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# Expression of an α-galactosidase from *Saccharomyces cerevisiae* in *Aspergillus awamori* and *Aspergillus oryzae*

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**A gene encoding α-galactosidase activity was isolated by polymerase chain reaction (PCR) from *Saccharomyces cerevisiae* NCYC686 and separately placed under the control of transcriptional elements regulating α-amylase expression in *Aspergillus oryzae* and glucoamylase expression in *A. awamori*. Following transformation of both *A. oryzae* and *A. awamori* with their respective expression vectors, induction of heterologous α-galactosidase from positively selected clones was effected through the addition of soluble starch (10% wt/vol) to the growth medium. Upon induction in *A. oryzae*, a transcriptional instability resulted in degradation of mRNA encoding heterologous α-galactosidase, thus preventing expression of the active enzyme. The use of a gene fusion strategy in *A. awamori* overcame this instability and resulted in stable expression of *S. cerevisiae* α-galactosidase. Subsequent to initial (shake flask) experiments, a series of scale-up and optimisation studies led to heterologous expression of the recombinant enzyme in batch fermentation at 51 U $mg^{-1}$ total extracellular protein. This was higher than previously published works, which reported extracellular levels of heterologous α-galactosidase up to 38 U $mg^{-1}$ total protein. Analysis of crude extracts of the fermentation medium revealed significant differences between the activity parameters reported previously in the literature for this enzyme and those observed here. The recombinant enzyme exhibited thermostability properties not previously reported for *S. cerevisiae* α-galactosidase, a trait which would make it suitable for use in processes requiring high temperatures.**
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**Keywords:** α-galactosidase; *Aspergillus awamori*; *Aspergillus oryzae*; heterologous expression; process development

## Introduction

α-Galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) catalyses the hydrolysis of α-D-galactosidic linkages present in oligosaccharides such as raffinose, stachyose and verbascose. The enzyme is of interest because of its many potential biotechnological applications, including molasses utilisation by baker's yeast, hydrolysis of raffinose in sugar beet and soy processing, serological conversion of blood group type and, most importantly, use as a dietary additive for humans and animals [2,9,10,12–14,19,20,27,41].

Raffinose, stachyose and verbascose are the galactooligosaccharides most commonly found in feedstuffs with relatively high levels of leguminous seeds [16,36]. These oligosaccharides are not digested in the small intestine of monogastric animals but are degraded to some degree by the microflora of the hindgut. Microbial fermentation of galactooligosaccharides in the lower gut generates short-chain fatty acids (SCFAs), but the contribution in net energy to the host animal from the SCFA is less than from the direct absorption of the oligosaccharides as monosaccharides. In addition, fermentation of galactosides is associated with the production of flatulence gases such as carbon dioxide, hydrogen and methane, which increases the rate of feed passage and consequently results in lower feed utilisation [8]. Dietary supplementation with α-galactosidase to bring about galactooligosaccharide hydrolysis in the upper part of the gastrointestinal tract is a possible solution to the poor utilisation of galactooligosaccharides by monogastric animals [3,32–34,40]. This aspect has been extensively researched and the use of α-galactosidase as a feed additive for improved animal growth and performance has been proven to be efficacious in situations where certain problematic feed ingredients are used [3,17,31,33].

α-Galactosidase has been cloned from a number of sources including *Escherichia coli* [45], *Cyamopsis tetragonoloba* [11], *Coffea* sp. [47], *Zygosaccharomyces cidri* [38] and *Saccharomyces cerevisiae* [25,28,29]. Indeed, *S. cerevisiae* has been shown to possess up to 10 separate genes encoding α-galactosidase, of which those that have been sequenced display greater than 80% nucleotide homology [28,29]. In this study, heterologous fungal strains were created in which an α-galactosidase gene from *S. cerevisiae* was placed under the control of highly inducible fungal regulatory elements. Two distinct expression constructs were generated in which a polymerase chain reaction (PCR)-amplified gene fragment encoding α-galactosidase was placed immediately downstream of both the α-amylase promoter from *Aspergillus oryzae* and the glucoamylase promoter from *Aspergillus awamori*. Screening of positively selected transformants resulted in the isolation of clone, which secreted significant levels of recombinant enzyme into the growth media. Subsequent scale-up and process parameter improvements utilising the most promising clone resulted in the production of levels of α-galactosidase up to 51 U $mg^{-1}$ total protein, noteworthy in comparison with reported extracellular expression levels up to 38 U $mg^{-1}$ in both genetically and nongenetically modified organisms [5,6,11,25,28,29,38,45–47].

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## Materials and methods

### Bacterial strains and plasmids

*A. awamori*, *A. oryzae* mutant, Ao7, and the expression vectors pPLF and pAG were a gift from Prof. Orla Conneely and Dr. Pauline Ward (Baylor College of Medicine, Houston, TX, USA) [43,44]. Both strains were maintained on growth media outlined previously [43,44]. *E. coli* strain Inv'αF and the plasmid PCRII™ were obtained from Invitrogen (Carlsbad, CA, USA). All manipulations for the growth and storage of *E. coli* were carried out according to Sambrook *et al* [30]. *S. cerevisiae* NCYC 686 was obtained from the National Collection of Yeast Cultures, AFRC Institute of Food Research (Norwich, UK). This strain was maintained on growth media as outlined by the culture collection.

### Chemicals and reagents

Restriction endonucleases, DNA-modifying enzymes and Digoxigenin System components were obtained from Roche (Mannheim, Germany). Specifically, DIG High Prime was used for random labelling of DNA probes. Taq DNA polymerase and PCR reagents were obtained from Perkin-Elmer Cetus (Foster City, CA, USA). Oligonucleotide primers were obtained from Sigma-Genosys (Cambridge, UK). Lodex 5 starch was obtained from Cerestar (Neuilly-sur-Seine, France). All other chemicals, reagents, molecular weight markers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and microbiological media constituents were obtained from Sigma (St. Louis, MO, USA).

### Nucleic acid manipulations

All nucleic acid manipulations were based on the methods of Sambrook *et al* [30]. Any deviations from these methodologies were in accordance with product manufacturer's instructions. Nonradioactive labeling of probes was carried out using the digoxigenin (DIG) labeling kit with subsequent Northern analyses being carried out according to the manufacturer's instructions. Total genomic DNA was isolated from *S. cerevisiae*, *A. oryzae* and *A. awamori* using a DNA isolation kit obtained from Roche. Total RNA was isolated from these organisms using the FastTrack 2.0 kit from Invitrogen.

### Isolation and manipulation of an α-galactosidase gene

PCR amplification of the α-galactosidase gene was carried out from total genomic DNA of *S. cerevisiae* NCYC 686 using the primer pair indicated below, which was based on previously published sequences [25]. Primers were designed to create an in-frame gene fusion when PCR-amplified products were subcloned into the expression vectors pAG and pPLF:

5′ end oligonucleotide (agaltp)
5′ TT*CCCGGG*TCTCCGAGTTACAATGGCCTTGGTC 3′
3′ end oligonucleotide (agalbm)
5′ T*CCCGGG*TTCAAGAAGAGGGTCTCAACTTATAGAA 3′

(the sequence that overlaps the *Sma*I sites used for manipulation of the DNA fragment is underlined). The PCR reaction contained the following: 1 μg of total genomic DNA or 50 ng of plasmid DNA; primers (500 ng each); $MgCl_2$ (2.5 mM); $(NH_4)_2SO_4$ reaction buffer at 1× concentration; dNTPs (500 μM each) and sterile distilled water, 98 μl. The enzyme (2 U) was added after an initial denaturation step. Reaction conditions for PCR consisted of the following: a 2-min denaturation step for total genomic DNA 94°C, followed by 1 min at 85°C for addition of polymerase. Subsequent to the addition of the polymerase, a total of 30 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C was carried out. A final 10-min step at 72°C was employed to ensure homogeneity of the amplified fragments.

Following amplification, the PCR fragment was subcloned directly into the PCRII™ vector and sequenced on this plasmid using standard oligonucleotides complimentary to the M13 Forward and M13 Reverse primer sites present adjacent to the insertion site. Fluorescence DyeDeoxy sequencing was used (ABIPRISM™ Dye Terminator Cycle Sequencing Core Kit; Applied Biosystems, Foster City, CA). Homology analysis of sequence data was carried out using the BLAST sequence analysis package, online at the National Centre for Bioinformatics (NCBI) homepage.

The α-galactosidase gene was subsequently liberated by *Sma*I digestion and purified using the high pure PCR product purification kit that was obtained from Roche.

Purified product was then ligated into *Nsi*I-restricted T4 DNA polymerase-treated pAG expression vector. In a similar fashion, this *Sma*I-digested gene fragment was also ligated into the expression vector pPLF, which had been *Not*I-restricted and blunt-ended with Mung Bean Nuclease prior to ligation. The expression vectors pAG and pPLF were purified using the high pure plasmid isolation kit obtained from Roche. Orientation analysis confirmation and reading frame integrity following ligation were confirmed carried by DNA sequencing.

### Growth and manipulation of A. oryzae and A. awamori

The *pyr*G mutant strain, Ao7, used in this study was derived from *A. oryzae* and was maintained on media as described by Ward *et al* [43]. The *A. awamori* strain used was grown and maintained as described by Ward *et al* [44].

Protoplast generation and transformation of both strains were carried out as described by Tilburn *et al* [37]. Genomic DNA from putative transformants was analysed by PCR to confirm integration of the expression construct into the respective host genome. PCR reaction conditions were the same as outlined previously with the exception of the use of a top strand, plasmid-specific primer, and a bottom strand gene-specific primer, the sequences of which are:

5′ end oligonucleotide (pplfseq)
5′ GGATCCGGCAGCGTGACCTCGACCAGCAAG 3′
3′ end oligonucleotide (agalbm)
5′ TCCCGGGTTCAAGAAGAGGGTCTCAACTTATAGAA 3′.

Induction of the heterologous α-galactosidase was carried out by growth of positive transformants in media containing 10% (wt/vol) Lodex 5 starch at 30°C. Initial work was carried out in 50-ml shake flasks with a rotation speed of 150 rpm. Larger investigations were performed in a 5-l Bioflo IIc batch/continuous fermentor (New Brunswick Scientific, Edison, NJ). Conditions for operation are outlined in the text.

### Characterisation of recombinant proteins

α-Galactosidase activity was assayed using *p*-nitrophenol-α-D-galactopyranoside as described by Dey and Pridham [10]. One unit of α-galactosidase activity can be defined as the amount of enzyme

which liberates 1 μmol of nitrophenol per minute per milliliter under standard assay conditions [10]. Protein quantification was determined using the Coomassie Protein Assay Reagent (Pierce, the Netherlands). SDS-PAGE analyses were performed using standard 7.5% polyacrylamide gels as outlined by Sambrook *et al* [30]. Cell-free crude extracts of fermentation medium were obtained by centrifugation at 14,000 rpm for 10 min. These extracts were then desalted using Centri-Spin$^{-10}$ columns available from Princeton separations (Adelphia, NJ, USA). Densitometric analysis of SDS-PAGE gels was performed using a Vilber Lourmat Bio-ID imaging system, version 6.11.

## Results

### α-Galactosidase gene isolation and manipulation

Using the primer pair and reaction conditions outlined, a 1368-bp PCR product was amplified. Sequencing of this PCR product and homology analysis showed the gene to have >90% homology with a number of deposited α-galactosidase gene sequences within the NCBI database. The isolated α-galactosidase gene sequence exhibited 94% homology with an α-galactosidase gene sequence, which had been previously deposited under the accession number M58484. The corresponding 1368-bp fragment was then subcloned into the *A. oryzae* expression vector (pAG), and placed under the control of the *A. oryzae* α-amylase promoter and signal sequence, highly inducible in the presence of starch. A second expression construct was created, in which the PCR-amplified gene was subcloned into the *A. awamori* expression vector (pPLF), and also placed under the control of a starch-inducible regulatory element, the *A. awamori* glucoamylase promoter. In this case, however, a gene fusion strategy was employed, whereby the α-galactosidase gene product was designed so as to be postranslationally cleaved from approximately 498 amino acids of the glucoamylase protein. This type of strategy has been widely employed and used successfully to both stabilise heterologous protein expression and increase recombinant protein yield in filamentous fungi [1].

### Expression in A. oryzae

A total of 20 positive transformants were induced for 96 h in growth media containing 10% (wt/vol) starch. A negative control (untransformed host *A. oryzae*) was also induced. Cell-free extracellular medium was then analysed for the expression of α-galactosidase; however, no activity was detected from any transformant or in the negative control. Intracellular contents from each of the transformants and the control were also assayed for α-galactosidase expression, and again no activity was detected. Analysis of the protein profiles produced on an intracellular and extracellular basis was performed by SDS-PAGE to determine if the heterologous protein was being produced in an inactive form. No differences between the protein profiles of the transformants and the negative control were noted. To determine if the problem lays at a transcriptional level, a Northern hybridisation analysis was performed.

Essentially, total RNA was extracted from an induced transformant and a negative control every 24 h over a 96-h period. A very-low-molecular-weight mRNA signal approximately 300 bp in size was detected at all time points in the transformant, but not in the negative control. In a similar fashion, Northern analyses on a number of other induced transformants produced the same result indicating the degradation of mRNA encoding heterologous α-galactosidase. From these analyses, it was apparent that the *A. oryzae* expression system was not suitable for the production of recombinant α-galactosidase due to transcriptional instability of the heterologous mRNA.

### Expression in A. awamori

Small-scale induction studies, using 50 ml of growth media supplemented with 10% (wt/vol) starch and inoculated with a total of $10^6$ spores, were carried out on 70 PCR-screened transformants and a negative control over a period of 11 days. Samples of extracellular media were analysed for production of α-galactosidase every 24 h. Using this expression system, activity was noted 48 h after inoculation in greater that 95% of the cultures induced. A low basal level of α-galactosidase was detected also in the negative control, indicating that the host secretes an endogenous form of the enzyme. A time course of enzyme production is illustrated in Figure 1. From this initial work, a transformant designated pPLF-686B3 was chosen for further study, as it was shown to secrete up to 1.65 U of α-galactosidase per milligram of total protein in these small-scale experiments.

Northern analysis of α-galactosidase mRNA expression in *A. awamori* detected a unique transcript of the anticipated size (data not shown) indicating the correct processing of mRNA encoding the recombinant enzyme.

### Optimisation of expression in A. awamori

Further small-scale experiments were carried out to optimise media for the production of the recombinant protein. The effects of inducer (starch) concentration, pH and autolysed yeast concentration on the expression levels were studied. The use of autolysed yeast significantly improves heterologous protein yield in this expression system (P. Ward, personal communication).

It was found that starch at a concentration of 100 g l$^{-1}$ was optimal for induction of expression, with concentrations greater than this having a negative effect. The pH of the media also had an influence on the production of the heterologous enzyme, with a pH of 5.0 being considered optimal. Finally, the use of autolysed yeast at a concentration of 35 g l$^{-1}$ had a beneficial effect.

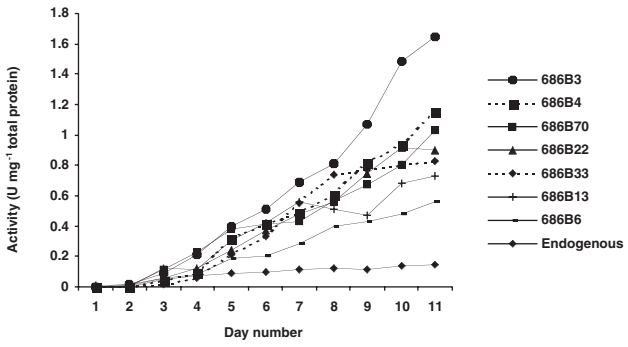


**Figure 1** Time course of heterologous α-galactosidase production in 50 mL shake flask cultures of *Aspergillus awamori*. Flask containing 50 mL of induction media were inoculated and grown at 30°C with a constant shake speed of 150 rpm. Samples were taken every 24 hours and assayed for α-galactosidase activity using p-nitrophenol α-D-galactopyranoside. Codes starting with the prefix 686B in the legend refer to a sample pool of the individual transformants which were assessed for their heterologous α-galactosidase production.

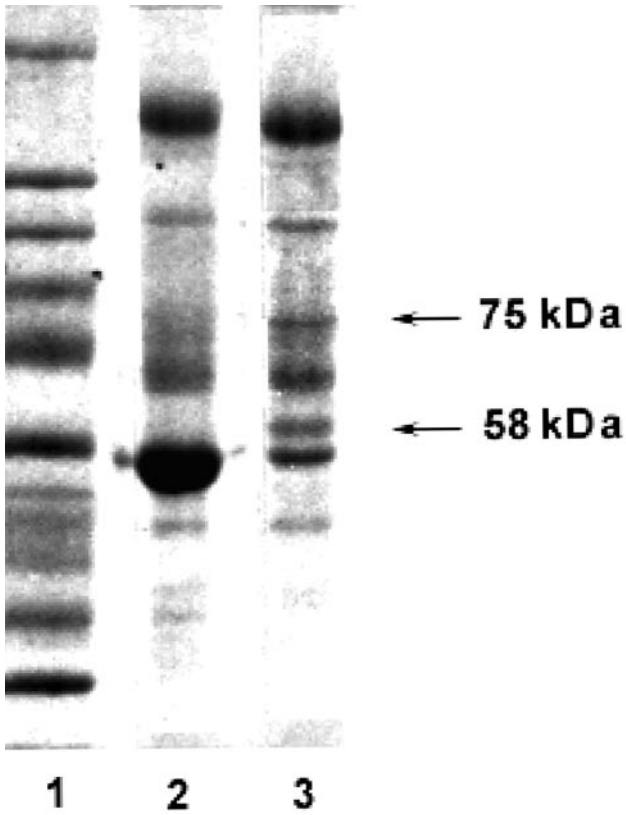


**Figure 2** SDS-PAGE analysis of total extracellular protein from transformed and control *A. awamori*. Lane 1: Molecular size markers, 20, 24, 29, 36, 45, 55, 66, 84, 97, 116, 205 kDa. Lane 2: Total extracellular protein from induced host *A. awamori*. Lane 3: Total extracellular protein from induced transformant pPLF-686B3. The presence of the fusion peptide (58 kDa) and the heterologous α-galactosidase (75 kDa) is indicated by the arrows.

From this work, the use of an induction medium at pH 5.0 with 100 g $l^{-1}$ inducer and 35 g $l^{-1}$ autolysed yeast was considered optimal for small-scale induction. An induction was then carried out using the combination outlined above, from which an expression level of 3.6 U $mg^{-1}$ total protein of enzyme activity was achieved. This was 90-fold higher than the level of endogenous enzyme produced by the host during the initial screening. As a control, untransformed *A. awamori* was cultivated using our defined medium and no additional increase in endogenous α-galactosidase activity was detected.

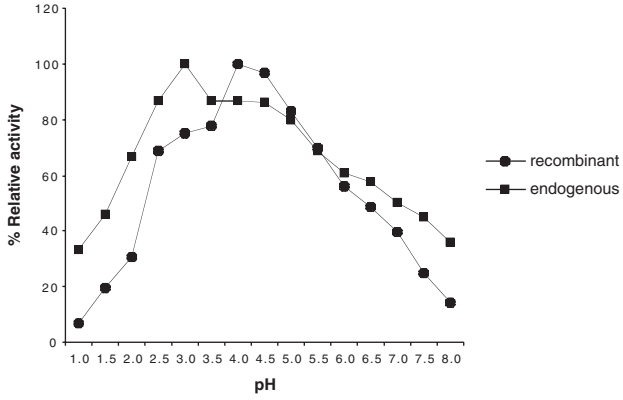


**Figure 3** pH versus activity profile of crude extracts from *Aspergillus awamori* containing heterologous α-galactosidase. Crude extracts of post-induction media containing recombinant α-galactosidase and media from the host strain containing endogenous α-galactosidase were assayed for relative enzyme activity at the pH indicated.

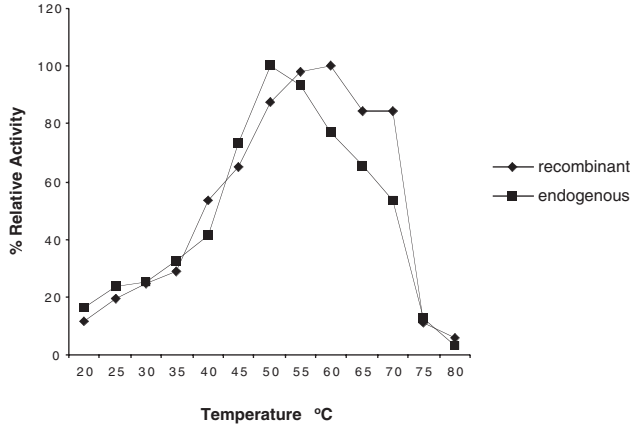


**Figure 4** Temperature versus activity profile of crude extracts from *Aspergillus awamori* containing heterologous α-galactosidase. Crude extracts of post-induction media containing recombinant α-galactosidase and media from the host strain containing endogenous α-galactosidase were assayed for relative enzyme activity at the temperature indicated.

Further optimisations were performed in which a series of 3-l batch fermentations was carried out in a 5-l Bioflo IIC fermentor using the previously defined conditions. In these studies, the effects of varying agitation speed and aeration rate on expression level were studied. Aeration rate and agitation speed played a critical role in this expression system. Essentially, optimal heterologous expression of α-galactosidase was achieved using an aeration rate of 350 mM $O_2$ $h^{-1}$ and an agitation speed of 25 rpm. Using these combined media and growth parameters, we readily attained assayable expression levels of 50.9 U $mg^{-1}$ total protein in a 3-l batch fermentation.

## *Characterisation of the recombinant enzyme*

Preliminary analysis of the recombinant enzyme was undertaken using crude extracts of cell-free fermentation media from the transformant pPLF-686B3. SDS-PAGE was used to estimate the molecular weight of monomeric α-galactosidase at 75 kDa

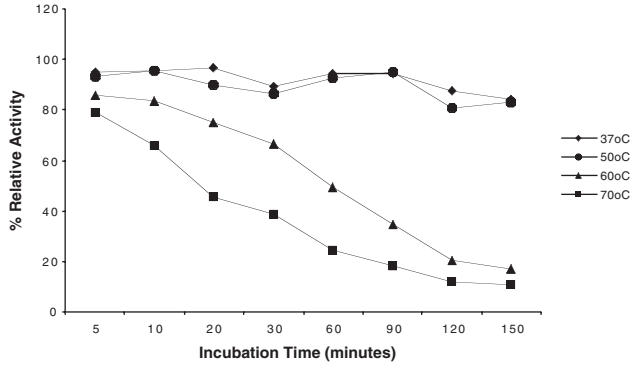


**Figure 5** Thermal stability profile of crude extracts of heterologous α-galactosidase produced in *Aspergillus awamori*. Crude extracts of post-induction media containing recombinant α-galactosidase were incubated at the indicated temperatures for time-periods ranging from 5 to 150 minutes and subsequently assayed for residual activity.

(Figure 2). This is in close agreement with previously published data [22]. The cleaved fusion peptide is also clearly visible as a unique 58-kDa protein. Densitometric analysis of this polyacrylamide gel indicated that the recombinant α-galactosidase represented approximately 10% of the total extracellular protein produced by the host.

Profiles of pH and temperature *versus* activity were prepared (Figures 3 and 4). To serve as a control, the same profiles were prepared for the endogenous α-galactosidase from the untransformed host, *A. awamori*. The optimal pH and temperature for crude extracts of the recombinant enzyme were 4.0–4.5 and 60°C, respectively. A thermal stability profile of the recombinant enzyme was also prepared (Figure 5). Approximately 65% of the original activity remained following heating at 70°C for 10 min. In contrast, a similar profile prepared for the endogenous enzyme showed that it became inactivated rapidly at temperatures above 60°C (data not shown).

## Discussion

α-Galactosidases occur widely in microorganisms. However, despite the wide occurrence of this enzyme, only a small number have been purified and extensively studied. As might be expected, research in this area has centered on sources of α-galactosidase that offer the greatest economic potential. In this study, an α-galactosidase gene from *S. cerevisiae* was cloned and expressed in an attempt to achieve economic viability *via* genetic engineering.

Filamentous fungi have been extensively employed on an industrial scale for many decades in the production of a variety of enzymes. Some industrial strains of *A. niger* have been reported to produce up to 20 g $l^{-1}$ of the enzyme glucoamylase into the fermentation medium [1]. As the molecular genetics of these organisms have been well defined, expression of α-galactosidase was attempted in *A. oryzae* and *A. awamori*. Initial attempts in *A. oryzae* at overexpression of this enzyme were prevented by undefined instabilities at a transcriptional level of the heterologous mRNA encoding α-galactosidase. Transcriptional instability of mRNA has been described as a troublesome parameter associated with filamentous fungal expression systems [18,39].

Developments in fungal expression systems have led to the use of fusion strategies whereby the gene product of interest is expressed as a chimera fused to a highly stable protein such as glucoamylase, thus overcoming instability problems [7,15,42,44]. The use of such a strategy in the fungus *A. awamori* led to the production of α-galactosidase at levels up to 51 U $mg^{-1}$ total protein following optimisation and process development. Initial small-scale work resulted in expression of the recombinant enzyme at a relatively high level of 1.65 U $mg^{-1}$ total protein. Subsequent process development involving the optimisation of media constituents resulted in an increased expression of the enzyme to 3.6 U $mg^{-1}$ total protein, and this value was further increased to 51 U $mg^{-1}$ total protein when aeration and agitation speeds were optimised in a 3-l batch fermentation. On comparison with previously published α-galactosidase expression levels, it was found that this was noteworthy in relation to previous attempts at heterologous expression of this enzyme. Expression of the enzyme from *Cyamopsis tetragonoloba* in *Hansenula polymorpha* led to the extracellular production of α-galactosidase activity up to 38 U $mg^{-1}$ total protein [11]. The α-galactosidase from a *Coffea* sp. was expressed at extracellular levels up to 33 U $mg^{-1}$ total protein [47], whereas the gene from *E. coli* was expressed only extracellularly in *Zymomonas mobilis* at 2.9 U $mg^{-1}$ total protein [45]. The intracellular expression of *E. coli* α-galactosidase in *E. coli* expression system led to production of active enzyme at levels up to 62.5 U $mg^{-1}$ total protein [45].

Scope also exists for industrial-scale optimisation work with the system described here, which could result in further increases in expression levels. Furthermore, it was demonstrated that the α-galactosidase expressed in this system represented up to 10% of the total extracellular soluble protein produced by the host organism.

An analysis of crude extracts of the recombinant enzyme estimated the pH optimum to be pH 4.0–4.5, a figure in close agreement with the reported value of pH 4.0 for the purified enzyme from *S. cerevisiae* [23]. The estimated temperature optimum of 60°C differed significantly in contrast to the results reported by Lazo *et al* [23] for *S. cerevisiae*. This high temperature optimum for *S. cerevisiae* α-galactosidase has not been reported elsewhere in the literature; however, it is of interest to note that *S. cerevisiae* possesses up to 10 different α-galactosidase genes exhibiting extensive (but not complete) nucleotide sequence homology. The protein products encoded by each of the 10 individual α-galactosidase genes have not been isolated and characterised; thus, it remains reasonable to expect that the protein products of these gene variants may have different activity properties. Of more interest, however, is the α-galactosidase produced by a closely related yeast, *Pichia guilliermondii* [4]. Purification analysis of this α-galactosidase showed it to have a temperature optimum of 70°C and to possess a remarkably similar thermal activity profile to the recombinant α-galactosidase described in this study. Cross-complementation of genes between species of *Saccharomyces* and *Pichia* has been demonstrated and subsequent investigations have shown that a high degree of homology exists between α-galactosidase genes from both species (manuscript in preparation).

The thermal stability of the heterologous enzyme is of interest also, and previous work has shown that, typically, α-galactosidases are inactivated rapidly when exposed to high temperatures for short periods [21,35]. A limited number of examples of thermal stability in the range 40–75°C have been reported for α-galactosidases from other organisms [10,24]. It was shown in this work that the recombinant enzyme was stable at 70°C for 10 min with only a loss of 35% of activity. Similar work, carried out on crude extracts of the endogenous enzyme from *A. awamori*, showed it to be inactivated rapidly at temperatures above 60°C. This is in close agreement with previously published work on an α-galactosidase from *A. awamori*, which demonstrated complete inactivation of the enzyme after 14 min at 70°C [26]. The high thermal stability of this *S. cerevisiae* enzyme may make it suitable for direct incorporation into diets whose preparation requires high temperature processing. Work is currently underway to purify and characterise the recombinant enzyme. It is hoped that through this work, the full extent and nature of the high thermal optimum and thermal stability of this enzyme will be elucidated.

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