Shake flask experiments proved that there are common nitrogen sources such as sodium nitrate and casein hydrolysate, which are less suitable for α-amylase production by *A. oryzae* compared to the novel nitrogen source PNC. These results correspond to the data of Pedersen and Nielsen (2), who showed that ammonium ions are a higher productive nitrogen source concerning enzyme activity compared to the nitrate ions. The reason for this might be in the need for additional energy for nitrate assimilation and in the lack of growth-stimulating substances in casein hydrolysate. Rather high levels of amylase can be achieved by supplementing media with additives rich in growth promoting compounds such as yeast extract or malt extract, but the obtained increase in enzyme yield (15–22 % with respect to PNC addition) economically hardly justifies the application of these supplements for an industrial production of amylase by *A. oryzae*. The final enzyme yields in media with PNC related to carbon source dry mass of 35 and 300 μkat/g for corn starch and lactose, respectively, as the carbon source differ strongly. Generally, amylase activities depend both on the strain and the medium used, where the influence of the medium again depends on the strain (*15,17*). Nevertheless, both values are within the range reported for α-amylase production, *e.g.* in optimized solid-state fermentation [150 μkat/g at 50 °C, pH=5.0 after 96 h on brewer's spent grains supplemented with various inorganic and complex compounds; Francis *et al.* (*18*)]. The use of PNC or CSL, both products of starch manufacturing from agricultural plants, as additives to media containing starch and ammonium as major carbon and nitrogen sources results in an increased productivity of amylase. Although there are supplements that provide high amylase titre when biomass is relatively low, significant indications that amylase formation is lower at high biomass concentrations, as described by Agger *et al.* (*19*) in continuous culture, can be seen neither from shake flask experiments nor in bioreactor cultivation. Kinetic analyses reveal that the enzyme titre increases almost linearly during cultivation. The application of a pretreatment step consisting in a clarification of the PNC or CSL solution has a beneficial effect on enzyme formation and leads to approx. 10 % higher yield of amylase activity. Small differences in enzyme yield on media supplemented with CSL or PNC may be due to differences in the content of compounds acting as carbon or nitrogen source or to substances having a promoting or inhibiting effect on growth or product formation. Thus, changes in the conditions of operations of the production process or a modified or additional pretreatment step may improve the applicability of PNC or CSL as supplement for media used in the industrial production of amylolytic enzymes by filamentous fungi.

Conclusions

Experiments with complex additives proved the necessity to supplement cultivation media containing starch and ammonium as main carbon and nitrogen sources with complex nitrogen-containing substrates. Studying shake flask experiments and especially cultivations in instrumented bioreactors, it could be shown that PNC, like CSL or yeast extract, might also represent a suitable alternative complex nitrogen-containing supplement for the production of fungal α-amylase. Cultivations with PNC as the sole nitrogen source elucidated that PNC might also be able to replace complex nitrogen sources, such as yeast extract, in the production of fungal α-amylase.

References

1. Z. Baysal, F. Uyar, Ç. Aytekin, Production of α-amylase by thermotolerant *Bacillus subtilis* in the presence of some carbon, nitrogen containing compounds and surfactants, *Ann. Microbiol. 53* (2003) 323–328.
2. H. Pedersen, J. Nielsen, The influence of nitrogen sources on the α-amylase productivity of *Aspergillus oryzae* in continuous cultures, *Appl. Microbiol. Biotechnol. 53* (2000) 278–281.
3. A.K. Kundu, S. Das, T.K. Gupta, Influence of culture and nutritional conditions on the production of amylase by the submerged culture of *Aspergillus oryzae*, *J. Ferment. Bioeng. 51* (1973) 142–150.
4. M. Carlsen, J. Nielsen, Influence of carbon source on α-amylase production by *Aspergillus oryzae*, *Appl. Microbiol. Biotechnol. 57* (2001) 346–349.
5. Q.D. Nguyen, J.M. Rezessy-Szabó, Á. Hoschke, Optimisation of composition of media for the production of amylolytic enzymes by *Thermomyces lanuginosus* ATCC 34626, *Food Technol. Biotechnol. 38* (2000) 229–234.
6. M.A. Malik, M.I. Chaudhary, Effect of carbon source on the production of α-amylase, *Pak. J. Biochem. 10* (1976) 14–18.
7. L.A. Underkofler, G. Reed: *Enzymes in Food Processing*, Academic Press, New York, USA (1966) p. 218.
8. B. Atkinson, F. Mavituna: *Biochemical Engineering and Biotechnology Handbook*, The Nature Press, New York, USA (1991) pp. 346–347.
9. S.A.Z. Mahmoud, A.M. Abdel-Hafez, W.A. Mashhoor, A.A.A. Refaat, Utilization of industrial and agricultural by-products for fungal amylase production, *Zentralbl. Bakteriol. Abt. 2, 133* (1978) 115–120.
10. M.A. Márquez, A.P. Balatti, L.A. Mazza, R.J. Ertola, Effect of several components of media on recovery of fungal amylase, *Rev. Esp. Quim. Apl. 32* (1972) 709–711 (in Spanish).
11. G. Volavsek, J. Meyrath, Effect of culture condition on the formation of α-amylase by *Aspergillus oryzae*, *Mitt. Versuchsstn. Gärungsgwrb. Wien, 27* (1973) 169–176 (in German).
12. G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Anal. Chem. 31* (1959) 426–428.

13. M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, *Anal. Chem. 28* (1956) 350–356.

14. S. Moore, W.H. Stein, A modified ninhydrin reagent for the photometric determination of amino acids and related compounds, *J. Biol. Chem. 211* (1954) 907–913.

15. B. Annadurai, P. Karunanidhi, S. Mahalingam, Effect of sugars on amylase activity of *Aspergillus oryzae*, *J. Ecotoxicol. Environ. Monit. 9* (1999) 209–212.

16. S. Ueno, M. Miyama, Y. Ohashi, M. Izumiya, I. Kusaka, Secretory enzyme production and conidiation of *Aspergillus oryzae* in submerged liquid culture, *Appl. Microbiol. Biotechnol. 26* (1987) 273–276.

17. M. Podhorsky, Factors influencing the production of amylase by *Aspergillus oryzae* in submerged cultures. II. The influence of carbon sources on the production of amylase, *Ceskoslov. Mikrobiol. 2* (1957) 383–385.

18. F. Francis, A. Sabu, K.M. Nampoothiri, S. Ramachadran, S. Gosh, G. Szakacs, A. Pandey, Use of response surface methodology for optimizing process parameters for the production of α-amylase by *Aspergillus oryzae*, *Biochem. Eng. J. 15* (2003) 107–115.

19. T. Agger, A.B. Spohr, J. Nielsen, α-Amylase production in high cell density submerged cultivation of *Aspergillus oryzae* and *A. nidulans*, *Appl. Microbiol. Biotechnol. 55* (2001) 81–84.