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SHORT COMMUNICATION

Production of the *Aspergillus aculeatus* endo-1,4-β-mannanase in *A. niger*

Petrus J. van Zyl · V. Moodley · S. H. Rose · R. L. Roth · W. H. van Zyl

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Abstract The β-mannanase gene (*man*1) from *Aspergillus aculeatus* MRC11624 (Izuka) was patented for application in the coffee industry. For production of the enzyme, the gene was originally cloned and expressed in *Saccharomyces cerevisiae*. However the level of production was found to be economically unfeasible. Here we report a 13-fold increase in enzyme production through the successful expression of β-mannanase of *Aspergillus aculeatus* MRC11624 in *Aspergillus niger* under control of the *A. niger* glyceraldehyde-3-phosphate dehydrogenase promoter (gpd_P) and the *A. awamori* glucoamylase terminator ($gla A_T$). The effect of medium composition on mannanase production was evaluated, and it was found that the glucose concentration and the organic nitrogen source had an effect on both the volumetric enzyme activity and the specific enzyme activity. The highest mannanase activity levels of 16,596 nkat ml^{-1} and 574 nkat mg^{-1} dcw were obtained for *A. niger* D15[*man*1] when cultivated in a process-viable medium containing corn steep liquor as the organic nitrogen source and high glucose concentrations.

Keywords β-mannanase · *Aspergillus aculeatus* · *Aspergillus niger* · Medium composition

P. J. van Zyl (✉) · V. Moodley · R. L. Roth
CSIR Biosciences, Private Bag X2,
Modderfontein 1645, South Africa
e-mail: pvanzyl@csir.co.za

S. H. Rose · W. H. van Zyl
Department of Microbiology, Stellenbosch University,
Private Bag X1, Stellenbosch 7602, South Africa

W. H. van Zyl
e-mail: whvz@sun.ac.za

Introduction

Endo-β-1,4-mannanase (β-mannanase, E.C. 3.2.1.78) belongs to the glycosyl hydrolase family #5 and has been cloned from bacterial and fungal origins [1–4]. β-mannanases catalyse the random hydrolysis of the β-1, 4-mannan main chains of mannans and heteromannans. Mannans are one of the major constituents of hemicellulosic softwoods and are also found as a minor portion of hemicelluloses in hardwoods as glucomannans [3]. Other types of heteromannans are found as storage polysaccharides in plant seeds, e.g. locust bean gum, a galactomannan polymer with a main chain of β-1,4-mannan with α-1, 6-bound galactosyl side groups.

β-mannanases are useful in several industrial processes, such as extraction of vegetable oils from leguminous seeds and the reduction of viscosity of coffee extracts during the manufacture of instant coffee [5]. They can also be used for biobleaching of softwood Kraft pulps to enhance extractability of lignin [6, 7].

Filamentous fungi such as *Aspergillus niger* have the ability to produce and secrete exceptionally large amounts of properly folded proteins and can produce proteins that contain *O*- and *N*-linked glycans without extensive hyperglycosylation [8–10]. Filamentous fungi are commonly used in the fermentation industry for the large-scale production of proteins, mainly industrial enzymes [11]. The main attraction of filamentous fungi is their natural ability to secrete large amounts of proteins into the growth medium.

The β-mannanase gene (*man*1) from *Aspergillus aculeatus* MRC11624 (Izuka) was originally cloned and expressed in *Saccharomyces cerevisiae* [2]. Both the alcohol dehydrogenase (*ADH*2) and phosphoglycerate kinase (*PGK*1) promoters and terminators were used, and

β-mannanase with activities of 521 and 379 nkat ml^{-1} respectively was secreted into the medium. The enzyme was subsequently evaluated for application in the coffee industry and found to increase the extraction yield of both pure and coffee chicory blends [12]. Here, the successful expression of *A. aculeatus* MRC11624's β-mannanase in *A. niger*, under control of the *A. niger* glyceraldehyde-3-phosphate dehydrogenase promoter (gpd_P) and the *A. awamori* glucoamylase terminator ($gla A_T$), is reported. The pH and temperature optima of the β-mannanase activity were determined and the effect of medium composition on enzyme production was evaluated resulting in a robust medium for production of the enzyme for application in the coffee industry.

Materials and methods

Strains and culture conditions

The genotypes of the microbial strains and plasmids used in the present study are summarised in Table 1.

Recombinant plasmids were constructed and amplified in *E. coli* JM109. *E. coli* was cultivated at 37°C in LM medium (1% yeast extract, 1% tryptone and 0.5% NaCl) on a rotary shaker at 200 rpm, or on LM agar (1.5% agar), supplemented with ampicillin at a final concentration of 100 μg ml^{-1}.

The *A. niger* fungal strains were maintained at 30°C in minimal media (MM) containing 0.5% yeast extract, 0.2% casamino acids, 1% glucose, 0.6% $NaNO_3$, 0.1% of 1,000× trace element solution [2.2% $ZnSO_4 \cdot 7H_2O$, 1.1% H_3BO_3, 0.5% $MnCl_2 \cdot 4H_2O$, 0.5% $FeSO_4 \cdot 7H_2O$, 0.17% $CoCl_2 \cdot 6H_2O$, 0.16% $CuSO_4 \cdot 5H_2O$, 0.15% $Na_2MoO_4 \cdot 2H_2O$ and 5% ethylenediaminetetraacetic acid (EDTA)] and 0.01 M uridine prior to spheroplast harvesting [10, 13]. Transformants were selected on MM containing 10 mM acetamide and 15 mM CsCl, but lacking casamino acids, uridine and $NaNO_3$. *A. niger* D15 transformants were cultivated in double-strength minimal media (2×MM) containing 10% glucose for initial transformant selection and heterologous enzyme characterisation, inoculated to a final spore concentration of 1×10^6 ml^{-1}. For medium evaluation experiments, *A. niger* D15[*man*1] was cultivated on selective agar medium containing 1.8% agar, 0.2% g neopeptone (Difco), 0.1% yeast extract, 0.04% $MgSO_4 \cdot 7H_2O$, 1% glucose and 0.2% casamino acids. Then 2% 50× AspA + N stock solution (30% $NaNO_3$, 2.6% KCl and 7.6% KH_2PO_4, pH 6.5) and 0.1% 1,000× trace element stock solution were added aseptically after sterilisation. The plates were cultivated at 30°C for 3–5 days, when the spores were harvested and resuspended in 0.9% (m/v) NaCl. Flasks containing 200 ml of medium were inoculated to a final spore concentration of 3×10^5 ml^{-1}. The pH of the media was adjusted to pH 5 with NH_4OH (33% v/v) and H_2SO_4 (20% v/v). Where included, glucose, casamino acids, AspA + N and trace elements were added aseptically after sterilisation. After inoculation, the flasks were incubated for 7 days at 30°C on a rotary shaker at 220 rpm.

Data shown were obtained from triplicate flasks in all cases.

Vector construction

Standard protocols were followed for DNA manipulations [14]. Plasmid pGT [15] was used as expression vector for the constitutive expression of the recombinant *A. aculeatus* MRC11624 *man*1 gene in *A. niger*. The *man*1 gene was retrieved from pBS-*man*1 [2] as a *Not*I-*Xho*I fragment and

Table 1 Microbial strains and plasmids used in the present study

Strain or plasmid	Relevant genotype	Reference
Strains		
Escherichia coli JM109	*end*A1 *rec*A1 *gyr*A96 *thi hsd*R17 ($r_k^- m_k^+$) *rel*A1 *sup*E44 Δ(*lac-pro*AB) [F' *tra*D36 *pro*AB *lac*I^qZΔM15]	Promega
Aspergillus niger D15	*pyr*G *prt*T *phm*A (non-acidifying)	Wiebe et al. 2001 [24]
A. niger D15[pGT]	*A. niger* D15 with gpd_P-$gla A_T$ integrated into the chromosome	Rose and Van Zyl 2002 [15]
A. niger D15[*man*1]	*A. niger* D15 with gpd_P-*man*1-$gla A_T$ integrated into the chromosome	This study
Plasmids		
pBLUESCRIPT SK	*bla*	Stratagene
pGT	*E. coli/A. niger* vector, *bla*, gpd_P-$gla A_T$	Rose and Van Zyl 2002 [15]
pBS-pyrGamdS	*bla, pyr*G_P-*pyr*G_T, *amd*S_P-*amd*S_T	Plüddemann and van Zyl 2003 [23]
pBS-*man*1	*bla, man*1	Setati et al. 2001 [2]
pGT-*man*1	*bla*, gpd_P-*man*1-$gla A_T$	This study

cloned into the *Not*I and *Sal*I sites of pGT, generating pGT-*man*1. The gene product of *man*1 was designated *man*1.

A. niger transformations and DNA hybridisations

A. niger D15 transformations were performed by means of spheroplasting using lysing enzymes (Sigma) in accordance with Punt and van den Hondel [16]. Southern hybridizations to confirm integration were carried out according to Sambrook et al. [14]. Chromosomal DNA was isolated after 3 days of cultivation using liquid nitrogen [17]. Integration was confirmed by digesting the genome of the *A. niger* D15 transformants overnight using *Eco*RV. DNA were separated on a 0.8% agarose gel and used for traditional Southern blot analysis. The entire 1.2-kb coding region of *man*1 was labeled with [α-^{32}P]ATP using the Random Primed Kit (Roche), according to the suppliers' specifications, for use as a DNA probe.

Characterisation of β-mannanase produced by *A. niger* D15[*man*1]

The supernatants of *A. niger* D15[pGT] and D15[*man*1] were collected after 7 days of cultivation in 2×MM containing 10% glucose and freeze-dried before determining the specific Man1 activity.

Supernatant from *A. niger* D15[*man*1] cultivated on medium 2 (see below) was separated by 10% SDS-PAGE using the PageRuler molecular weight marker (Fermentas) to estimate the size of the produced protein. Coomassie brilliant blue was used to visualise the protein bands [18].

The effect of media composition on mannanase production by *A. niger* D15[*man*1]

Three different nitrogen sources were evaluated for Man1 production. Yeast extract medium (YEM) had the following composition: 1% yeast extract, 0.2% $MgSO_4 \cdot 7H_2O$, 2% glucose, 0.2% casamino acids, 4% of 50× AspA + N stock solution and 0.2% of 1,000× trace element solution. Corn steep liquor (CSL^{SD}) medium (CSLM) had the following composition: 0.4% Roquette CSL^{SD} (spray-dried), 0.5% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 2% glucose, 0.2% casamino acids, 4% of 50× AspA + N stock solution and 0.2% of 1,000× trace element solution. Phytase-treated-CSL medium (PCSLM) had the following composition: 1.3% (v/v) Roquette CSL^{PT} (spray-dried, phytase-treated), 0.5% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 2% glucose, 0.2% casamino acids, 4% of 50× AspA + N stock solution and 0.2% of 1,000× trace element solution. The effect of different glucose concentrations on Man1 production was evaluated in CSLM with different concentrations of glucose.

Analysis

Flasks were sampled daily, samples filtered and the supernatant used for sugar and enzyme analysis. Residual glucose was determined using Accutrend (Boehringer Mannheim). The fungal biomass was dried at 100°C to constant dry weight.

The temperature and pH optima of the Man1 enzyme were determined using 0.25% galactomannan (Sigma-Aldrich, St Louis, MO, USA) in 50 mM citrate phosphate buffer [19]. The amount of reducing sugars released during the degradation of mannan was determined by the dinitrosalicylic acid method using mannose as standard [20]. After pH and temperature optima determination, further assays were carried out at 75°C, in 50 mM citrate phosphate buffer (pH 3.8). Enzyme activity is defined as the amount of enzyme required to convert one mole of substrate per second and reported in katal.

Results

Construction of expression vectors and recombinant strains

Plasmids pGT and pGT-*man*1 were individually co-transformed with plasmid pBS-pyrGamdS [11] into *A. niger* D15, resulting in transformants *A. niger* D15[pGT] and *A. niger* D15[*man*1]. Plasmid *pBS-pyr*G*amd*S contains the *amd*S and the *pyr*G marker genes that were used for the double selection of the transformants. *Pyr*G enabled the transformants to grow in the absence of uridine, whereas *amd*S allowed the utilisation of acetamide or acrylamide as the sole carbon and nitrogen source. Transformant *A. niger* D15[pGT] was used as reference strain in all experiments.

The *A. niger* D15[*man*1] transformant that produced the highest level of activity was selected for further study. Chromosomal DNA was isolated from *A. niger* D15[pGT] and *A. niger* D15[*man*1]. The DNA was digested overnight with *Eco*RV, an enzyme which does not cut within the coding region of the *man*1 gene. Southern blot analysis revealed the presence of at least one copy of the *man*1 expression cassette integrated into the genome of *A. niger* D15 (Fig. 1).

pH and temperature optima of *A. aculeatus* β-mannanase expressed in *A. niger* D15[*man*1]

The production of the heterologous Man1 by *A. niger* D15[*man*1] was followed over a time period of 10 days in 2×MM with 10% glucose and compared to the mannanase activity of the host strain (Fig. 2). The highest level of Man1 activity (6,800 nkat ml^{-1}) was produced on day 8

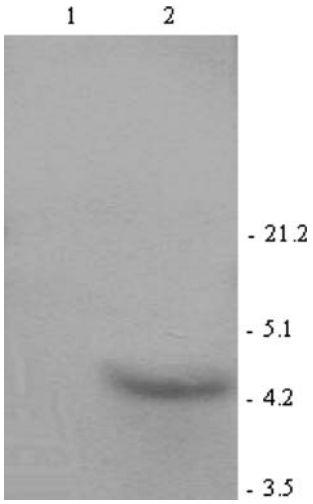

Fig. 1 Southern blot analysis showing the presence of the *man*1 gene on the genome of *A. niger* D15[*man*1]. The marker sizes are indicated *on the right* in kilobits. The ^{32}P-highlighted DNA species represents at least one copy of the *man*1 gene

although the rate of enzyme production started to decrease from day 6 at a level above 6,000 nkat ml^{-1}. The mannanase activity of the host strain was at background levels.

The activity of Man1 was determined using 0.25% galactomannan at different pH values and temperatures. The pH optimum of the Man1 was around pH 3.8 (Fig. 3a), while the temperature optimum was found to be between 75 and 80°C (Fig. 3b). The Man1 enzyme retained 80% mannanase activity after 10 min incubation and more than 50% activity after 5 h at 60°C. It lost all activity after 2 min at 80°C and after only 1 min at 90°C (data not shown).

The supernatant of *A. niger* D15[*man*1], cultivated on CSLM, was separated by 10% SDS-PAGE (Fig. 4). The recombinant Man1 protein constituted a major portion of

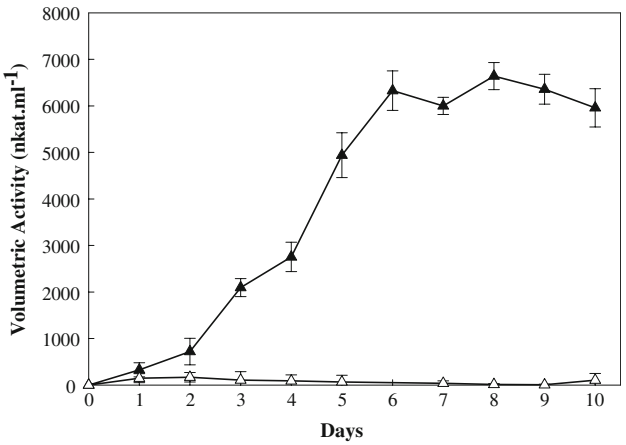

Fig. 2 The heterologous production of Man1 (*closed triangles*) by *A. niger* D15[*man*1] in 2×MM medium + glucose and compared to the activity produced by *A. niger* D15[pGT] (*open triangles*)

the total amount of protein produced extracellularly and was present as a slightly diffuse band with a molecular size ranging from 45 to 50 kDa, whereas the rest of the natively secreted proteins were barely visible.

The effect of media composition on Man1 production by *A. niger* D15[*man*1]

The effect of nitrogen supplementation on the production of Man1 by *A. niger* D15[*man*1] was evaluated in shake flasks in two different types of CSL, namely, Roquette CSL^{PT} (spray dried, phytase-treated and centrifuged; PCSLM) and Roquette CSL^{SD} (spray-dried; CSLM), and in yeast extract-containing medium (YEM). Stationary growth phase was reached after 1 day irrespective of the growth medium evaluated. The highest biomass of 35 g L^{-1} was obtained in the YEM medium. This was 6% higher than the maximum biomass of 33 g l^{-1} obtained in CSLM and 16% higher than the maximum biomass obtained with PCSLM (Table 2). The limiting nutrient in all cases was not glucose, as this was only depleted by day 4 in all flasks (data not shown). The biomass concentrations decreased steadily after the initial peak in concentration, most probably due to autolysis.

Extracellular Man1 activity was observed after day 2 and increased until day 7. It is unclear whether the production of Man1 only occurs after day 2 or if the residual high glucose concentration in the medium masks the measurable mannanase activity. The maximum Man1 activity of 4,175 nkat ml^{-1} was obtained in CSLM. This was 23% higher than the maximum activity of 3,199 nkat ml^{-1} in PCSLM and 46% higher than the 2,848 nkat ml^{-1} obtained in YEM. The highest specific Man1 activity of 159 nkat mg^{-1} dcw was achieved with *A. niger* D15[*man*1] cultivated in CSLM and was 35% higher than that achieved in YEM, which had the lowest specific activity of 103 nkat mg^{-1} dcw. Based on this data, CSL^{SD} was chosen as the organic nitrogen supplementation of choice for further media evaluation.

The effect of glucose concentration in CSLM on Man1 production was subsequently evaluated. Decreasing the glucose concentration resulted in a concomitant decrease in biomass (Table 3). When the glucose concentration was reduced from 100 to 25 g l^{-1}, the yield of biomass on glucose fed increased to 0.40 from 0.33 g g^{-1}, indicating that some metabolic overflow of carbon was present at the higher glucose concentration. The yield of biomass on glucose was very high on 2 g l^{-1} glucose, but it is assumed that at this low glucose concentration, the organism will start consuming protein for carbon and energy requirements. Decreasing the glucose concentration by 75% from 100 to 25 g l^{-1} resulted in a 78% decrease in the volumetric Man1 activity from 16,596 to 3,542 nkat ml^{-1} at

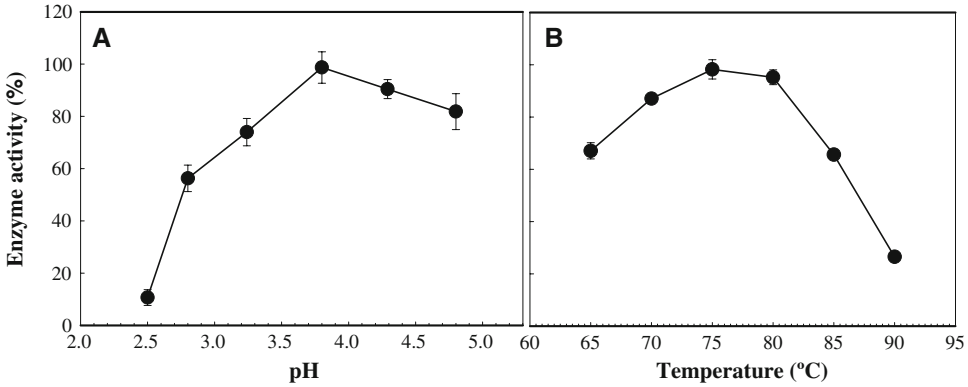

Fig. 3 The effect of pH (**a**) and temperature (**b**) on the enzymatic activity of Man1 when produced by *A. niger* D15[*man*1]

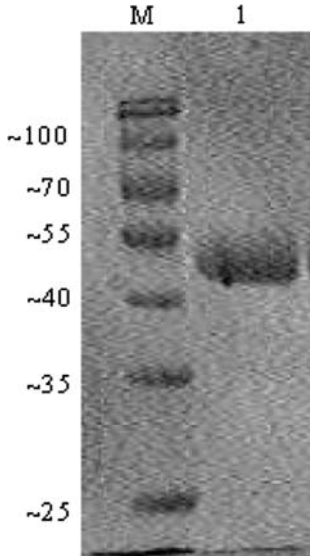

Fig. 4 Separation of the total extracellular protein fractions of *A. niger* D15[*man*1] on 10% SDS-PAGE

day 7, when the maximum activity was detected (Fig. 5a). The decrease in the glucose concentration from 100 to 25 g l^{-1} had very little effect on the specific activity of Man1, with maximum specific enzyme activities of 574 and 515 nkat mg^{-1} dcw, respectively (Fig. 5b).

Discussion

The *A. aculeatus* MRC11624's β-mannanase was successfully expressed in *A. niger*, under control of the *A. niger* glyceraldehyde-3-phosphate dehydrogenase promoter (gpd_P) and the *A. awamori* glucoamylase terminator ($gla A_T$). The pH and temperature optima of the β-mannanase enzyme produced by *A. niger* D15[*man*1] were determined and found to be pH 3.8 and 75–80°C respectively. This differs from the optimum pH and temperature of pH 3 and 60°C observed for β-mannanase produced in *A. awamori* or *S. cerevisiae* [2].

Initial Man1 production by *A. niger* D15[*man*1] in 2×MM with 100 g l^{-1} glucose resulted in a volumetric activity of 6,800 nkat ml^{-1}. These levels of activity exceeded the best levels of activity obtained with expression of *man*1 in *S. cerevisiae* (521 nkat ml^{-1}) by approximately 13-fold [2]. Further media development increased the maximum Man1 activity a further 2.4-fold to 16,596 nkat ml^{-1} at a specific activity of 574 nkat mg^{-1}.

The highest reported mannanase activity produced is 18,403 nkat ml^{-1} and was achieved by producing a *B. subtilis* mannanase in *P. pastoris* in a fermenter [4]. This compares favourably with the shake-flask-produced mannanase activity of 16,596 nkat ml^{-1} we report in this study.

The increase in enzyme activity during stationary growth phase was not expected, as the *gpd* promoter is constitutive and part of the Embden–Meyerhof pathway. The specific enzyme activity increased during the stationary growth phase, indicating a metabolic shift away from biomass production towards enzyme production. This is in contrast to results published by Talabardon and Yang [21] who reported that the production of a glucoamylase-green fluorescent protein fusion by *A. niger* is growth-dependent. However, it should be noted that the strong starch-induced

Table 2 The effect of nitrogen supplementation on heterologous production of Man1 by *A. niger* D15[*man*1]

Standard deviations are shown *in brackets*

Nitrogen supplementation	Biomass (g l^{-1} dcw)	Volumetric activity (nkat ml^{-1})	Specific activity (nkat mg^{-1} dcw)
Yeast extract	35 (±2.6)	2,848 (±164)	103 (±8)
Roquette CSL^{SD}	33 (±5.2)	4,175 (±323)	159 (±17)
Roquette CSL^{PT}	30 (±0.9)	3,199 (±529)	119 (±28)

Table 3 Yield of biomass per glucose fed (g g^{-1}) for *A. niger* D15[*man*1] in shake flasks with different glucose concentrations

Medium	Glucose (g l^{-1})	AspA + N salts	Biomass (g)	Yield of biomass per glucose fed (g g^{-1})	Volumetric activity (nkat ml^{-1})	Specific activity (nkat mg^{-1})
1	100	2×	33	0.33	16,596	574
2	25	2×	10	0.40	3,542	515
3	2	2×	4	2	684	342

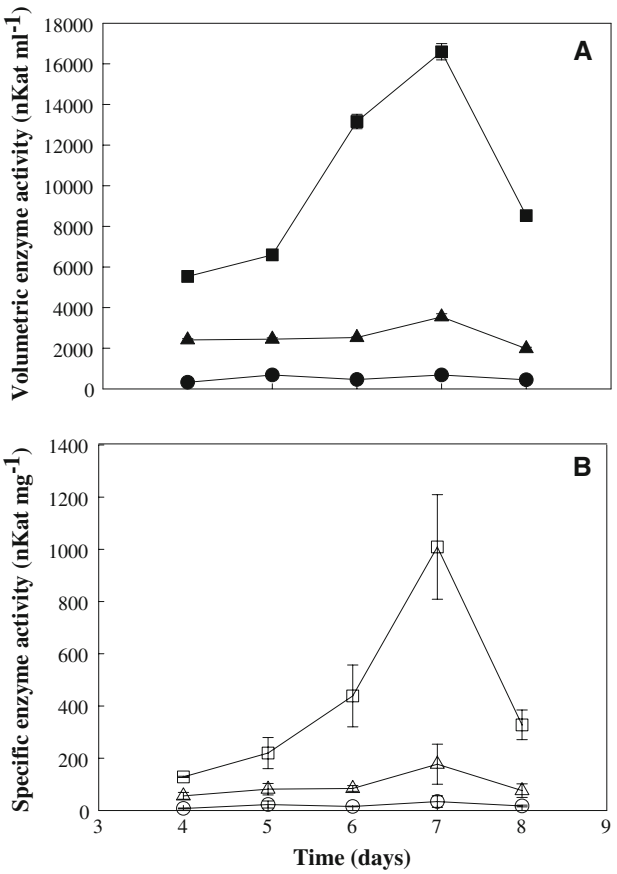

Fig. 5 The effect of glucose concentration on the heterologous production of Man1 by *A. niger* D15[*man*1] over a period of 7 days in medium containing 100 g l^{-1} glucose (*squares*), 25 g l^{-1} glucose (*triangles*) and 2 g l^{-1} glucose (*circles*). The volumetric activity (nkat ml^{-1}) is represented by closed symbols in **a** and the specific activity (nkat mg^{-1} dcw) by open symbols in **b**

promoter of the glucoamylase *glaA* gene of *A. niger* was used, compared to the constitutive *gpd* promoter used in this study. Prolonged release of man1 could also be due to slow secretion of the heterologous enzyme produced intracellularly [13, 22].

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