Exploitation of *Aspergillus niger* for the Heterologous Production of Cellulases and Hemicellulases

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Abstract: Filamentous fungi of the *Aspergillus niger* group are native soil saprophytic fungi. Industrial strains of this group have been extensively used for the production of plant degrading enzymes for the food and beverage, animal feed and paper-and-pulp industries. Recombinant DNA technology allows for the overproduction of these enzymes in copious amounts. The advantages and limitations of *A. niger* as recombinant host for enzyme production are briefly discussed. Specific attention is devoted to the overproduction of several cellulases and hemicellulases to high homogeneity in the protease-deficient strain *A. niger* D15. The size, temperature and pH optima of the heterologous enzymes were shown to be similar to that of their natively produced counter parts. The optimization of enzyme production in dilute sugar cane molasses, using a recombinant strain producing the xylanase II of *Trichoderma reesei* as example, was also demonstrated.

Key Words: *Aspergillus niger*, heterologous expression, cellulase, xylanase, mannanase.

INTRODUCTION

Filamentous fungi belonging to the genus *Aspergillus* are commonly associated with biomass degradation and produce a wide range of secreted hydrolases, including native endo- and exo-acting enzymes involved in the degradation of plant cell walls. Detailed overviews of these enzymes, their characteristics and the regulatory systems involved for their production have been presented by Aro *et al*. [1], De Vries [2] and De Vries and Visser [3]. Strains of particularly the black aspergilli (*Aspergillus niger* group including well-known industrial strains of *A. niger*, *Aspergillus aculeatus*, and *Aspergillus awamori*) [4] have been used successfully as industrial host for the production of various plant cell wall degrading enzymes with cost-effective applications in the food and beverage, animal feed and paper-and-pulp industries [2]. Specific features that favour *Aspergillus* spp. for the production of industrial enzymes include its high secretion capacity, GRAS (Generally Regarded As Safe) status, rapid growth on inexpensive media and the large range of native enzymes produced. These features render *Aspergillus* spp. ideal for commodity enzyme applications on inexpensive media, however, the concomitant production of a large variety of native enzymes by host strains, together with the production of undesirable proteases, limit the large scale production of single enzymes or enzyme cocktails for specific applications.

During the past two decades much progress has been made towards the development of *Aspergillus* spp. as host for the production of heterologous proteins in concentrations of up to grams per liter [5-8]. *Aspergillus* has been established as efficient host for the production of a variety of enzymes, including proteases, lipases, phytases, numerous glycosyl hydrolases, as well as pharmaceuticals [9-12]. Although *Aspergillus* spp. have much to offer as recombinant hosts, several limitations still hampers its optimum use for recombinant protein production. The production of proteases is one of the major hurdles, more so for heterologous than for homologous proteins [8]. *Aspergillus* spp. can produce a range of diverse proteases, of which the acid proteases predominate since medium acidification normally occurs during cultivation. Approaches taken to limit protease production include medium optimization, classical mutagenesis to generate protease-deficient strains or pH mutants with decrease protease production, and selective disruption of protease genes on the genome [13].

Being natural plant degraders, A*spergillus* spp. produce a large variety of cellulases and hemicellulases, when cultivated on plant polysaccharides [3]. Enzyme cocktails are routinely used for industrial applications in the technical, food and feed enzyme markets, but the purification of specific enzyme activities from cellulase and hemicellulase mixtures remains costly and time-consuming. Furthermore, the production of cellulases and hemicellulases are subject to transcriptional activation *via* XlnR in the presence of lignocellulosics, carbon catabolite repression in the presence of simple sugars mediated by CreA and induction under nitrogen-limiting conditions by AreA [1]. Carbon catabolite repression is especially problematic for large scale production of enzymes on inexpensive cultivation medium (such as cane and sugar beet molasses) that contains easily metabolised sugars as carbon source.

Plant cell wall degrading enzymes have a broad spectrum of industrial applications with a world market estimated to grow to $2 billion by [14]. The largest group (about 65%) is technical enzymes used in the detergent, textile, leather and pulp and paper industry, second by food enzymes (about 27%), followed by enzymes used in the animal feed industry (about 6%) [2, 3, 15-19]. Xylanases found applications in the baking industry by modifying cereal flours [20], improvement in the nutritional value of animal feeds for ruminants (cattle and sheep) [21] and monogastrics (chickens and pigs)

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[22, 23], and the pre-bleaching of pulp for paper making [24]. Xylanases and cellulases (together with pectinases) are used for the clarification of wine and fruit juice [25], liquefying of fruits and vegetables for the making of puree and the treatment of textiles [26, 14]. More than three-quarter of the enzymes are hydrolases produced by filamentous fungi (particularly *Aspergillus* spp.). Industrial scale enzyme production by fungi accounts for more than 40% of the commercial enzyme market [14, 27]. The saccharification of plant biomass for the production of fuel ethanol has the potential of outgrowing all three these markets, taking enzymes applications in the industrial biotechnology era to new heights [28]. With the recent development of cost-effective lignocellulosic enzyme mixtures, cellulases and hemicellulases will enter the major biofuel markets for the efficient enzymatic conversion of plant biomass to fermentable sugars for bioethanol production [28].

Currently, the production of specific cellulase and hemicellulase activities to high homogeneity without additional native enzyme activities from wild-type strains of *Aspergillus*, remains a challenge**.** In this study we constructed genetically engineered strains of *A. niger* to constitutively overproduce different hydrolases to address the problem of homogeneity. The production of cost-effective hydrolases requires the use of inexpensive cultivation medium such as molasses based medium. Whereas growth and production of primary metabolites on molasses based industrial medium has been reported in literature [29, 30], few studies have considered production of recombinant proteins on such medium. Therefore, we also explored the impact of cultivation conditions and strain properties on growth and production of recombinant Xyn2 β-xylanase produced by *A. niger* D15 in a medium consisting solely of diluted molasses (diluted to 20% molasses in water).

MATERIALS AND METHODOLOGY

Strains, Plasmids, Media and Cultivation Conditions

The genotypes of the bacterial and fungal strains as well as the plasmids used in this study are summarized in Table **1**. The construction of the plasmids and the fungal strains was similar to that described in detail in Rose and van Zyl (2002) [31]. The plasmid, pBS-pyrGamdS, was introduced and integrated into the genomes of *A. niger* D15[pGT] and *A. niger* D15[*xyn2*], resulting in the prototrophic *A. niger* D15[pGT] $PyrG^+$ and *A. niger* D15[*xyn2*]$PyrG^+$ strains (Table **1**). The

Table 1. The Genotype and Sources of the Strains and Plasmids Used in this Study

Strains	Genotype	Enzyme's Native Host
A. niger D15[a]	*pyrG prtT phmA*	–
A. niger D15[pGT][b]	*A. niger* D15 with gpd_P-$glaA_T$ integrated (reference strain)	–
A. niger D15[*man1*]	*A. niger* D15 with gpd_P-*man1*-$glaA_T$ integrated into genome	*Aspergillus aculeatus*
A. niger D15[*acegI*]	*A. niger* D15 with gpd_P-*acegI*-$glaA_T$ integrated into genome	*Aspergillus carneus*
A. niger D15[*eglA*]	*A. niger* D15 with gpd_P-*eglA*-$glaA_T$ integrated into genome	*Aspergillus niger*
A. niger D15[*eg1*][b]	*A. niger* D15 with gpd_P-*eg1*-$glaA_T$ integrated into genome	*Trichoderma reesei*
A. niger D15[*eg2*]	*A. niger* D15 with gpd_P-*eg2*-$glaA_T$ integrated into genome	*T. reesei*
A. niger D15[*xyn2*][b]	*A. niger* D15 with gpd_P-*xyn2*-$glaA_T$ integrated into genome	*T. reesei*
A. niger D15[*xynC*]	*A. niger* D15 with gpd_P-*xynC*-$glaA_T$ integrated into genome	*Aspergillus kawachii*
A. niger D15[*xyn2*]pyrG	*A. niger* D15[*xyn2*] with fungal *pyrG* integrated into genome	*T. reesei*
A. niger D15[*xyn2*]SA	Salt adapted strainto of *A. niger* D15[*xyn2*]	*T. reesei*
Plasmids:		**Gene Product**
pGT[b]	*bla* gpd_P-gla_T	–
pBS-pyrGamdS[c]	*bla pyrG amdS*	PyrG & AmdS
pGT-manI	*bla* gpd_P-*man1*-$glaA_T$	ManI
pGT-acegI	*bla* gpd_P-*acegI*-$glaA_T$	AcegI
pGT-eglA	*bla* gpd_P-*eglA*-$glaA_T$	EglA
pGT-eg1[b]	*bla* gpd_P-*eg1*-$glaA_T$	EgI
pGT-eg2	*bla* gpd_P-*eg2*-$glaA_T$	TregII
pGT-xyn2[b]	*bla* gpd_P-*xyn2*-$glaA_T$	XynII
pGT-xynC	*bla* gpd_P-*xynC*-$glaA_T$	xynC

[a] [33], [b] [31], [c] [34].

procedure for salt adaptation was followed as described in Redkar *et al*. (1998) [32]. The fungus was plated out twice onto spore plates containing 0.5 M KCl and allowed to grow and sporulate for 4 - 6 days. The spores were subsequently transferred to plates containing 1 M, 1.5 M and 2 M KCl. Finally, spores were transferred three times to plates containing 2 M KCl. Spores of the salt adapted strains of *A. niger* D15[pGT]PyrG$^+$ and *A. niger* D15[*xyn2*]PyrG$^+$, subsequently called *A. niger* D15[pGT]SA and *A. niger* D15[*xyn2*]SA, respectively, were stored in a 2 M KCl solution. Strains were inoculated to a final concentration of $1x10^6$ spores/ml in 2xMM [31] unless stated otherwise. Cultivation took place at 30°C, in 125 ml flasks containing 20 ml medium, with aeration (mixing) at 100 rpm.

Optimisation of Molasses as Cultivation Medium

The molasses used in this study was supplied by Tongaat-Hulett (South Africa). Routinely, 20 ml of 20% molasses (native pH of 5.5) was inoculated to a final concentration of $1x10^5$ spores per ml, cultivated at the above mentioned conditions. Although the *A. niger* D15[*xyn2*] and *A. niger* D15[pGT] strains require uridine for growth in synthetic media, they grow well in molasses without the addition of uridine and uridine was subsequently omitted from the media. The optimal concentration of the molasses required for optimal Xyn2 production, was determined by using flasks containing 20 ml of 10, 20, 30, 40 and 50% molasses (v/v) inoculated with $1x10^5$ spores/ml of the *A. niger* D15[pGT] and *A. niger* D15[*xyn2*] strains, respectively. The ideal inoculum concentration of *A. niger* D15[pGT] and *A. niger* D15[*xyn2*] spores was determined by inoculating 20 ml of 20% molasses, to a final concentration of $1x10^4$, $1x10^5$ and $1x10^6$ spores per ml, respectively. The optimal temperature of cultivation was determined by inoculating $1x10^5$ spores/ml of *A. niger* D15[pGT] and *A. niger* D15[*xyn2*] in 20 ml of 20% molasses and cultivating at 20, 25 and 30°C, respectively. The optimum pH required for the germination of the spores was determined by inoculating $1x10^5$ spores/ml of the A. *niger* D15[pGT] and *A. niger* D15[*xyn2*] strains in 20 ml of 20% molasses. The initial pH of the molasses was set at pH 5.5, 6.5, 7.5 and 8.5 by the addition of 10 M NaOH aseptically after autoclaving.

The *A. niger* D15[pGT], *A. niger* D15[*xyn2*] and *A. niger* D15[*xyn2*]PyrG$^+$ strains were inoculated to a spore concentration of $1x10^6$ spores/ml in 20 ml of 20% molasses at pH 6.5 and cultivated under optimal conditions in order to determine if the uridine dependency of *A. niger* D15*[xyn2*] plays a significant role in the β-xylanase activity or biomass production. The salt adapted strain *A. niger* D15[*xyn2*]SA was compared to *A. niger* D15[*xyn2*]PyrG$^+$ (which was not salt adapted) by inoculating and cultivating them under optimal conditions to determine whether the salt adaptation had any effect on the amount of β-xylanase activity produced.

Enzyme Assays

The recombinant strains were cultivated in shake flasks containing 2xMM [31] and the extracellular production of the heterologous enzymes monitored over a period of 10 days. Individual recombinant enzymes were determined using 1% CMC (carboxymethyl cellulose) as substrate for the β-endoglucanases, 1% birchwood xylan for the β-xylanases and 1% locust bean gum for the β-mannanase. The reducing sugars released from these substrates where determined with the dinitrosalicylic acid method [35, 36]. The pH and temperature optima of the individual recombinant enzymes were also determined (Fig. **1a-d**). One unit of enzyme was defined as the activity producing 1 μmol per minute of reducing sugars in xylose equivalents under these assay conditions.

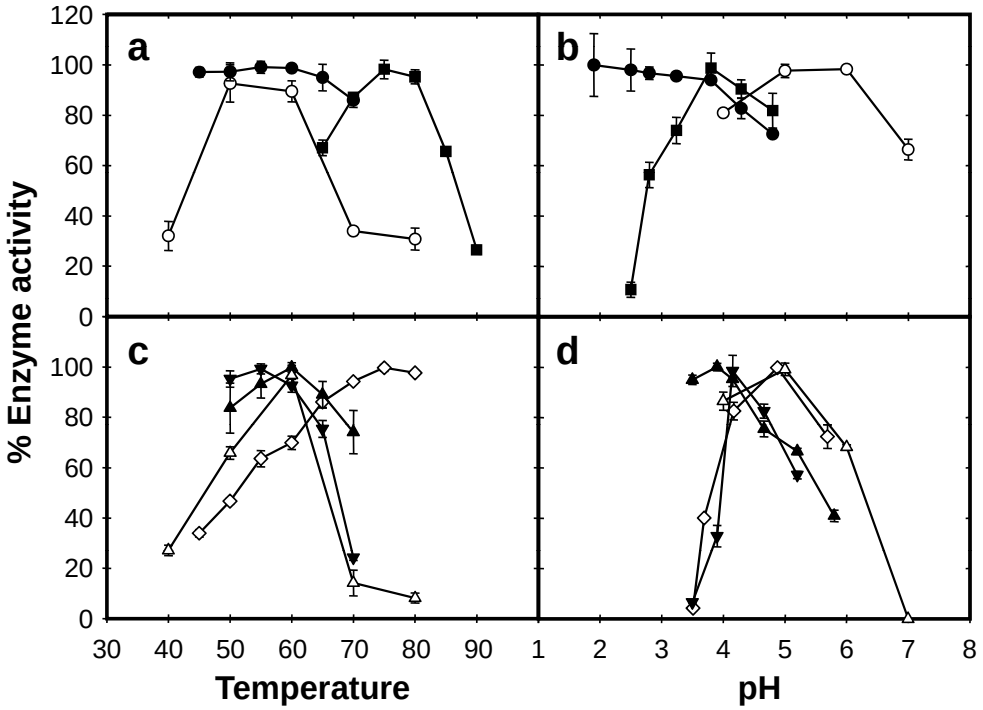

Fig. (1). The effect of (**a** and **c**) temperature and (**b** and **d**) pH on the enzymatic activity of the heterologous enzymes (ManI (■), xynC (●), EglA (▲), AcegI (▼) from *Aspergillus* species, and Xyn2 (○), TregI (△) and TregII (◇) from *T. reesei*) when produced by *A. niger* D15. The enzymatic assays were performed in triplicate with the error bars indicating the deviation between the different samples.

RESULTS AND DISCUSSION

Selective Production of Cellulases and Hemicellulases by Recombinant *A. niger* Strains

The strain *A. niger* D15 have previously proven to be an excellent host for the production of heterologous proteins [31]. Strain *A. niger* D15 was derived through successive mutagenesis events from the industrial strain N402 (ATCC 9029). Strain AB4.1, a *cspA1 pyrG1* derivative of *A. niger* N402 [37], was subjected to UV mutagenesis and a protease-deficient mutant AB1.13 obtained that produced only 1-2% of the intracellular protease activity observed in the parental strain, AB4.1 [38]. The *A. niger* D15 strain was finally isolated as a non-acidifying, protease-deficient (*prtT*) strain of AB1.13 [11, 33, 39]. These characteristics enable the fungus to produce large quantities of protein with diminished degradation by natively produced acid proteases.

The construction of a successful expression system has previously been described in Rose and van Zyl [31] where the β-1,4-xylanase 2 (*xyn2*) and β-1,4-endoglucanase I *(egI*) genes of *Trichoderma reesei* were successfully expressed in *A. niger* D15 under the transcriptional control of the glyceraldehyde-6-phosphate dehydrogenase (*gpdA*) promoter from *A. niger* and the *glaA* terminator of *A. awamori*. The strong glycolytic promoter was chosen to enable heterologous expression of individual cellulase and hemicellulase genes in *A. niger* D15 using simple fermentable sugars with limited production of the native hydrolases and proteases. Using the same expression system, several other cellulases and hemicellulases have also been overproduced in the laboratory strain of *A. niger* D15. These genes include the β-1,4-endoglucanase A (*eglA*) of *A. niger*, β-1,4-endoglucanase I *(acegI*) of *Aspergillus cardeus*, β-1,4-xylanase (*xynC*) of *Aspergillus kawachii*, the β-1,4-mannanase (*man*I) of *A. aculeatus*, as well as the β-1,4-endoglucanase II (*eg2*) of *T. reesei*. The genes were cloned with their native secretion signals intact, leading to the successful secretion of all the heterologous enzymes. Protein secretion was preferred to simplify downstream processing [27].

In most cases, with the exception of the β-mannanase I, the characteristics of the heterologous enzymes were similar to that of their natively produced counter parts (Table **2**). In all cases the levels of activity expressed in *A. niger* was significantly higher than that produced by the native host or by recombinant *S. cerevisiae* (Fig. **2a,b**). With the exception of the β-endoglucanase I of *T. reesei* [31], there was little evidence of overglycosylation (data not shown), which is normally associated with foreign gene expression in *S. cerevisiae* [40, 41].

Submerged Cultivation on Simple Sugar Syrup (Molasses) as Substrate

The product yield, production cost, and the cost of the feedstock in particular, are critically important determinants of the economical viability of applying these enzymes in industrial processes [53]. It is, therefore, important to produce enzymes from inexpensive and readily available substrates, in order to keep the production cost to a minimum. Since South Africa is a prominent producer of cane sugar, it was fitting to investigate the possible use of molasses C (a major by-product of the sugar cane industry) as carbon source. Molasses C is the condensed residue that remains after repeated removal of crystallized sucrose from the concentrated sugar juice. Molasses C is a heterogeneous product, containing approximately 46.7% total sugars of which 33.2% is sucrose, 2.1% chlorine, 3.3 % potassium, with a variety of amino acids and spore elements [54]. It is readily available at low cost and used extensively in the manufacturing of bakers' yeast, in the fermentation industry for the production of potable alcohol and fuel alcohol, as well as the production of citric acid by *A. niger* [55].

The β-xylanase activity of recombinant *A. niger* D15 [*xyn2*]pyrG$^-$ was monitored over a period of 10 days under different cultivation conditions to determine the optimum conditions required for extracellular Xyn2 production in molasses media. The total biomass production (dry weight) after 10-day incubation periods was measured to determine the specific productivity (enzyme yield per biomass). The highest xylanase activities of 226 and 209 U/ml were produced in 20 and 30% molasses, respectively (Fig. **3a**), suggesting it being the preferred concentration for optimal enzyme production. Higher molasses concentrations sustained more biomass production, but less enzyme production. The maximum biomass (58.4 g/l) was obtained at 40% molasses, but drastically declined at 50% with growth being visibly slower at this concentration (Table **3**). The viscosity at 50% molasses limited agitation in shake flasks and most probably did not allow for efficient oxygen transfer. Molasses also contains a variety of components, some (such as heavy metals present) of which could be inhibitory. The β-xylanase production by *A. niger* D15[pGT] in all the experiments did not exceed 29 U/ml and was not included in Fig. (**3a-d**).

An increase in spore inoculums concentration from $1x10^4$ spores/ml to $1x10^5$ spores/ml and $1x10^6$ spores/ml gave 10 and 18% higher levels of heterologous β-xylanase production, respectively, however the biomass yields only increased slightly (Table **3**). Protein secretion in filamentous fungi is believed to predominantly take place at the hyphal tips where proteins can pass through the newly synthesised cell wall [27, 56]. The secretion capacity of a fungus is thus directly proportional to the number of hyphal tips present in the culture [38, 57]. Higher spore concentrations yield more germinating spores, thus more individual mycelial masses with more hyphal tips form. The total biomass (dry weight, DW) yields were dependent on the utilizable sugars in the medium and did not increase significantly. We conclude that higher concentrations ($1x10^6$ spores) are preferred for high heterologous β-xylanase production per biomass produced.

The efficiency of *Aspergillus* spore germination, the proteolytic system and morphology (pellet formation) were found to be affected by the pH of the growth media [11]. The optimum pH for spore germination was between pH 7 – 8, whereas the natural pH of molasses is 5.5. Yet, the effect of pH at the time of inoculation was found to be less critical when considering both enzyme and biomass production (Table **3**). The effect of cultivating at different temperatures on the heterologous production of β-xylanase was determined since MacKenzie *et al.* [57] found that lowering the cultivation temperature decreased biomass production, while increasing enzyme production. A temperature of 30°C yielded the highest β-xylanase production and productivity in the shortest time period (Fig. **3b**, Table **3**). Cultivation at lower

Table 2. Comparison of the Enzyme Characteristics when Produced by Different Hosts

Enzyme	Native Host	*S. cerevisiae*	*A. niger*
AcegI			
Activity (U/ml)	nd	nd	77.98
pH optimum	nd	nd	4.2
Temp. optimum °C	nd	nd	55
Protein size (kDa)	nd	nd	27
AnegA			
Activity (U/ml)	nd	nd	179.96
pH optimum	nd	nd	3.8
Temp. optimum °C	nd	nd	60
Protein size (kDa)	nd	nd	27
EgI			
activity (U/ml)	88.15 [42[a]]	7.14[b]	137.97
pH optimum	4-5 [43, 44]	6 [44]	5 [4[c] in 43]
Temp. optimum °C	60°C [44]	60 [44]	60
Protein size (kDa)	50 [43, 45]	60-100 [43]	62-100 [67[c] in 43]
TregII			
Activity (U/ml)	11.2 U/mg [46]	0.02 [46]	380.14
pH optimum	nd	6[d] [46]	4.8
Temp. optimum °C	nd	50[d] [46]	76
Protein size (kDa)	25 [46]	28 [46]	27
ManI			
Activity (U/ml)	nd	31.25 [41]	359.9
pH optimum	3 [41]	3 [41]	3.8
Temp. optimum °C	60 [41]	60 [41]	76-80
Protein size (kDa)	45 [41]	50 [41]	45 - 50
XynII			
Activity (U/ml)	323.94 [47[e]]	71 98 [40]	485.9
pH optimum	5-5.5 [48]	6 [40]	5 - 6
Temp. optimum °C	56-60[e] [49]	60 [40]	50 - 60
Protein Size (kDa)	20 [50]	27 [40]	21
XynC			
Activity (U/ml)	nd	18 [51]	55.78
pH optimum	nd	<3 [51, 52]	< 3
Temp. optimum °C	nd	60 [51]	55 - 60
Protein size (kDa)	19.8 (theoretical) 29 (glycosylated) [52]	24.5 [51]	22

[a]The cellulolytic mutant RUT C-30 can produce levels of up to 225 IU/ml [42]
[b]Values obtained in this study using a multicopy episomal plasmid and the *ADH2* promoter for expression in SC^{-URA} medium [40]
[c]Expression in *Aspergillus oryzae* as host
nd - not determined (no information available)
[d]assays were performed at conditions mentioned, but were not determined to be the optimal conditions.
[e]The combined action of all the xylanases produced by *T. reesei*.

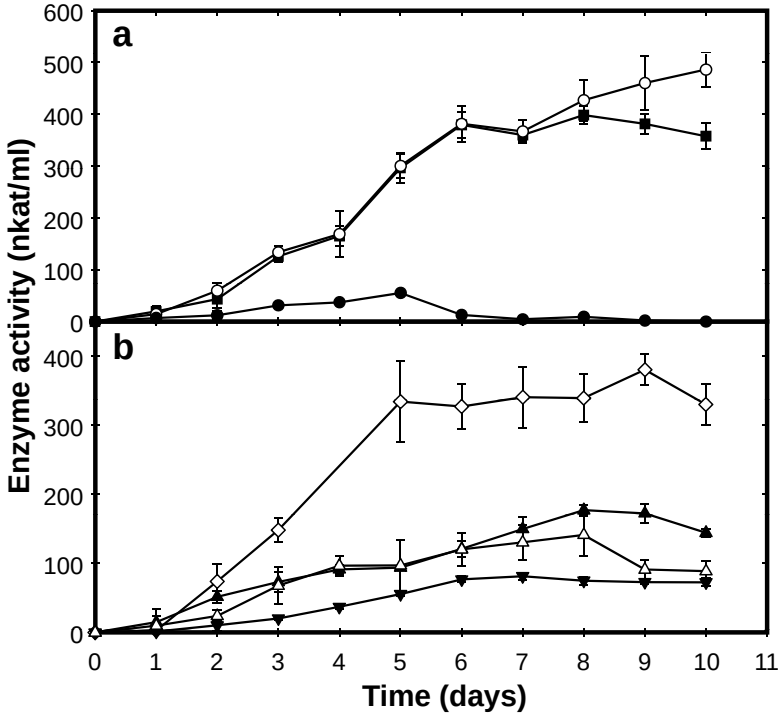

Fig. (2). The heterologous production of (**a**) hemicellulases and (**b**) endoglucanases by recombinant *A. niger* D15 strains. The production of ManI (■) of *A. aculeatus*, xynC (●) of *A. kawachii*, EglA (▲) of *A. niger*, AcegI (▼) of *A. carneus*, and Xyn2 (○), TregI (△) and TregII (◇) of *T. reesei* was monitored over a period of 10 days in 2xMM medium. The values in this figure were obtained using six parallel cultures with the error bars indicating the deviation between the different flasks.

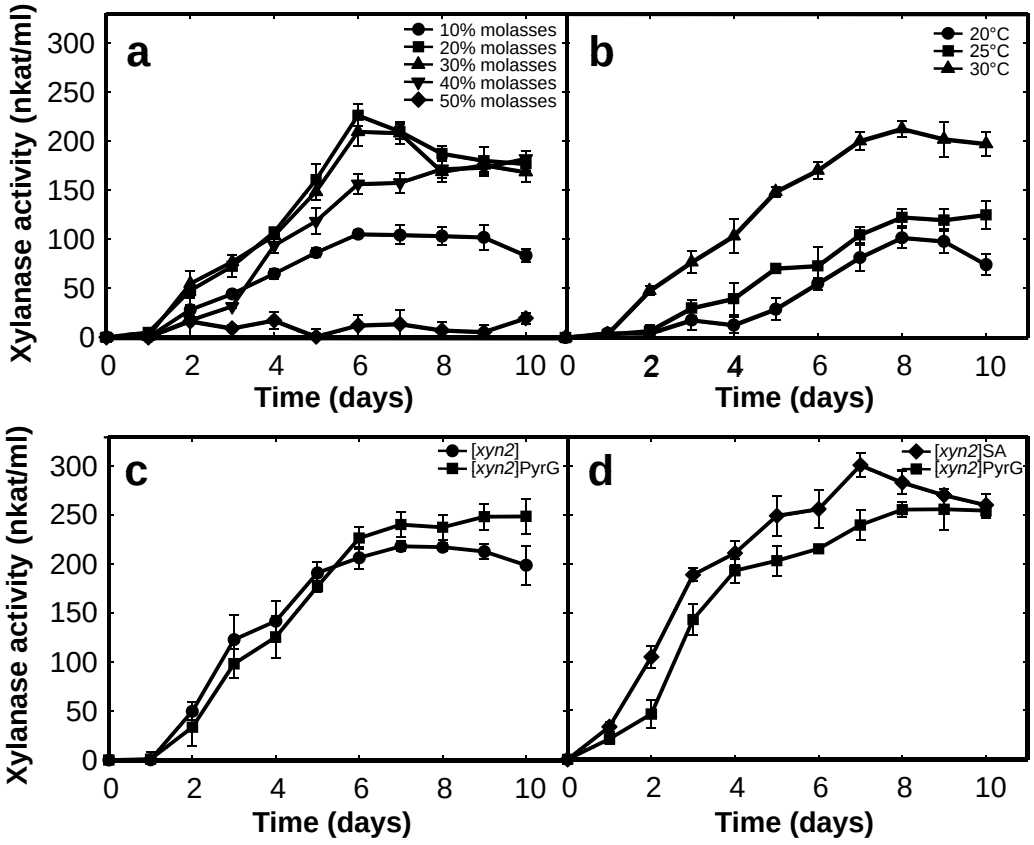

Fig. (3). The heterologous production of Xyn2 by recombinant *A. niger* D15 strains was monitored over a period of ten days in different molasses media formulation and under different growth conditions: (**a**) media formulations containing 10-50% molasses concentration, (**b**) cultivation temperatures ranging from 20°C-30°C, (**c**) introduction of additional pyrG gene copies and (**d**) salt adaptation to 2M KCL. The values in this figure were obtained using six parallel cultures with the error bars indicating the deviation between the different flasks.

Table 3. The Maximum β-Xylanase Activity (U/ml), Total Biomass (g/L) and β-Xylanase Productivity (U/g) Recombinant *A. niger* Cultures were Determined after Cultivation of 10 Days in Different Media Formulations and Under Different Growth Condition

Cultivation Condition	β-Xylanase Activity (U/ml)	Biomass (Dry Weight) (g)	β-Xylanase Productivity (U/g)
Molasses concentration			
10%	105.6 ± 3.9	19.7 ± 1.2	5338.9 ± 347.9
20%	226.4 ± 11.1	31.3 ± 2.5	7258.5 ± 545.9
30%	209.7 ± 14.4	47.6 ± 4.4	4433.1 ± 407.9
40%	182.0 ± 8.0	58.4 ± 9.5	3191.3 ± 593.9
50%	19.4 ± 5.8	3.7 ± 0.4	5266.9 ± 66.0
Spore concentration			
$1x10^4$ spores/ml	185.3 ± 9.4	28.9 ± 0.7	6886.6 ± 192.0
$1x10^5$ spores/ml	226.7 ± 21.7	30.2 ± 2.3	7552.5 ± 605.9
$1x10^6$ spores/ml	250.4 ± 10.3	30.8 ± 2.2	8152.4 ± 593.9
Initial pH			
5.5	178.2 ± 11.1	32.7 ± 1.9	5476.9 ± 323.9
6.5	223.0 ± 21.9	35.4 ± 2.2	6322.7 ± 395.9
7.5	184.9 ± 13.0	36.6 ± 2.7	5069.0 ± 383.9
8.5	210.3 ± 1.6	34.9 ± 3.5	6070.8 ± 611.9
Cultivation temperature			
20°C	101.6 ± 10.4	20.6 ± 0.7	4883.0 ± 144.0
25°C	125.0 ± 14.3	25.4 ± 2.9	4949.0 ± 473.9
30°C	213.0 ± 7.9	28.1 ± 2.8	7618.4 ± 731.9
Prototrophic strains			
D15[*xyn2*]	248.6 ± 1.1	37.9 ± 2.1	6562.7 ± 365.9
Control(D15[*xyn2*]pyrG$^-$)	217.9 ± 5.1	34.4 ± 2.3	6358.7 ± 455.9
Salt adaptation			
D15[*xyn2*]SA	300.8 ± 11.87	34.9 ± 1.0	8620.3 ± 263.9
Control(D15[*xyn2*])	255.6 ± 20.7	35.5 ± 1.9	7216.6 ± 401.9

The values in this table were obtained using six parallel cultures.

temperatures yielded less biomass, accompanied by a significant drop in the β-xylanase productivity.

The recombinant strain *A. niger* D15[*xyn2*]pyrG$^-$ is an uridine deficient strain that normally requires the addition of uridine to synthetic growth media. Molasses is derived from crushed sugarcane plant material and therefore contains, apart from sugars, enough other nutrients, including uridine and uridine precursors, to support growth of uridine deficient strains. Although the *A. niger* D15[*xyn2*]pyrG$^-$ strain grew well on molasses without the addition of uridine (Fig. **3a,b**), the *pyrG*$^-$ mutation still could have a negative effect on the biomass produced when the uridine or uridine precursors / substitute in the molasses or transport into the cell became limited. Additional *pyrG* gene copies were integrated into the genome of strain *A. niger* D15[*xyn2*]pyrG$^-$ (generating strain *A. niger* D15[*xyn2*]) to complement the *pyrG*$^-$ mutation. Strain *A. niger* D15[*xyn2*] was compared to *A. niger* D15[*xyn2*]pyrG$^-$ with regard to β-xylanase production and productivity (Fig. **3c**). The β-xylanase and biomass production increased with 14% and 10%, respectively, corresponding to a productivity increase of 3%. These results underline the importance of using prototrophic strains independent of nutrient supplements.

Molasses (76% dry mass) contains significant amounts of potassium and chlorine [54]. Redkar *et al.* [32] found that salt adaptation affects gene expression levels. The effect of adapting strain *A. niger* D15[*xyn2*] to 2 M KCl on β-xylanase and biomass production was evaluated. The salt-adapted strain *A. niger* D15[*xyn2*]SA gave the highest maximum β-xylanase activity (300.8 U/ml) and β-xylanase productivity (8620.3 U/g dry weight) obtained for any of the recombinant strains (Fig. **2d**, Table **3**). This represents an improvement of

more than 30% on strain *A. niger* D15[*xyn2*]pyrG$^-$ before the addition of the *pyrG* gene copies and salt adaptation.

CONCLUSIONS

This study illustrated the successful overproduction of several cellulases and hemicellulases in *A. niger* using media rich in fermentable sugars, thus limiting the production of *A. niger's* native enzymes. Secretion of the enzymes was preferred in order to simplify harvesting of the enzymes. The heterologous enzymes were produced as a significant portion of the total extracellular protein fraction with little additional protein contaminants [31] to simplify downstream process. All enzymes produced high levels of activity and in most cases the characteristic of the heterologous enzymes were similar to that of their native equivalent (Table **3**).

The use of a pH mutant of *A. niger* as host allowed for the production of significant amounts of extracellular proteins without the problem of proteolytic degradation. The pH of the growth media was monitored throughout the study, and never dropped below pH 4.5, thus preventing activation of production of the remaining native *A. niger* extracellular acid proteases. No visible degradation of the recombinant enzymes could be detected by SDS-PAGE. This overproduction of recombinant proteins in the absence of other hydrolases and proteases yields recombinant enzymes at high homogeneity.

The effect of cultivation condition on the heterologous protein production by recombinant *Aspergillus* strains using molasses as growth medium was also illustrated. The use of the constitutive *gpd* promoter enabled the strain to produce the recombinant enzymes upon germination without being effected by the sugar concentration in the media [31]. Production of the recombinant enzymes using the *gpd* promoter does not require the addition of an inducer, thus simplifying the production of recombinant enzymes in bulk.

Optimal conditions for the production of *T. reesei* Xyn2 β-xylanase can be summarized as follows: inoculate 20% molasses at pH 6.5 with a spore inoculum of at least $1x10^6$ spores/ml and cultivate at a temperature of 30ºC. The importance of using prototrophic recombinant strains that are salt adapted was also demonstrated. Although up-scaling of production of recombinant proteins remain a formidable bioengineering challenge, functional production of several cellulases and hemicellulases to high homogeneity could be demonstrated, paving the way to their large-scale production.

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