Acta Alimentaria, Vol. 29 (1), pp. 71–79 (2000)

SCREENING THE STRAINS OF THERMOPHILIC FUNGUS *THERMOMYCES LANUGINOSUS* FOR AMYLOLYTIC ACTIVITIES

G. RÓNASZÉKI[a], Q. D. NGUYEN[a], J. M. REZESSY-SZABÓ[a], Á. HOSCHKE[a,c] and M. K. BHAT[b]

[a] Department of Brewing and Distilling, University of Horticulture and Food Science, H–1118 Budapest, Ménesi út 45. Hungary

[b] Institute of Food Research, Reading Laboratory, Whiteknights Road, Reading, RG6 6B. U.K.

(Received: 25 May 1999; accepted: 8 September 1999)

Eighteen strains of thermophilic fungus *Thermomyces lanuginosus* were screened for amylolytic activities. All of them produced extracellular amylolytic enzymes. Three strains were selected for detailed studies on their production of glucoamylase and α-amylase. The optimum conditions for the assays of amylolytic activities were selected. The optimum parameters were found to be the following: 70 °C and pH 4.6 for glucoamylase and 70 °C and pH 5.0 for α-amylase.

Keywords: *Thermomyces lanuginosus*, amylolytic activity, glucoamylase, α-amylase, screening, thermophilic fungus

Over the last few decades, there has been an increasing demand for polysaccharide-degrading enzymes all over the world. At present, most of the commercially available polysaccharidases have many disadvantages. Therefore, to meet the current demand and improve the economics of enzyme production, there is a need for enzymes which can catalyse the desired reactions under industrial conditions.

The use of amylolytic enzymes for processing starch is preferred over acid hydrolysis because of the high specificity of enzymes and the mild reaction conditions, lower energy requirements and the absence of undesirable side reactions associated with their use. However, the efficient hydrolysis of natural starch not only requires amylolytic enzymes, but also requires these enzymes to be stable and active at temperatures around 90–95 °C. α-Amylases from *Bacillus* species are stable and active between 90–95 °C (JANECEK, 1993) and are used widely in food industries and others. Their narrow pH optimum makes them less suitable for industrial processing of starch from an economic viewpoint. Furthermore, the bacteria do not produce appreciable amounts of

[c] To whom the correspondence should be addressed: Tel: +36-1-372-6215; Fax:+36-1-372-6214; E-mail:hoschke@hoya.kee.hu

0139-3006/2000/$ 5.00

glucoamylase, so this has to be produced from an additional source. Currently, the glucoamylase from *Aspergillus* is used in industry, but this enzyme is stable and active only up to 55–65 °C and between pH 4.0–6.0 (UNDERKOFLER, 1976; GODFREY, 1983). Therefore, a thermophilic fungus, which produces thermostable α-amylase and glucoamylase appeared to be an ideal source for developing an economically attractive method for the industrial processing of starch.

Thermomyces lanuginosus, formerly known as *Humicola lanuginosa* (DOMSCH et al., 1980), had been suggested as an excellent test organism for the study of extracellular amylase from a thermophilic fungus (BARNETT & FERGUS, 1971).

So far some strains of the thermophilic fungus *Thermomyces lanuginosus* have been investigated, but there has not been any comprehensive screening for evaluation of the amylolytic enzyme production of the individual strains. In the present study, we have collected and screened as many strains as possible and determined the optimal conditions for amylolytic enzymes.

1. Materials and methods

All chemicals used were of analytical grade and purchased either from Merck, Sigma, Reanal or other companies.

1.1. Microorganisms

Thermomyces lanuginosus strains were originated from various culture collections. The investigated strains are the following: IMI 084400/ATCC 22070, IMI 110803, IMI 158749, ATCC 38905, ATCC 46882, IMI 140524, IMI 096213, CBS 218.34, CBS 224.63, CBS 288.54, CBS 395.62, ATCC 28083, ATCC 16455, IMI 131010, ATCC 34626, ATCC 36350, ATCC 44008 (RM-B), DSM 5828.

1.2. Maintenance of stock cultures

The strains were maintained in yeast-powder/soluble starch (YPSS) agar medium (COONEY & EMERSON, 1964), and stored under refrigeration.

1.3. Iodine vapour method for detecting amylolytic activities

The screening experiments were first carried out by the iodine vapour method based on diameter measurements. The clear zones around the colonies indicated starch degrading capability. The YPSs medium was poured into Petri dishes. The fungus was inoculated onto the surface of the media and, after cultivation of the microbe, the diameters of the colonies and the clear zones, indicated by iodine vapour, were

determined. The ratio of the clear zone to the colonies was calculated for the evaluation of the results.

1.4. Cultivation and production of amylolytic enzymes by Thermomyces lanuginosus

A three-stage cultivation technique was used. In the first stage, the fungus was grown on YPSS slant agar for 8 to 10 days at 50 °C under humified incubation. In the second stage a suspension of spores was prepared using 0.1% Triton X-100 solution. Five ml of it was added to 100 ml of glucose-asparagine medium, pH 6.0, to initiate the cultivation at 50 °C and 220 r.p.m. in an orbital shaker for 1 to 2 days to obtain a homogeneous mycelium growth. In the third stage, 10 ml of the mycelial suspension was used as inoculum for initiating the production of amylolytic enzymes in 150 ml of starch-asparagine medium (soluble starch 40 g, L-asparagine 4 g, KH_2PO_4 3 g, K_2HPO_4 2 g, $MgSO_4.7H_2O$ 0.5 g and 1 ml of VOGEL's (1956) trace elements solution was dissolved in 1 l of distilled water). Samples (10 ml) were taken under laminar box from duplicate flasks at varying times. The samples were filtered and the α-amylase and glucoamylase activities were assayed in the filtrate.

1.5. Enzyme assays

α-Amylase. A reaction mixture containing 1 ml of 0.1 mol sodium-acetate buffer (pH 5.0) and 1 ml of 0.5% (w/v) soluble starch solution was mixed and pre-incubated at 50 °C for 10 min before adding 1 ml of appropriately diluted culture filtrate as an enzyme source. After 5 min the reaction was stopped by adding 1 ml of 0.5 mol HCl. The unhydrolysed starch in this aliquot was estimated by the iodine method described below. One unit of α-amylase activity was defined as the amount of enzyme that hydrolyses 1 mg of soluble starch in 1 min.

Glucoamylase. One ml of reaction mixture containing 0.25 ml of 0.1 mol phosphate buffer (pH 4.6) and 0.25 ml of 1% (w/v) soluble starch solution was pre-incubated at 50 °C for 10 min. Half a ml of appropriately diluted culture filtrate as an enzyme source was added, and the incubation was continued for further 15 min. The reaction was stopped by placing the tubes in a boiling bath for 30 min. After cooling the released glucose concentration was estimated by the glucose-oxidase/peroxidase method (MCCOMB & YUSHOK, 1957; WOOD & BHAT, 1988). One unit of glucoamylase activity was defined as the amount of the enzyme that releases 1 μmol of glucose in 1 min.

1.6. The iodine method for starch estimation

An aliquot of the sample (starch hydrolysate) was mixed with 1 ml of iodine reagent and this mixture was adjusted up to 2 ml by distilled water. The iodine reagent contained 0.02% (w/v) of iodine and 0.2% (w/v) of KI in 0.5 *N* HCl. Five ml of distilled

water was added to this mixture and the colour developed was read at 590 nm against blank. The amount of starch was estimated using a standard curve prepared with potato soluble starch (Merck) under the same conditions.

2. Results and discussion

2.1. Morphological observations of different Thermomyces lanuginosus *strains*

All investigated *Thermomyces lanuginosus* strains were found to be capable of growing in the following culture media: PDA, OA, YPSS, PSA, MA2, NUT and PYS (ATCC, 1984). The morphology of individual strains showed deviations within the range of the species. Their colonies developed in PDA and reached diameters of 6–8 cm at 45 °C within 3 days. The structures and the colours of their surfaces were very different. Pigments diffusing into the nutrient agar varied in colour from amber to purple. Variation of colony morphology was observed both among the different strains and during the growth of some strains, their fructification properties have segregation (ATCC 36350, IMI 084400). With respect to micro-morphological properties, the tested strains showed only slight differences. Their mycelia consisted of branched, partitioned hyphae with diameters between 1.5–4.0 μm in the nutrient agar. The aerial hyphae rise at right angles to the basal hyphae, which run along the surface of the medium. The unicellular spherical asexual spores appeared at the tips of the air hyphae.

2.2. Rapid screening of different strains of Thermomyces lanuginosus

Based on the results of iodine vapour method, all investigated strains showed a ratio of clear zone-to-colonies higher than 1 (Fig. 1). This indicated that all of them produced amylolytic enzymes. The strains ATCC 34626, ATCC 44008 (RM-B) and IMI 084400, which have ratios higher than 2, showed clear differences between clear zone and colony. To confirm these results, shaken flask experiments were carried out and their results (see later) validated the previous findings.

2.3. Screening of Thermomyces lanuginosus *strains for the production of amylolytic enzymes*

The production of α-amylase and glucoamylase activities were evaluated at different times. The activity measurements were done at 50 °C and at pH 5.0 (50 mmol sodium-acetate buffer). The maximum activities reached are shown in Fig. 2. The production of α-amylase was in the range of 4.6–46.6 U ml^{-1}. With respect to α-amylase activity the strains ATCC 28083 and ATCC 34626 looked promising (Fig. 2A). In the case of glucoamylase activity two strains (ATCC 44008 and ATCC

34626) were selected (Fig. 2B). They showed higher activities than 1.0 U ml^{-1}. Based on the experimental results three strains ATCC 28083, ATCC 34626 and ATCC 44008 deserve a more detailed study. It should be stressed that *Thermomyces lanuginosus* ATCC 34626 strain performed well with respect to both α-amylase and glucoamylase.

2.4. Determination of optimum parameters for activity assays

The effects of pH and temperature on the activities of α-amylase and glucoamylase were investigated. The optimum pH was determined to be in the range of 4.0–5.5 (0.1 mol sodium-acetate buffer) in the case of glucoamylase activity. 0.1 mol sodium-acetate buffer, pH 4.0–5.6, and 0.1 mol citrate/Na_2HPO_4 buffer, pH 5.6–7.0, were needed in order to determine the pH optimum of α-amylase. The optimum temperature was defined in the range of 50–85 °C at the optimum pH of glucoamylase and α-amylase activities.

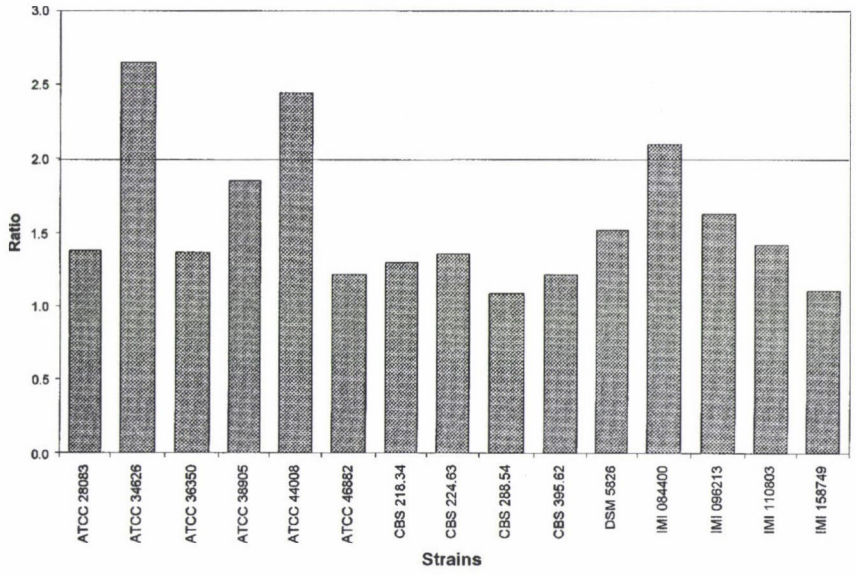

Fig. 1. Amylolytic activity of *Thermomyces lanuginosus* strains determined by iodine vapour rapid screening (YPSS medium, 50 °C)

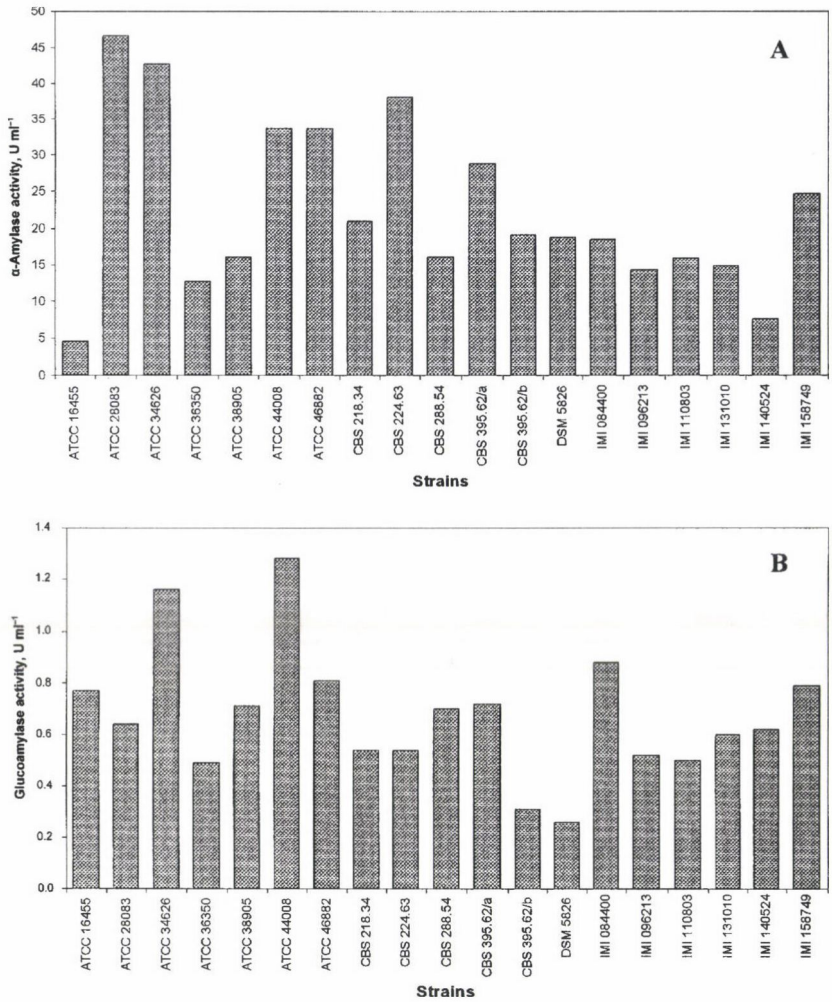

Fig. 2. Production of individual amylolytic enzymes by *T. lanuginosus* strains (with basic parameters: pH 5.0 and temperature 50 °C) A: α-amylase; B: glucoamylase

On the basis of activity assays the pH optima of α-amylase and glucoamylase activity were 5.0 and 4.6, respectively (Fig. 3A, B). TAYLOR and co-workers (1978) showed that glucoamylase from *Thermomyces lanuginosus* can be completely stable in the pH range 5–10 for 24 h at room temperature while losing activity at pH 4.0. Our results are consistent with their findings.

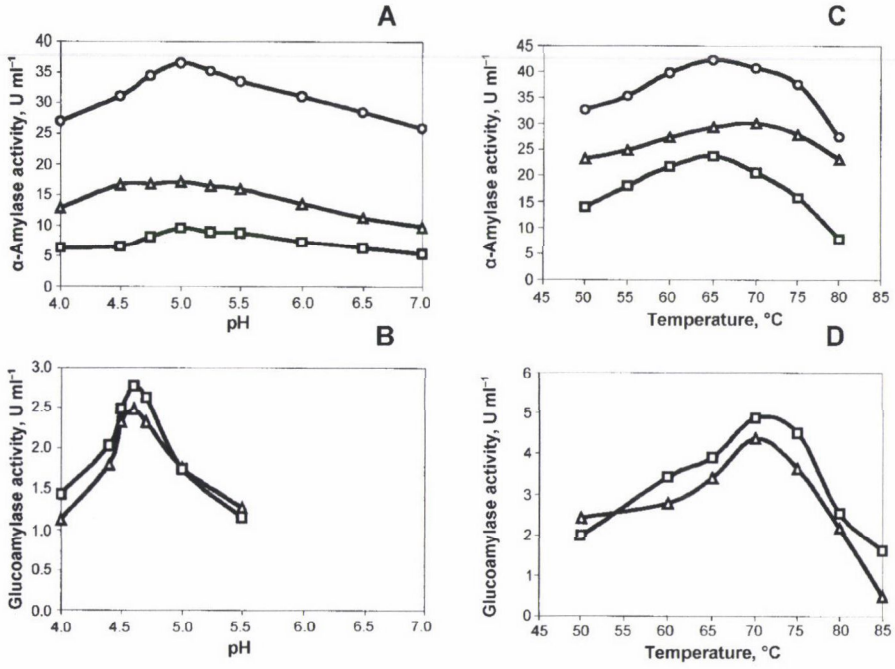

Fig. 3. Effect of pH (A, B) and temperature (C, D) on amylolytic activities from *T. lanuginosus*. —O—: ATCC 28083; —Δ—: ATCC 34626; —□—: ATCC 44008

The crude ferment broth from different strains of *Thermomyces lanuginosus* showed different optimal temperature values at pH optimum. In case of α-amylase the optimal temperature was between 65–70 °C (Fig. 3C), depending on the strain used. With respect to glucoamylase it was 70 °C (Fig. 3D). This means that the optimal temperature of glucoamylase originating from *Thermomyces lanuginosus* is 10 °C higher than that from *Aspergillus niger* (60 °C, NIGAM & SINGH, 1995). Some glucoamylase preparations produced from *Aspergillus niger* have an optimal temperature at 70 °C (SAHA & ZEIKUS, 1989), but they are not very stable (MUNCH & TRITSCH, 1990). This optimum was exactly the same as that found by BASAVESWARA RAO and co-workers (1981) and HAASUM and co-workers (1991). The activities of both amylolytic enzymes have decreased drastically above 75 °C.

Applying the optimal parameters in the activity assays resulted in 30% higher and 2.0–2.5 times higher activity in case of α-amylase and glucoamylase, respectively.

2.5. Effect of Ca^{2+} ion on α-amylase activity

In investigating the effect of Ca^{2+} ion on α-amylase activity, $CaCl_2$ was applied in different concentrations ranging from 25 mmol to 200 mmol in the buffer used. The experiment was carried out under optimal conditions. The activity of α-amylase was almost twice higher than in the control without Ca^{2+} ion and reached the maximum value at 75 mmol (data not shown). These results indicated the existence of α-amylase in the ferment broth. These results are in agreement with JENSEN and co-workers (1987), who successfully separated extracellular amylolytic enzymes (α-amylase, glucoamylase and α-glucosidase) from a ferment broth of *Thermomyces lanuginosus*.

*

The authors would like to acknowledge the financial support of the European Commission through the Copernicus Programme Project No. CT94-0232. This work was also supported by grant from the Hungarian National Scientific Research Fund through Project No. OTKA T017228.

References

ATCC (1984): *Media handbook*. American Type Culture Collection, Rockwille, MD.

BASAVESWARA RAO V., SASTRI N. V. C. & SUBBA RAO P. V. (1981): Purification and characterisation of a thermostable glucoamylase from the thermophilic fungus *Thermomyces lanuginosus*. *Biochem. J., 193*, 379–387.

BARNETT, E. A. & FERGUS, C. L. (1971): The relation of extracellular amylase, mycelium and time in some thermophilic and mesophilic *Humicola* species. *Mycopathol. Mycol. Appl., 44*, 131–141.

COONEY, D. G. & EMERSON, R. (1964): Thermophilic fungi: an account of their biology, activities and classification. W. H. Freemen. San Francisco, p. 188.

DOMSCH, K. H., GRAMS, W. & ADERSON, T. H. (1980): *Compendium of soil fungi*. Vol. 1, Academic Press, London, pp. 778–779.

GODFREY, T. (1983): Comparison of key characteristics of industrial enzymes by type and source. -in: GODFREY, T. & REICHELT, K. *Industrial enzymology*. Nature Press, New York, p. 466.

HAASUM, I., ERIKSEN, S. H., JENSEN, B. & OLSEN, J. (1991): Growth and glucoamylase production by the thermophilic fungus *Thermomyces lanuginosus* in a synthetic medium. *Appl. Microbiol. Biotechnol., 34*, 656–660.

JANECEK, S. (1993): Strategies for obtaining stable enzymes (Review). *Process Biochemistry, 28*, 435–445.

JENSEN, B., OLSEN, J. & ALLERMENN, K. (1987): Purification of extracellular amylolytic enzymes from the thermophilic fungus *Thermomyces lanuginosus*. *Can. J. Microbiol., 34*, 218–223.

MCCOMB, R. B. & YUSHOK, W. D. (1957): Colorimetric estimation of D-glucose and 2-deoxy-D-glucose with glucose-oxidase. *J. Franklin Inst., 265*, 417–422.

MUNCH, O. & TRITSCH, D. (1990): Irreversible thermoinactivation of glucoamylase from *Aspergillus niger* and thermostabilization by chemical modification of carboxyl groups. *Biochim. Biophys. Acta*, *1041*, 111–116.

NIGAM, P. & SINGH, D. (1995): Enzyme and microbial systems involved in starch processing. *Enzyme Microbial Technol.*, *17*, 770–778.

SAHA, B. C. & ZEIKUS, J. G. (1989): Microbial glucoamylase: biochemical and biotechnological feature. *Starch/Stärke*, *41*, 57–64.

TAYLOR, P. M., NAPIER, E. J. & FLEMING, I. D. (1978): Some properties of a glucoamylase produced by the thermophilic fungus *Humicola lanuginosa*. *Carbohydrate Res.*, *61*, 301–308.

UNDERKOFLER, L. A. (1976): Production of amylolytic enzymes. Chapter 16. -in: RADLEY, J. A. (Ed.) *Starch production technology*. Applied Science Pub. Ltd., London, p. 295.

VOGEL, J. (1956): A conventional growth medium for *Neurospora crassa* (medium N). *Microb. Genet. Bull.*, *13*, 42–43.

WOOD, T. M. & BHAT, M. K. (1988): Methods for measuring cellulase activities. *Meth. Enzymol.*, *160*, 87–112.