Influence of the Nitrogen Source on the Productions of α-Amylase and Glucoamylase by a New *Trichoderma* sp. from Soluble Starch

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Original scientific paper
Received: November 21, 2003
Accepted: May 24, 2004

The influence of the nitrogen source on the production of extra–cellular amylolytic enzymes by a new *Trichoderma* sp. isolate was investigated. Preliminary tests performed using a mixture of urea and ammonium sulfate demonstrated that this microorganism was able to effectively degrade ungelatinized soluble starch (20 g l^{-1}) yielding 420±30 U l^{-1} α–amylase and 130 ± 20 U l^{-1} glucoamylase. Tests were also performed using different nitrogen sources at the optimum level of 0.80 g l^{-1} nitrogen, specifically ammonium citrate, yeast extract, urea, ammonium sulfate, ammonium sulfate plus urea, peptone, and corn steep liquor. Corn steep liquor was shown to be the best nitrogen source for the simultaneous activities and productivities either of α–amylase ($a_{a,max}$ = 520 ± 4 U l^{-1}; P_a = 5.0 ± 0.5 U l^{-1} h^{-1}) or glucoamylase ($a_{g,max}$ = 1100 ± 70 U l^{-1}; P_g = 15 ± 1 U l^{-1} h^{-1}).

Key words:
α–amylase, glucoamylase, nitrogen source, soluble starch, *Trichoderma* sp.

Introduction

Filamentous fungi constitute an heterogeneous group of microorganisms able to use a great variety of carbon and nitrogen sources for growth through different metabolic pathways, which makes them well-known amylase producers. Members of *Aspergillus* and *Rhizopus* genera are often utilized to degrade starch.[1] However, due to their polycrystalline structure, starch granules are insoluble in cold water; therefore, to improve the susceptibility of starch to enzyme action, it is mainly used after gelatinization by hot water. Nevertheless, to save the high cost of gelatinization, attempts were also made to look for microorganisms able to grow on ungelatinized starch.[1–4] Their use would in fact allow reducing the duration of the expensive warm pre-treatment to the time required only for sterilization.

Amylases find use in many industrial applications, among which the preparations and/or manufactures of foods, textile products, beverages, paper, adhesives, sugar derivatives, and distillates.[5] In particular, they are utilized in food industry for the preparation of glucose and fructose syrups (through the action of glucose isomerase after starch saccharification), for baking, etc. In distillate beverage industry, amylases are used as a supplement of malts having low diastase activity or to saccharify starchy raw materials. Moreover, amylases are employed in agro–industry to hydrolyze starchy raw materials for ethanol production.

The production of amylolytic enzymes depends on the microbial species, the composition of the medium, the cultivation methods, and the nitrogen source. Compounds whose molecules contain amino groups, such as nucleic acids and amino acids, constitute the main nitrogen sources. A certain influence of, both, nitrogen and crude carbon sources on the production of amylolytic enzymes was already observed mainly in *Aspergillus* sp.[6]

Besides being a good cellulase producer, like all the members of the *Trichoderma* genus, the new isolate utilized in this work was already shown to effectively degrade different *in natura* starches without preliminary gelatinization.[4] In the present work, the nitrogen source was varied and its effect on the productions of α–amylase and glucoamylase by *Trichoderma* sp. from ungelatinized soluble starch, was investigated.

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Materials and methods

Microorganism

The microbial strain of *Trichoderma* sp. used in this work was isolated from the environment at the Faculty of Pharmaceutical Sciences, University of São Paulo, Brazil, by successive transfers and enrichments in Petri plates.[4] The microorganism was maintained in a medium containing, in 1.0 liter of distilled water, glucose (10 g), peptone (5.0 g), meat extract (3.0 g), and agar-agar (20 g). A loopful of cells from the plates was used for pre-cultures. To this purpose, according to *Brown* et al.,[7] a medium having the following composition (per liter of distilled water) was used: 20 g soluble, ungelatinized potato starch, 8.0 g KH_2PO_4, 0.84 g urea, 3.9 g $(NH_4)_2SO_4$, 1.2 g $MgSO_4 \cdot 7H_2O$, 8.2 mg $MnSO_4 \cdot 4H_2O$, 20 mg $FeSO_4 \cdot 7H_2O$, 15 mg $CoCl_2 \cdot 6H_2O$, and 5.6 mg $ZnSO_4 \cdot 7H_2O$.

Cultivation media

A soluble starch solution was prepared according to the methodology reported by *Okolo* et al.[3] and sterilized using absolute ethanol to avoid gelatinization.[8] Preliminary tests were performed on a medium having the same composition as that used for pre–cultures to check the ability of this microorganism to produce extra–cellular α–amylase and glucoamylase.

The influence of nitrogen source on the production of amylolytic enzymes was investigated in a medium containing soluble starch (20 g l^{-1}) and prepared similarly to that used for preliminary tests, but using different nitrogen sources at concentrations equivalent to the optimum nitrogen level previously suggested (0.80 g l^{-1}),[7] specifically: urea (1.7 g l^{-1}), $(NH_4)_2SO_4$ (3.8 g l^{-1}), ammonium citrate (6.5 g l^{-1}), peptone (5.7 g l^{-1}), yeast extract (7.6 g l^{-1}), corn steep liquor (23.5 g l^{-1}), and a mixture of ammonium sulfate (2.6 g l^{-1}) plus urea (0.55 g l^{-1}). Salt and nitrogen source solutions were sterilized at 121 °C for 10 min, except for urea, which was sterilized by microfiltration through membranes with 0.2 μm pore diameter.

Experimental conditions

Tests were performed at 28 °C and initial pH 5.3 in 500 ml–Erlenmeyer flasks containing 100 ml of the medium, agitated in rotary shaker at 180 min^{-1}, and inoculated with $1.0 \cdot 10^7$ spores ml^{-1}.[4] The results were reported as the average of five replicates and the experimental errors were expressed as standard deviations with respect to the mean value.

Analytical procedures

Concentrations of glucose and total reducing sugars (TRS) were determined in supernatant samples according to the procedures of glucose oxidase[9] and *Somogyi*.[10]

The glucoamylase activity was determined by measurement of the initial rate of glucose release from the hydrolysis of excess starch under standard conditions. After 1 h incubation at 45 °C of 100 μl supernatant samples with 1.0 ml of the soluble ungelatinized starch suspension (40 g l^{-1}) in 50 mmol l^{-1} sodium citrate buffer (pH 4.8), the reaction was stopped by boiling for 5 min[4] and the concentration of glucose released was measured as described above. To make interference of glucoamylase negligible, the α–amylase activity was determined, according to *Bergmann* et al.,[1] after 30 min incubation at 45 °C of 100 μl supernatant samples with 900 μl soluble starch suspension (11.1 g l^{-1}) in 50 mmol l^{-1} sodium citrate buffer (pH 5.5) containing 2.0 mmol l^{-1} $CaCl_2$.[1,2] The reaction was stopped by addition of 1.0 ml of the *Somogyi* solution[10] and the TRS concentration was measured as previously mentioned. According to the literature,[11] one unit of glucoamylase or α-amylase activities were defined as the enzyme amounts required to release 1 μmol glucose or TRS (expressed as glucose) per min.

Starch concentration was determined on supernatant samples according to the anthrone method,[12] while biomass concentration was determined by filtration of the medium through paper 1, Whatman (Maidstone, UK), drying the resulting solid at 100 °C up to constant weight.

Results and discussion

Preliminary tests

Preliminary tests were performed using a mixture of urea and ammonium sulfate as nitrogen source in order to check the capability of soluble starch (20 g l^{-1}) to induce extra–cellular α–amylase and glucoamylase activities in *Trichoderma* sp. The results of these tests, illustrated either in terms of enzyme activities or substrate consumption and TRS formation (Figure 1), demonstrate that, although in different proportions and with different kinetics, both α–amylase and glucoamylase formed during the cultivation. As already suggested,[4] the relatively long times of maximum α–amylase (96 h) and glucoamylase activities (120 h) were likely due to the inducible character of these enzymes in the selected microorganism and agree with the results of *Fowler* et al.,[13] who observed an inducing effect of maltose in *Aspergillus niger*. The simultaneous

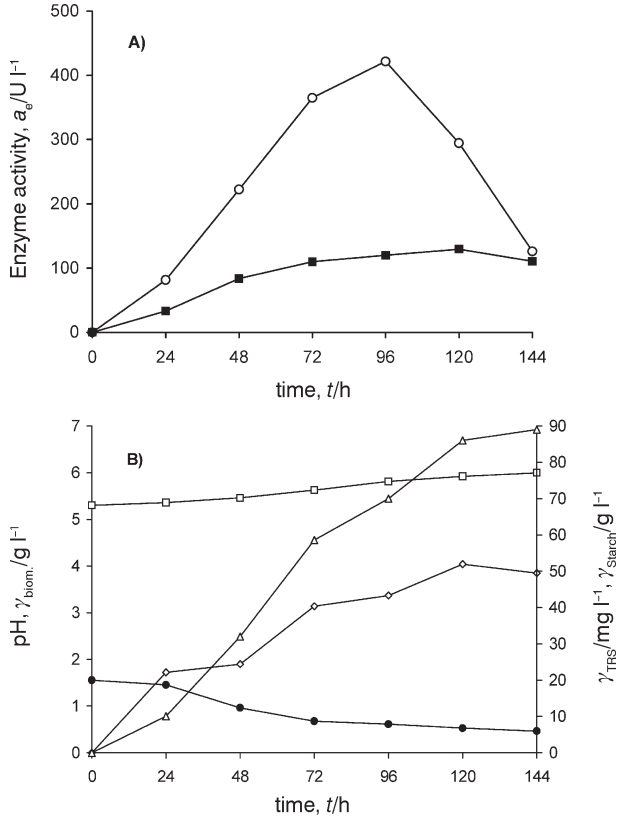

Fig. 1 – *Preliminary tests of α–amylase and glucoamylase productions by Trichoderma sp. on soluble starch. A) Enzyme activities: (○) α–amylase, (■) glucoamylase; B) (□) pH, (◇) biomass concentration, (●) starch mass concentration and (△) TRS mass concentration*

production of glucoamylase and α–amylase could be a synergistic process, influenced by the interaction between some components of the enzyme mixture and the native carbon source, during the action on crude starch.[3] We have recently detected the same displacement of activities when this isolate was grown on different starches,[4] thus confirming previous observations made with different *Aspergillus* species.[14]

In spite of the fact that the selected carbon source (soluble potato starch) is notoriously difficult to be digested,[15] the new *Trichoderma* sp. isolate exhibited maximum activities and productivities either of α–amylase ($a_{a,max}$ = 420 ± 30 U l^{-1}; P_a = 3.9 ± 0.5 U l^{-1} h^{-1}) or glucoamylase ($a_{g,max}$ = 260 ± 40 U l^{-1}; P_g = 1.1 ± 0.2 U l^{-1} h^{-1}) comparable to those obtained from gelatinized corn starch with engineered strains,[11] thereby demonstrating its potential.

Effect of the nitrogen source

The results summarized in Tables 1 and 2 were obtained using soluble starch and different nitrogenous compounds at the optimum nitrogen level of 0.80 g l^{-1}.[7] Figure 2 shows that, regardless of the nitrogen source, the use of optimal concentration of this element allowed *Trichoderma* sp. to produce

Table 2 – *Influence of the nitrogen source on pH, total reducing sugars, glucose and residual starch concentrations at the end of Trichoderma sp. cultivations on soluble starch*

Nitrogen source	pH_f	γ_{TRS} / mg l^{-1}	γ_G / mg l^{-1}	γ_S / g l^{-1}
Ammonium sulfate	3.8±0.2	56±5	36±3	6.6±0.4
Ammonium citrate	7.6±0.2	51±5	41±4	5.8±0.1
Peptone	7.1±0.3	56±6	32±3	5.6±0.5
Ammonium sulfate plus urea	6.5±0.3	77±5	40±3	5.8±0.2
Corn steep liquor	7.6±0.3	239±28	105±9	5.6±0.2
Yeast extract	7.5±0.2	201±27	95±20	9.7±0.3
Urea	7.8±0.3	105±10	62±6	4.2±0.2

pH_f = final pH value; γ_{TRS} = final mass concentration of total reducing sugars expressed as glucose; γ_G = final glucose mass concentration; γ_S = residual starch mass concentration.

Table 1 – *Influence of the nitrogen source on the productions of α–amylase and glucoamylase by Trichoderma sp. from soluble starch*

Nitrogen source	$a_{a,max}$ / U l^{-1}	$t_{a,max}$ / h	$a_{g,max}$ / U l^{-1}	$t_{g,max}$ / h	P_a / U l^{-1} h^{-1}	P_g / U l^{-1} h^{-1}
Ammonium sulfate	500±40	112±10	330±10	112±12	4.6±0.6	3.1±0.4
Ammonium citrate	470±30	96±0	250±4	96±0	4.8±0.4	2.5±0.1
Peptone	410±30	128±18	220±3	96±8	3.3±0.4	2.5±0.3
Ammonium sulfate + urea	410±20	88±8	210±20	112±10	4.8±0.6	2.1±0.3
Corn steep liquor	520±4	104±10	1100±70	72±0	5.0±0.6	15±1
Yeast extract	400±130	80±14	190±30	120±42	4.4±1.7	1.7±0.4
Urea	480±10	120±0	220±10	144±0	3.9±0.2	1.5±0.1

$a_{a,max}$ = maximum α–amylase activity; $t_{a,max}$ = time of maximum α–amylase activity; $a_{g,max}$ = maximum glucoamylase activity; $t_{g,max}$ = time of maximum glucoamylase activity; P_a = α–amylase productivity calculated at $t_{a,max}$; P_g = glucoamylase productivity calculated at $t_{g,max}$.

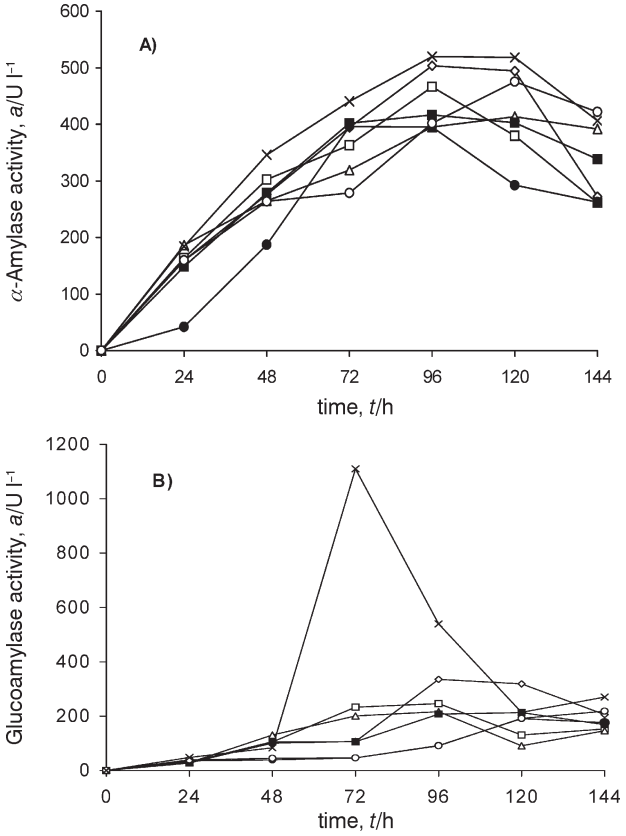

Fig. 2 – *Influence of the nitrogen source on the productions of A) α–amylase and B) glucoamylase by Trichoderma sp. on soluble starch. (□) Ammonium citrate, (●) yeast extract, (○) urea, (×) corn steep liquor, (◇) ammonium sulfate, (△) peptone, (■) ammonium sulfate plus urea*

α–amylase and mainly glucoamylase at higher levels than in preliminary tests. According to the observations of *Rao* and *Modi* with *Aspergillus nidulans*,[16] the enhanced productions of both enzymes with respect to preliminary tests could be the result of an increased synthesis of lipids in the cell membrane, which could have been responsible for an easier transfer of nitrogen to the cell. The data of Table 1 also show that the use of optimum nitrogen level in the medium reduced by 7–8% of the time of maximum expressions of both α–amylase ($t_{a,max}$ = 88 ± 8 h) and glucoamylase ($t_{g,max}$ = 112±10 h).

Corn steep liquor (CSL) was shown to be the best nitrogen source to sustain the α–amylase activity, followed by ammonium sulfate, urea, ammonium citrate, peptone, ammonium sulfate plus urea, and yeast extract (Table 1). Almost the same sequence was observed for glucoamylase activity, with the exception of the ammonium citrate that behaved better than urea. The final decreases in, both, amylolytic activities and productivities, associated to high cell concentrations at the end of fermentations, may be due either to the secretion of proteases or to different time patterns of nutrient consumption.[17,18]

Compared to preliminary tests, the use of CSL ensured glucoamylase activity and productivity almost one order of magnitude higher, whereas those of α–amylase were only 20–30 % higher. The results of Table 1 show, for the other nitrogen sources, that their organic or inorganic nature did not influence appreciably the productivities either of glucoamylase or α–amylase. CLS seemed to accelerate glucoamylase formation, shifting its maximum activity from 120 to 72 h, while that of α–amylase kept nearly unvaried. As a consequence, this nitrogen source showed the best performance, ensuring glucoamylase and α–amylase productivities of P_g = 15 ± 1 and P_a = 5.0 ± 0.6 U l^{-1} h^{-1}. Possible explanation of the strong stimulating effect of CLS on glucoamylase activity should be searched in its origin from the corn wet–milling process, when the dry corn is soaked (steeped) in a warm sulfurous acid solution at 50 – 55 °C for about 30 h.[19,20] Although, the composition of CLS was shown to depend on the process, the variety of corn, the season and even the soil, the fraction of carbohydrates (expressed as glucose) usually varies in the range 6 – 21 % of total dry matter, the rest consisting of proteins, lipids, lactic acid, vitamin and mineral salts. The most abundant sugars are glucose, fructose and galactose which account for 22 – 42 %, 17 – 31 % and 5 – 16 % of total sugars, respectively, whereas pentoses (arabinose and xylose), disaccharides (melibiose, sucrose and maltose), and trisaccharides (trehalose and raffinose) are present at lower levels.[20] These figures suggest that glucoamylase synthesis was likely induced by maltose, which is the most common amylase inducer,[14] even if a similar effect by any other oligosaccharide released during starch hydrolysis cannot be excluded. In addition, since the monosaccharides contained in CLS, mainly glucose, provided the system with directly–metabolizable carbon sources, biomass growth was stimulated particularly at the start of cultivations (results not shown), thus contributing to favor the whole enzyme release.

Final glucose and TRS concentrations as well as residual starch level (S) are listed in Table 2. Compared to preliminary tests, these fermentations exhibited in general better results. Depending on the nitrogen source, while glucose release passed from 40 to 32 – 105 mg l^{-1}, and residual starch mass concentration increased from 4.2 up to 4.2 – 9.7 g l^{-1}, maximum α–amylase and glucoamylase activities did in fact shift from 420 to 400 – 520 U l^{-1} and from 130 to 190 – 1100 U l^{-1}, respectively, and their respective productivities from 3.9 to 3.3 – 5.0 U l^{-1} h^{-1} and from 1.1 to 1.5 – 15 U l^{-1} h^{-1}. In the meanwhile, TRS level passed from 89 to 51 – 239 mg l^{-1}. These results on the whole demonstrate a clear influence of the nitrogen source upon the for-

mation of these enzymes and confirm the significance of nitrogen optimum level on their expression.

As expected by the improved productivities of amylolytic enzymes, the release of glucose (105 ± 9 mg l^{-1}) and TRS (239 ± 28 mg l^{-1}) was higher using CSL than with the remaining nitrogen sources (Tab. 2). With regard to the concentration of residual starch, this carbon source was hydrolyzed up to 51 – 79 % depending on the nitrogen source, with the highest and lowest yields being obtained with urea and yeast extract, respectively. Such unexpected findings were probably due to some adaptation of the microorganism to the medium, which could have led to variations in its ability to metabolize soluble starch for the growth.

Conclusions

Preliminary cultivations demonstrated that the use of a mixture of urea and ammonium sulfate as nitrogen source could promote the production of α–amylase and glucoamylase by *Trichoderma* sp. even using a poor carbon source as soluble starch. Tests performed, using different nitrogenous compounds, revealed a significant influence of the nitrogen source on the productivities of both amylolytic enzymes. The best results either in terms of enzyme activity or productivity were obtained using corn steep liquor, likely because of an inducing effect of some sugar component of this low cost nitrogen source mainly on glucoamylase synthesis.

ACKNOWLEDGMENTS

The authors would like to thank FAPESP (Sno Paulo State Foundation for the Support of Research, Brazil) for the financial support. This work is published in memory of PhD Rachel A. Pacheco Chávez, who unfortunately died before her PhD thesis dissertation.

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