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Overproduction of Glucoamylase by a Deregulated Mutant of a Thermophilic Mould *Thermomucor indicae-seudaticae*

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Abstract *Thermomucor indicae-seudaticae*, a glucoamylase-producing thermophilic mould, was mutagenised using nitrous acid and gamma (^{60}Co) irradiation in a sequential manner to isolate deregulated mutants for enhanced production of glucoamylase. The mutants were isolated on Emerson YpSs agar containing a non-metabolisable glucose analogue 2-deoxy-D-glucose (2-DG) for selection. The preliminary screening for glucoamylase production using starch–iodine plate assay followed by quantitative confirmation in submerged fermentation permitted the isolation of several variants showing varying levels of derepression and glucoamylase secretion. The mutant strain *T. indicae-seudaticae* CR19 was able to grow in the presence of 0.5 g l^{-1} 2-DG and produced 1.8-fold higher glucoamylase. As with the parent strain, glucoamylase production by *T. indicae-seudaticae* CR19 in 250-ml Erlenmeyer flasks attained a peak in 48 h of fermentation, showing higher glucoamylase productivity (0.67 U ml^{-1} h^{-1}) than the former (0.375 U ml^{-1} h^{-1}). A large-scale cultivation in 5-l laboratory bioreactor confirmed similar fermentation profiles, though the glucoamylase production peak was attained within 36 h attributable to the better control of process parameters. Although the mutant grew slightly slow in the presence of 2-DG and exhibited less sporulation, it showed faster growth on normal Emerson medium with a higher specific growth rate (0.138 h^{-1}) compared to the parent strain (0.123 h^{-1}). The glucoamylase produced by both strains was optimally active at 60 °C and pH 7.0 and displayed broad substrate specificity by cleaving α-1,4- and α-1,6-glycosidic linkages in starch, amylopectin, amylose and pullulan. Improved productivity and higher specific growth rate make *T. indicae-seudaticae* CR19 a useful strain for glucoamylase production.

Keywords 2-Deoxy-D-glucose · Mutagenesis · Deregulated mutant · Glucoamylase · γ-Irradiation · *Thermomucor indicae-seudaticae*

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Introduction

Glucoamylase (α-1, 4-D-glucan glucohydrolase, E.C. 3.2.1.3) has been traditionally used for hydrolysing starch and related substrates to glucose [1, 2]. The enzyme finds application in a number of food and beverage industries where glucose is preferred over other sugar substrates. Although a large number of microorganisms including bacteria, yeasts and fungi are known to produce glucoamylase, filamentous fungi particularly the *Aspergillus* spp. are the major source of glucoamylase for industrial application because of higher production levels and ease in recovery of secreted enzyme [3–5]. *Aspergillus* glucoamylase is, however, relatively less thermostable and optimally active under acidic conditions that limit its performance. Therefore, there has always been a room for improved enzymes which could suit better the processing parameters [6–8].

Screening environmental isolates is a viable option to isolate novel proteins/metabolites for specific applications. A few thermophilic fungi have been isolated and characterised with respect to glucoamylase production [9–12]. Most thermophilic fungal glucoamylases, like their mesophilic counterparts, are active in the acidic pH range. Another serious problem with environmental isolates is their low production levels. Although optimisation of culture conditions and using different fermentation strategies could help in improving the production levels to some extent, strain improvement is critical for making any fermentation process economical. Although there are a number of recent reports on molecular and genetic approaches for strain improvement [13], hardly there is any for modifying regulatory genes in industrial production strains by a genetic engineering approach [14]. Induced mutagenesis using physical or chemical mutagens followed by selection still remains a powerful strain improvement technique in industrial microbiology that has successfully been used for generating mutants with elevated productivity of metabolites, and this has greater public acceptance.

The thermophilic fungus *Thermomucor indicae-seudaticae* produces a neutral and thermostable glucoamylase, which has been suggested to offer potential advantages in starch hydrolysis [12, 15]. The glucoamylase production levels in the wild-type strain of *Thermomucor indicae-seudaticae* are low, and thus, the present investigation was carried out with the aim of isolating derepressed mutants of *T. indicae-seudaticae* to overproduce glucoamylase.

Materials and Methods

Source of the Thermophilic Fungal Culture

T. indicae-seudaticae Subrahmanyam, Mehrotra and Thirumalachar (CBS 104.75) was isolated from municipal compost at Pune, India [16]. The thermophilic mould was routinely grown on Emerson YpSs agar [17] at 40 °C. The culture was preserved at 4 °C and also in glycerol at −20 °C.

Glucoamylase Production

The spore suspension of a 4-day-old culture grown on Emerson YpSs slants was prepared by dispensing 20–30 ml of 1 N saline containing Tween 40 (0.1%, *v*/*v*) followed by shaking and filtration. Spores were counted under compound microscope using haemocytometer, and their viability was checked by plating 0.1 ml of a suitable dilution on Emerson YpSs

agar plates incubated at 40 °C for 24 h. Glucoamylase production was carried out by inoculating 250-ml Erlenmeyer flasks containing 50 ml sucrose–yeast extract medium with ~5×10^6 sporangiospores and incubating at 40 °C in an incubator shaker at 250 rpm [18]. After 48 h, the fermented broth was filtered through Whatman no. 1 filter paper, and the cell-free filtrate was used in glucoamylase assays.

Glucoamylase Assay and Protein Determination

Glucoamylase was assayed according to Kumar and Satyanarayana [12] by measuring the amount of glucose liberated by its action on soluble starch (Merck) using dinitrosalicylic acid reagent [19]. One unit of glucoamylase is defined as the amount of enzyme that liberates 1 μmol of reducing sugar as glucose per minute per millilitre under the assay conditions. For determining the intracellular glucoamylase activity, the fresh mould biomass was homogenised in phosphate buffer (pH 7.0, 50 mM) using bead beater, and the extract was centrifuged at 8,000×*g* for 10 min. The supernatant was collected and used for estimating the intracellular glucoamylase activity. One gram wet mould biomass was oven-dried to calculate the dry weight.

Soluble protein content in the cell-free culture filtrate was determined according to Lowry et al. [20] using bovine serum albumin as the standard.

Random Mutagenesis

Preparation of Culture for Mutagenesis

Sporangiospore suspension from a 4-day-old culture of *T. indicae-seudaticae* was prepared to inoculate sucrose–yeast extract broth and incubated in a rotary shaker at 40 °C and 250 rpm. After 6 h, the germinating sporangiospores were separated by centrifugation and washed twice with normal saline. A spore suspension containing approximately 10^6 germinating spores was used for mutagenesis.

Mutagenesis

A two-step mutation experiment was carried out involving nitrous acid treatment and γ-irradiation (^{60}Co). The mutant strain selected at the end of first mutation experiment served as the parent strain for the next step of mutagenesis. Nitrous acid treatment was carried out as described by Azin and Noroozi [21] by mixing the sporangiospore suspension into a 0.07 M solution of $NaNO_2$ prepared in acetate buffer (0.2 M, pH 4.5). The samples were drawn periodically from the above mixture and diluted with phosphate buffer (0.1 M, pH 7.0) to stop the reaction. The next step of mutagenesis was performed by exposing the spore suspension to ^{60}Co γ-irradiation in a dose range of 0–150 KR. During each phase of the two-step mutagenesis, the mutagenised samples were drawn at different time intervals and plated on Emerson's YpSs agar plates and incubated at 40 °C. After 24 h, the number of surviving colonies was calculated to generate the survival curve against each mutagen. A mutagen dose resulting in 99% reduction in viable population was used for isolating mutant strains.

Selection of Derepressed Mutants

The derepressed mutants were isolated by plating mutagenised spore suspension on Emerson's YpSs agar containing 0.2 g l^{-1} 2-deoxy-D-glucose (2-DG), a non-metabolisable

glucose analogue, as selection marker. Sodium deoxycholate (0.1%, *w*/*v*) was included in the isolation medium to restrict mycelial growth. The colonies growing in the presence of 2-DG were considered derepressed mutants. The individual colonies were point-inoculated to fresh starch agar plates using sterile toothpicks and incubated at 40 °C. After 24 h, the plates were flooded with Lugol's iodine (I_2 0.3% and KI 3.0%) to locate the zone of starch hydrolysis around mutant colonies. The colonies showing broader zones of starch hydrolysis were selected and quantitatively tested for glucoamylase production in 250-ml Erlenmeyer flasks as described above.

Characterization of the Mutant Strain

Morphological Examination

The mutant and parent strains were grown on Emerson's YpSs agar plates and incubated at 40 °C. The colony morphology of two strains was compared by visual and microscopic examinations. The morphological examination of mutant revealed less sporulation in the medium containing 2-DG (0.5 g l^{-1}). This observation was confirmed by preparing spore suspensions from the cultures grown in the presence and absence of 2-DG and counting the spores in a haemocytometer. The number of spores was calculated per square centimetre surface area of the agar slant. Parent strain cultivated on Emerson's YpSs agar was considered as the control.

Glucoamylase Production and Fermentation Profile

The growth and glucoamylase production profile of the mutant strain was studied by cultivating in 250-ml Erlenmeyer flasks as mentioned above for 72 h. Different parameters like sucrose consumption, biomass production and pH of the fermentation medium were analysed and compared with the parent strain. Glucoamylase production was also carried out in shake flasks at different temperatures (25 to 60 °C) and pH (3 to 10) and in the chemically defined medium (g l^{-1}: sucrose 20, K_2HPO_4 0.5, $MgSO_4$ 1.3, NH_4Cl 1.0, pH 7.0) to find out any altered growth requirement of the mutant strain. The specific growth rates of parent and mutant strains were determined by cultivating them in a 5-l autoclavable glass bioreactor (Bio Console ADI 1025, Applikon, The Netherlands).

Kinetic Studies

The crude glucoamylase preparation was partially purified by acetone precipitation (80% saturation) and used in enzyme assays. The glucoamylase assay was carried out at different temperatures (30, 40, 50, 60, 70, 80, 90 and 100 °C) and pH (3, 4, 5, 6, 7, 8, 9, 10 and 11). A number of α-linked substrates (starch, amylopectin, amylose, pullulan, dextrin, maltose, α-cyclodextrin) containing α-1,4- and α-1,6-glycosidic linkages were used in glucoamylase assays. The glucoamylase activity against the routinely used substrate, soluble starch (Merck), was considered 100 to calculate the relative enzyme activity values for different substrates.

Results

The dose–response curves of *T. indicae-seudaticae* for the two mutagens, viz. nitrous acid and γ-irradiation (^{60}Co), were generated to find out the appropriate dosage for mutant

Table 1 Glucoamylase production by 2-deoxy-D-glucose resistant mutants generated by HNO_2 treatment.

Strain	Glucoamylase production (U ml^{-1})
Parent strain	18.20
H-I	21.80
H-II	18.64
H-III	18.74
H-IV	25.25
H-V	22.08

isolation. Nitrous acid treatment led to a 99% reduction in viable mould population after 15 min, whilst a similar effect was observed with a dose of 150 KR of γ-irradiation. After nitrous acid mutagenesis, 19 colonies were found to grow on the selection medium containing the anti-metabolite 2-DG (0.2 g l^{-1}). Five of the 19 colonies showing comparatively larger zones of starch hydrolysis were tested for glucoamylase production in submerged fermentation (Table 1). One of the derepressed mutants designated H-IV secreted around 38% higher glucoamylase (25.25 U ml^{-1}) than the parent strain (18.24 U ml^{-1}) and was selected for the next step of mutagenesis. In the next step, the nitrous acid mutant H-IV was mutagenised by γ-irradiation (^{60}Co). By starch–iodine plate assay, five mutants displaying broader zones of starch hydrolysis were selected. Most of the mutant strains selected after γ-irradiation were glucoamylase hyperproducers (Table 2). The strain *T. indicae-seudaticae* CR19 produced approximately 1.8-fold higher glucoamylase (33.03 U ml^{-1}) than the parent strain. *T. indicae-seudaticae* CR19 also displayed higher anti-metabolite resistance than the nitrous acid mutant. *T. indicae-seudaticae* CR19 grew much faster in the presence of 2-DG (0.2 g l^{-1}) than *T. indicae-seudaticae* H-IV, suggesting its higher anti-metabolite resistance, as is evident from the colony size of the two mutants after 96 h of incubation at 40 °C (Fig. 1).

Characterization of the Mutant Strain

The selected mutant strain *T. indicae-seudaticae* CR19 grew slightly slow in the presence of 2-DG; however, it displayed faster growth compared to parent *T. indicae-seudaticae* on normal medium (without 2-DG). Both the parent and mutant strains, however, grew equally well on Emerson YpSs medium agar and covered whole plate within 48 h of incubation. The growth pattern and colony morphology of both strains appeared more or less similar, with young mycelia being white coloured that turned pale brown to black after 48 h due to the formation of sporangia. The microscopic examination revealed a similar mycelial growth, hyphal extension and zygospore formation in the parent and mutant strains (Fig. 2).

Table 2 Glucoamylase production by mutant strains isolated after γ-irradiation (^{60}Co).

Strain	Glucoamylase production (U ml^{-1})
Parent strain	18.95
CR3	27.70
CR8	26.54
CR11	30.15
CR19	33.03
CR21	23.34

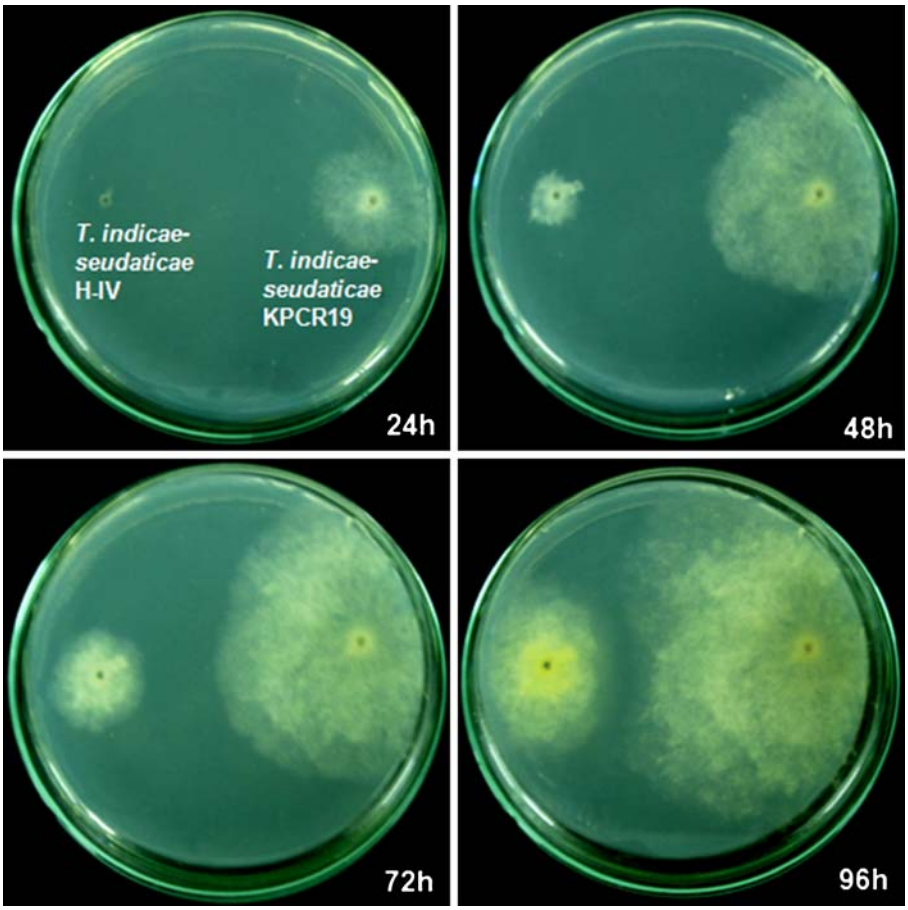

Fig. 1 Comparison of 2-deoxy-D-glucose resistance in *T. indicae-seudaticae* CR19 and *T. indicae-seudaticae* H-IV mutants during 96 h of incubation at 40 °C

T. indicae-seudaticae CR19 displayed less sporulation on 2-DG-supplemented Emerson YpSs medium, whilst faster growth and sporangiospore formation than the original strain of *T. indicae-seudaticae* were observed on normal medium. This was confirmed by sporangiospore counting; *T. indicae-seudaticae* CR19 produced approximately 1.1×10^7 sporangiospores per square centimetre of the normal Emerson YpSs agar, whilst only 5.64×10^5 spores per square centimetre were observed on Emerson YpSs agar containing 2-DG. The parent strain produced around 8.9×10^6 sporangiospores per square centimetre surface area of Emerson YpSs agar, suggesting faster growth of *T. indicae-seudaticae* CR19 on the normal YpSs medium as compared to the parent strain.

Glucoamylase Production by *T. indicae-seudaticae* CR19

T. indicae-seudaticae CR19 secreted very low glucoamylase titres during the initial 6 h of fermentation that gradually increased and reached a peak in 48 h, followed by a slight decline thereafter (data not shown). Around 32 U ml^{-1} glucoamylase titre was attained after

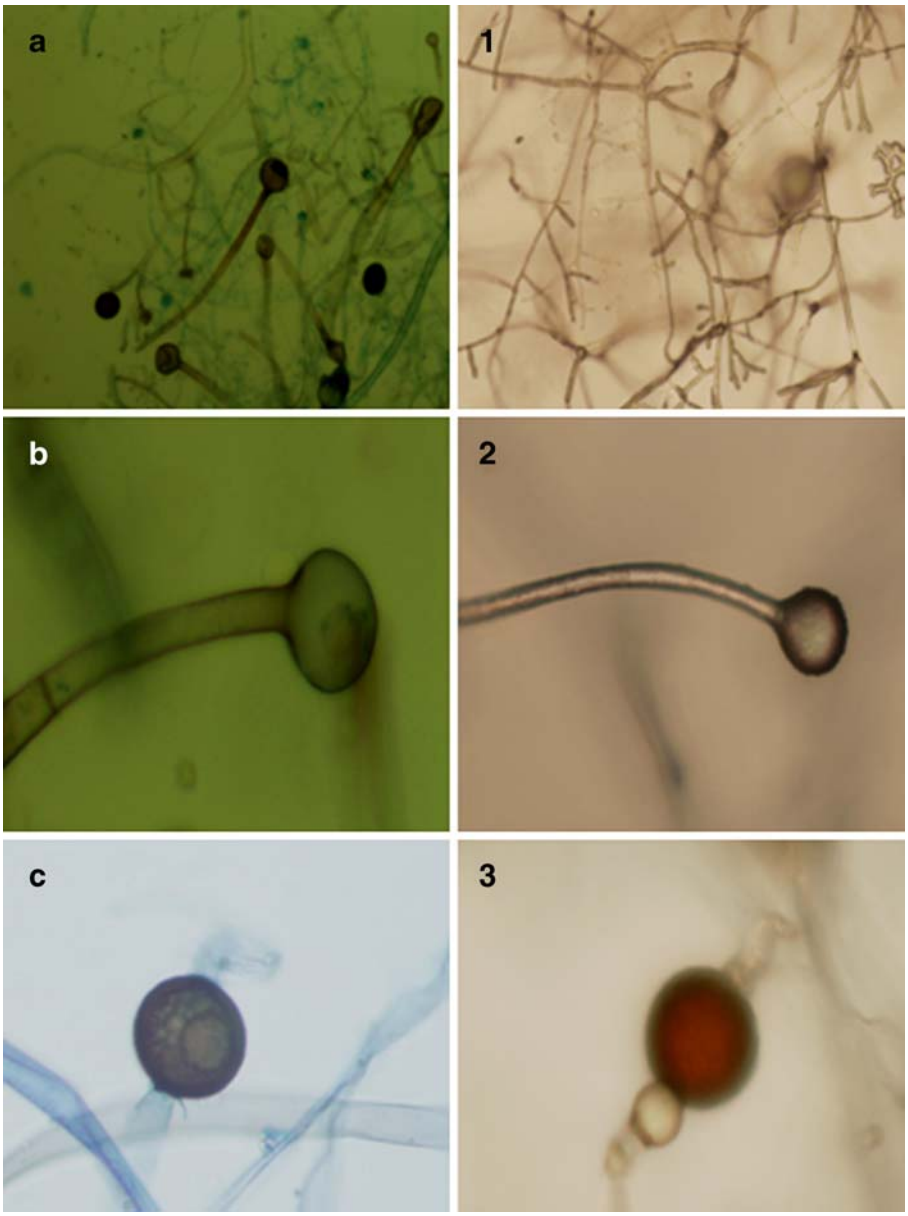

Fig. 2 Comparison of morphological features of parent and mutant strains cultivated on Emerson YpSs agar (*a–c* mycelial growth, sporangiospore and zygospore formation in parent strain, while *1–3* the same in mutant *T. indicae-seudaticae* CR19)

48 h of incubation, with a productivity of 0.67 U ml^{-1} h^{-1}. The glucoamylase titre in the intracellular fractions of *T. indicae-seudaticae* CR19 was found to be 10.95 U g^{-1} dry weight, which was almost comparable to that recorded in parent strain (10.27 U g^{-1} dry weight). As the fermentation progressed, a gradual decrease in sucrose concentration and an

increase in fungal biomass were recorded. After 72 h, fungal biomass reached a level of 6.5 g l^{-1}, whilst residual sucrose content in the fermented medium was 27% of the total initial concentration (20 g l^{-1}).

Both the parent and mutant strains displayed similar fermentation profiles, as in 250-ml Erlenmeyer flasks, when cultivated in a 5-l bioreactor. A slight variation in the pH profile of the parent and mutant strains was recorded, although a peak in the enzyme production was attained within 36 h in both (Fig. 3). The generation time of the parent strain was 5.65 h (specific growth rate 0.1225 h^{-1}), whilst that for *T. indicae-seudaticae* CR19 was 5.02 h (specific growth rate 0.138 h^{-1}).

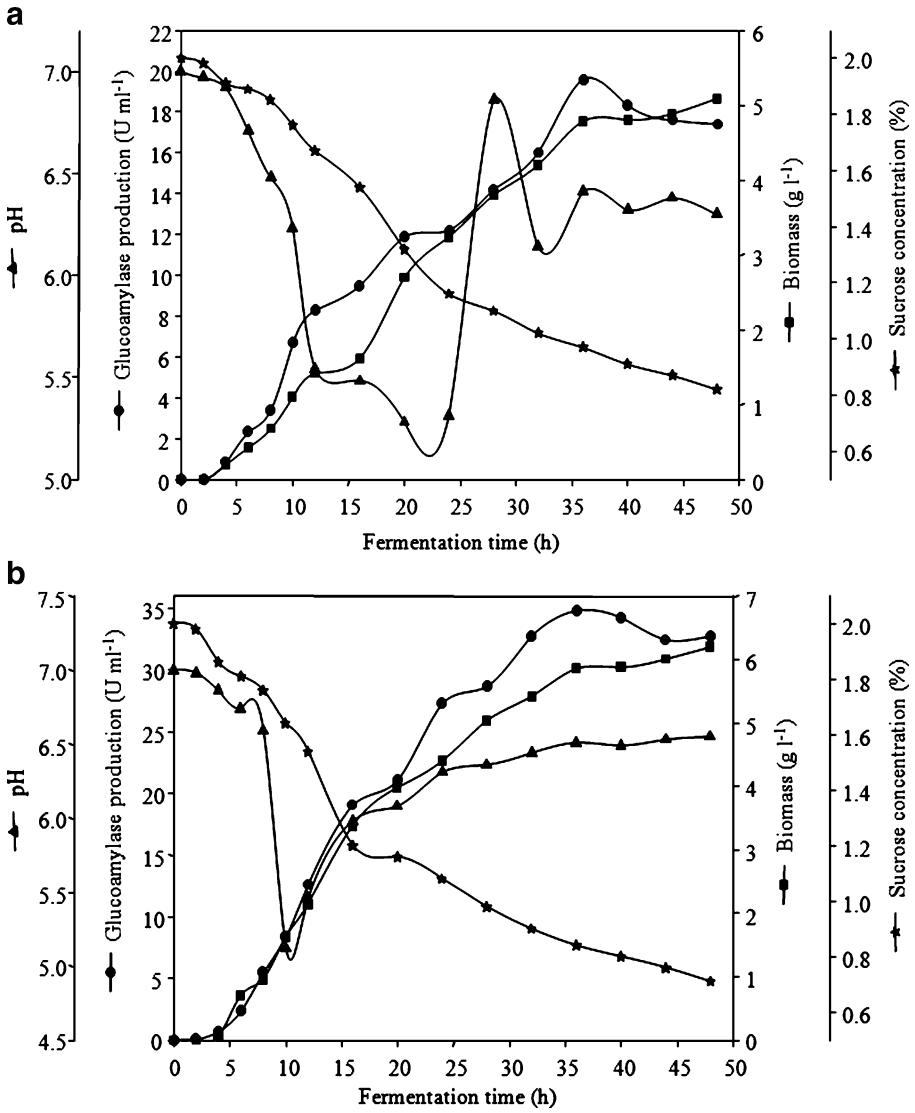

Fig. 3 Glucoamylase production profiles of parent strain *T. indicae-seudaticae* (**a**) and *T. indicae-seudaticae* CR19 mutant (**b**) in a 5-l laboratory bioreactor

Effect of Temperature and pH on Glucoamylase Production

The mould failed to grow below 25 °C and above 55 °C. The biomass and enzyme production gradually increased with a rise in temperature up to 40 °C. Although the optimum temperature for growth and glucoamylase production was 40 °C, a comparable biomass and enzyme production were recorded at 45 and 50 °C. The highest glucoamylase production was recorded when the initial pH of production medium was set at 7.0. Enzyme production declined on either sides of this pH, and there was no growth and glucoamylase production at pH 10.0 and above and <3.0 (Fig. 4).

Glucoamylase Production in a Defined Medium

Both the parent and mutant strains grew well and produced glucoamylase when cultivated in a defined medium. A glucoamylase titre of 17.8 U ml^{-1} was attained with the parent strain, whilst that with the mutant *T. indicae-seudaticae* CR19 was 27.2 U ml^{-1}.

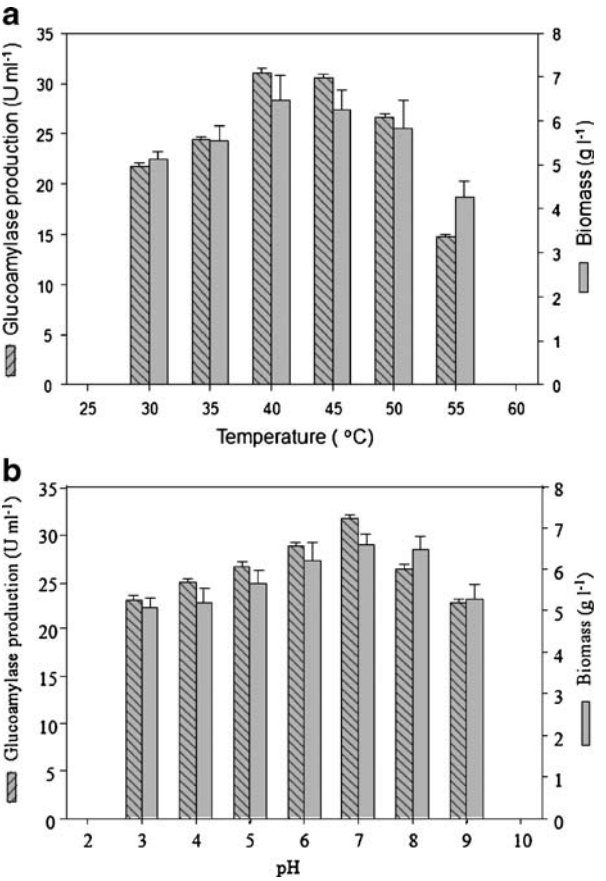

Fig. 4 Effect of temperature (**a**) and pH (**b**) on glucoamylase production by *T. indicae-seudaticae* CR19

Glucoamylase Kinetics

Glucoamylase produced by the parent and mutant strains was optimally active at 60 °C and displayed activity over a broad pH range with optimum at pH 7.0 (Fig. 5). Glucoamylase hydrolysed a variety of substrates such as starch, amylopectin, amylose, pullulan, dextrin and maltose. No activity was, however, observed on α-cyclodextrin. The relative glucoamylase activity values on different substrates are presented in Table 3.

Discussion

T. indicae-seudaticae is a potential producer of a thermostable glucoamylase that exhibits optimum activity at neutral pH, having a significant potential in starch hydrolysis [15]. Glucoamylase production in *T. indicae-seudaticae* is relatively low and it was repressed by the presence of anti-metabolite 2-deoxy-D-glucose; this was confirmed by the observation that the mould utilised glucose, but not starch, in the presence of non-metabolisable glucose analogue 2-DG even at very low concentration (0.1 g l^{-1}). Therefore, an attempt was made

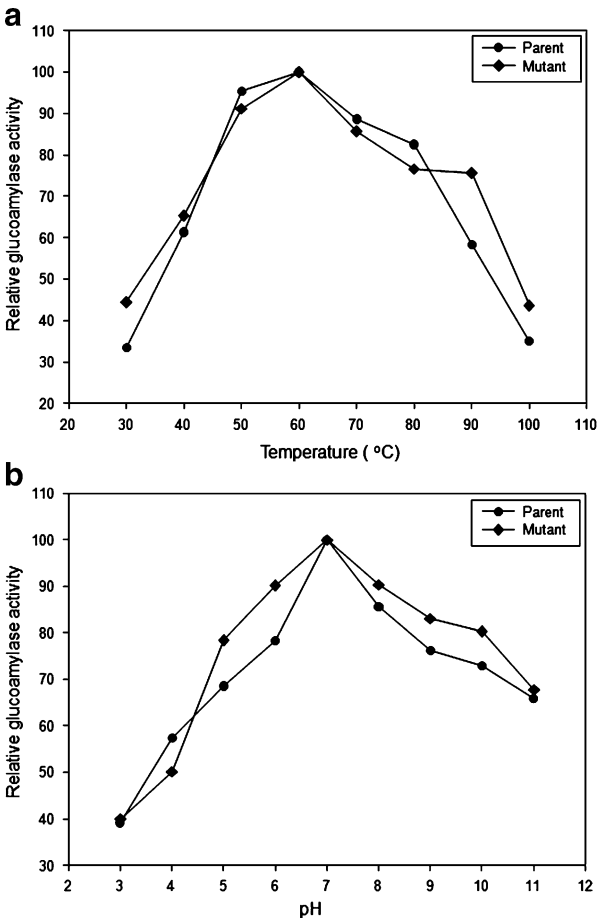

Fig. 5 Effect of temperature (**a**) and pH (**b**) on the glucoamylase activity of *T. indicae-seudaticae* CR19

Table 3 The relative activity of glucoamylases produced by *T. indicae-seudaticae* CR19 and the wild type *T. indicae-seudaticae* on different substrates.

Substrate	Relative enzyme activity (%)	
	Wild type	Mutant strain
Soluble starch[a]	100.00	100.00
Potato starch	65.98	68.93
Rice starch	59.52	61.11
Amylose	86.46	97.42
Amylopectin	92.92	92.80
Pullulan	60.09	68.97
α-Cyclodextrin	–	–

[a] Manufactured by Merck, India. All other substrates were procured from Sigma Chemicals.

to generate deregulated mutant strains of *T. indicae-seudaticae* for enhanced glucoamylase production via random mutagenesis using 2-DG for selection. The use of anti-metabolites such as 2-DG in the isolation medium enables easy screening and selection of derepressed hyperproducing mutants [22–24].

The young microbial cultures are more susceptible to the action of a mutagen, and therefore, 6-h-old germinating sporangiospores of *T. indicae-seudaticae* were used for mutagenesis. The mutants were isolated from the few surviving colonies after a strong mutagen treatment leading to high death rate (99%). The advantage of isolating mutants at low survival rate (1%) is because of the higher probability of one or several mutations in the surviving cells [25]. The increase in the size of zone of starch hydrolysis by the genetic variants surviving in the presence of 2-DG is a good indication of mutants with improved glucoamylase production. The starch–iodine assay may sometimes lead to the screening of false positives, and hence, glucoamylase hyperproduction was confirmed by cultivating the variants in submerged fermentation followed by quantitative enzyme assay.

A two-step mutagenesis enabled the selection of a glucoamylase hyperproducing mutant *T. indicae-seudaticae* CR19. The ability of *T. indicae-seudaticae* CR19 to grow on starch medium in the presence of glucose analogue 2-DG could be due to the partial derepression that might have upregulated glucoamylase production. The cultivation in submerged fermentation and quantitative enzyme assays also confirmed improved glucoamylase production by the mutant strain. The intracellular glucoamylase accumulation in both the parent and mutant strains was almost the same, whilst the latter secreted higher glucoamylase into the extracellular medium, suggesting an enhanced glucoamylase synthesis in *T. indicae-seudaticae* CR19. The colonies that survived in the presence of 2-DG exhibited a wide variation in their glucoamylase production efficiency, indicating different levels of derepression among various mutants. Some of the surviving colonies produced glucoamylase comparable to the parent strain. These colonies might have survived on the isolation medium due to their reduced cell membrane permeability to 2-DG or the low catabolite repression by 2-DG [24]. 2-Deoxy-D-glucose resistance has also been exploited for generating mutants of *Aspergillus niger* [26] and *Penicillium echinulatum* [24] with enhanced titres of glucose oxidase, and cellulase and β-glucosidase, respectively.

The mutant strain *T. indicae-seudaticae* CR19 was maintained on Emerson YpSs medium containing 2-DG and further characterised with respect to its morphology and fermentation behaviour. Like the parent strain, the mutant *T. indicae-seudaticae* CR19 produced maximum glucoamylase titre at pH 7.0 and 40 °C. A slight decline in glucoamylase titre after 48 h of fermentation in 250-ml shake flasks could be due to the depletion of nutrients and autolysis, whilst the pH drop could be attributed to the deamination reactions during fermentation. *T. indicae-seudaticae* CR19 followed a similar

fermentation profile as reported for parent strain [27], although the enzyme titre was 1.8-fold higher in the former than the latter. Ghosh et al. [28] and Rubinder et al. [29] have also reported 1.8-fold and threefold higher glucoamylase production by mutants of *Aspergillus terreus* and *Thermomyces lanuginosus* than the parent strains, respectively. *T. indicae-seudaticae* CR19 displayed a similar trend for glucoamylase production in a 5-l fermentor, but a peak in enzyme production was attained in 36 h as compared to that of 48 h in shake flasks. The parent and mutant strains, however, exhibited a slight variation in pH profile during fermentation, which could possibly be due to the altered metabolism in the latter. A reduction in fermentation time or higher yields are generally expected in bioreactors than shake flasks because of the better control of process parameters including improved aeration, proper mixing of nutrients and maintenance of pH. The parent as well as the mutant strains produced glucoamylase in the chemically defined medium, suggesting that mutation has not resulted in auxotrophy.

Glucoamylase displayed a broad substrate specificity and hydrolysed a variety of α-glucans containing α-1,4- as well as α-1,6-glycosidic linkages. A marginally higher activity of *T. indicae-seudaticae* CR19 glucoamylase on amylose suggested a mutation in the enzyme itself. Glucoamylase was not active on α-cyclodextrin, which could be due to the cyclic nature of the substrate. This observation is consistent with the glucoamylase of *Paecilomyces varioti* AHU 9417, which did not exhibit activity on β-cyclodextrin [30]. The extracellular glucoamylase of *Lyophyllum shimeji* hydrolysed a variety of substrates, but not cyclodextrin [31]. On the contrary, β-cyclodextrin exhibited a slight inhibitory effect on *Trichoderma reesei* glucoamylase [32]. A high relative glucoamylase activity on amylopectin and pullulan indicated that *T. indicae-seudaticae* glucoamylase is capable of hydrolysing α-1,6-glycosidic bonds. The use of such an enzyme during commercial starch processing could eliminate/reduce the additional requirement of de-branching enzymes like pullulanase and/or isoamylase to hasten starch hydrolysis.

The mutant strain *T. indicae-seudaticae* CR19 displayed good stability in morphology and glucoamylase production on repeated sub-culturing. This is a glucoamylase hyper-producing strain that overcomes the repressive effect of 2-DG. Furthermore, glucoamylase produced by the mutant strain retained the desirable features like optimum activity at neutral pH, thermostability and broad substrate specificity, which benefit starch processing [15]. *T. indicae-seudaticae* CR19 is a useful strain due to its higher enzyme productivity and specific growth rate than the parent strain.

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