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Research Article

Effect of dimorphic regulation on heterologous glucose oxidase production by *Mucor circinelloides*

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Abstract

The production of *Aspergillus niger* glucose oxidase (GOX) and native amylase by the recombinant *M. circinelloides* KFA199 strain under conditions of dimorphic growth was investigated. The recombinant KFA199 strain was compared to its parental ATCC 1216b strain and a wild-type CBS 232.29 strain under similar morphology-controlled conditions. Cultivation in Vogel's medium supplemented with ergosterol/Tween-80 and sparged with nitrogen gas was most suitable for yeast-like biomass production under anaerobic conditions. Anaerobic growth was characterized by high levels of ethanol formation and linear growth rates of 0.24–0.05/h, indicating metabolic stress. Subsequent to anaerobic growth, cultures were shifted to aerobic conditions to induce aerobic mycelial growth. GOX produced by the recombinant KFA199 after the shift to aerobic conditions was poorly secreted and accumulated intracellularly to 0.56 U/ml$_{culture}$. Amylase production by the KFA199, ATCC12b and CBS 232.29 strains was determined during growth on starch after the shift to aerobic culture. Growth-associated amylase production by the ATCC 1216b (0.63 U/ml$_{culture}$) and wild-type CBS 232.29 (0.33 U/ml$_{culture}$) strains was substantially higher than by the recombinant KFA199 strain (0.07 U/ml$_{culture}$), which may be related to the leucine auxotrophy of the transformation host, or genetic changes induced during transformation of the KFA199 strain.

Keywords: ***Mucor circinelloides*; glucose oxidase; dimorphic fungi**

Introduction

Filamentous fungi are commonly used in the industrial production of pharmaceuticals, bulk chemicals and enzymes (Alexopoulos and Mims, 1979). Various wild-type and recombinant strains of *Aspergillus* and *Trichoderma* have been used as hosts for the production of heterologous proteins (Nevalainen *et al*., 2005). However, the mycelial morphology presents various engineering challenges during submerged fermentations (Wang *et al*., 2003). The presence of mycelia in fermentations results in highly viscous broths, which affects agitation, pumping and supply of oxygen to cultures. It also causes non-ideal mixing of the broth and poor oxygen and nutrient transfer to the fungi, which leads to lower product yields (Li *et al*., 2000). For this reason, yeast morphology is preferred by the fermentation industry because it allows higher biomass levels in submerged cultivation and easier separation of cells from the medium (Papp *et al*., 2006).

Dimorphic fungi represent a potential solution to problems associated with mycelial morphology due to their morphological variations in response to environmental variables (Orlowski, 1991). The morphology of dimorphic fungi such as *M. circinelloides*, a zygomycete in the order Mucorales, can be controlled in the yeast-like or filamentous form by means of the culture conditions, in particular through the presence of fermentable hexose and gas atmospheres (McIntyre, 2002; Wolff *et al*., 2002).

M. circinelloides can be cultivated in the yeast-like morphology under anaerobiosis to obtain sufficient biomass under lower medium viscosities, followed by a switch to aerobic conditions that induce filamentous growth (Lübbehüsen *et al*., 2003a). Anaerobic conditions for yeast-like morphology can be maintained by sparging with pure nitrogen or a gas mixture of carbon dioxide and nitrogen (Lübbehüsen *et al*., 2003b). Medium supplementation with ergosterol and Tween 80 (E/T80) has been found necessary for growth during anaerobiosis when sparging with nitrogen only (Lübbehüsen *et al*., 2003b). Exposure to trace amounts of oxygen during anaerobiosis induces the formation of filaments (Phillips and Borgia, 1985). However, during anaerobic growth, the yeast-like *Mucor* utilizes the hexose in the medium for growth and ethanol formation (Lübbehüsen *et al*., 2004), which may prove unfavourable for biomass yields and heterologous protein production. Various strains of the dimorphic fungus *Aureobasidium pullulans* (Thornewell *et al*., 1995), *Arxula adeninivorans* (Wartmann *et al*., 2002), *Yarrowia lipolytica* (Madzak *et al*., 2004) and *Mucor circinelloides* (Syn. *racemosus*) (Wolff and Arnau, 2002) have been studied as hosts for production of heterologous proteins (Larsen *et al*., 2004) and carotenoids (Papp *et al*., 2006).

The present study quantified the production of heterologous glucose oxidase (GOX) and native amylase by the recombinant *M. circinelloides* KFA199 during the control of dimorphic behaviour, in comparison to the parental host (ATCC 1216b) and a wild-type *Mucor* strain (CBS 232.29) with a different genetic background. The physiology of these strains during bioreactor cultivation with morphological control was also investigated.

Materials and methods

Micro-organisms

The recombinant strain *M. circinelloides* KFA199 was provided by Jose Arnau, Department of Fungal Biotechnology, Biotechnology Institute, Hørsholm, Denmark. The recombinant strain was derived by inserting the glucose oxidase (E.C. 1.1.3.4) gene (*gox*) from *A. niger* with the full-length (741 bp) glyceraldehyde phosphate dehydrogenase (*gpd1*) promoter of *M. circinelloides* into the chromosome of *M. circinelloides* R7B (ATCC 90 680, a leucine auxotrophic derivative of ATCC 1216b) to yield strain UPO1171 (GOX^+ phenotype; $leuA^+$, *crgA*) (Larsen *et al*., 2004). Strain UPO1171 was subjected to a series of random mutagenesis and screening steps, to derive *M. circinelloides* strain KFA199, with the same genotype and *gpd1* promoter, but improved GOX production (Jose Arnau, personal communication). *M. circinelloides* ATCC 1216b was obtained from the American Type Culture Collections. The wild-type strain CBS 232.29 of *M. circinelloides janssenii* was acquired from Stellenbosch University culture collection.

Cultivation media

Various media were used for conducting shake-flask experiments: YPG (McIntyre *et al*., 2002); YNB without amino acids (6.7 g/l yeast nitrogen base, 1.5 g/l ammonium sulphate; Difco); Hansson's medium (Hansson and Dostalek, 1988); and Vogel's medium with all the carbon sources described by McIntyre *et al*. (2002). The carbon sources galactose, gluconic acid and lactone were screened in Vogel's medium. All carbon sources were filter-sterilized and used at 10 g/l and 20 g/l concentrations. Biotin solution (0.5 mg/ml) was prepared in distilled water. Addition of two or three drops of 10 M NaOH to biotin solution ensured complete dissolution. The ergosterol/Tween 80 stock (500×) solution was prepared by dissolving 2 g ergosterol in 100 ml ethanol. Subsequently, 42 g Tween 80 was added and the solution was made up to 200 ml by adding ethanol. This was stored at −20 °C. For anaerobic growth, Vogel's medium was supplemented with ergosterol/Tween 80 solution at final concentrations of 0.02 and 0.42 g/l, respectively.

Preparation of spores

The recombinant strain KFA199 was initially grown on YNB plates (0.67% w/v yeast nitrogen base, 0.15% w/v ammonium sulphate, 2% w/v glucose, 2.5% w/v agar) to select for leucine prototrophic (leu^+) transformed colonies, to ensure that only cells with the *gpd1* $_P$*-gox* gene construct were used for sporulation in rice flasks. Sporulation and storage of leu^+ KFA199 was carried out as described by McIntyre *et al*. (2002). Sporulation of the parental *Mucor* ATCC 1216b strain was done as described by Lübbehüsen *et al*. (2004). The

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wild-type strain CBS 232.29 was grown on malt extract agar plates for sporulation as described by McIntyre *et al*. (2002).

Shake-flask conditions

Shake-flask studies were conducted to determine the enzyme and biomass production in Vogel's (Lübbehüsen *et al*., 2003b), Hansson's (Hansson and Dostalek, 1988), YPG (Lübbehüsen *et al*., 2003b) and YNB (Papp *et al*., 2006) media. In the cases of YPG and YNB, 60 ml medium was autoclaved in 250 ml baffle Erlenmeyer flasks at 121 °C for 15 min. For Vogel's medium, the carbon source (usually 20 g/l), casamino acids and glutamate, were autoclaved, and Vogel's salts, vitamin and biotin solutions were filter-sterilized and added afterwards to make up the final volume to 60 ml. The media was inoculated with 10^6 spores/ml medium. Shake flasks were incubated at 26 °C on an orbital shaker at approximately 100 rpm for up to 140 h.

Fermentations

Glucose solution (500 ml) was autoclaved in the fermenter vessel at 121 °C for 15 min. Other medium components for Vogel's medium were added after filter-sterilizing through a 0.22 μm filter. For anaerobic growth, ergosterol and Tween 80 (E/T80) were injected into the fermenter to reach a fermentation volume of 700 ml. Fermenters were inoculated with spore solution to a concentration of 1×10^6 spores/ml. During 'shift fermentations', cultures were shifted from anaerobic to aerobic conditions once they had reached the stationary phase. At the shift, 200 ml 4.5× medium (with either 20 g/l glucose or 10 g/l starch) was added to make up the total fermentation volume to 900 ml.

Cultivation conditions

All the strains were cultivated in shift fermentations to ensure the yeast-like morphology during initial biomass production under anaerobic conditions, followed by mycelial growth during aerobic conditions for enzyme production. The morphology, physiology and enzyme production of the recombinant strain, its parental strain and the wild-type strain were compared on the basis of cell growth, productivities and by-product formation. Fermentations were performed in 1.3 l fermenter (Bioflow 110 Non-Jacketed Vessels, New Brunswick Scientific Co., NJ, USA) with a working volume of 900 ml. The impellor arrangement consisted of one six-blade Rushton turbine fixed above a pitched blade impellor (New Brunswick Scientific Co.). Anaerobic fermentations were conducted by using nitrogen (99.99% N_2, instrument grade, Afrox, South Africa) or mixtures of gases containing 30% carbon dioxide and 70% nitrogen. When specified, a Model 1000 CRS oxygen trap (Chromatography Research Supplies, USA) was used to remove traces of oxygen from the sparge gas. During anaerobiosis in shift fermentations, nitrogen was sparged into the culture at a rate of 0.3 vvm ($volume_{gas}.volume_{culture}^{-1}.minute^{-1}$) and the agitation rate was maintained at 200 rpm. In the aerobic phase, air was sparged at 1 vvm and the agitation was increased gradually at 100 rpm/h from 200 to 500 rpm over 3 h. The DO levels were monitored using a Mettler Toledo oxygen probe. Gas flow rates were controlled by rotameters. pH was controlled by addition of 2 M KOH or 2 M HCl. Antifoam A (Sigma) at a concentration of 2 ml/l was pumped in to suppress foaming when deemed necessary. Exhaust gas from the fermenter was analysed by an Innova 1313 fermentation monitor (Innova Airtech Instruments, Copenhagen, Denmark).

Fermentation sampling

During fermenter cultures, 25 ml samples were taken every 4 h, of which 20 ml was used for dry weight estimation. Metabolite estimations and glucose and enzyme assays were conducted with 5 ml of the sample centrifuged at 6500 rpm for 3 min and the supernatant was stored at −20 °C until further analyses. For sampling the mycelial broth, 25 ml of the sample was filtered through Miracloth (Calbiochem, cat. no. 475 855) for dry weight determination, and the filtrate was stored at −20 °C until further analysis.

Dry weight estimation

For estimating dry weights of yeast cells, duplicates of 10 ml culture broth was filtered through 0.45 μm acetate filters and rinsed twice with RO (reverse osmosis) water. Mycelial dry weights were determined by filtering duplicates of approximately

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10 ml culture broth through Miracloth filters. The Miracloth filters were rinsed twice and squeezed gently to remove excess liquid. The filters were dried at 65 °C until constant weights were attained. All dry weight determinations were done in duplicate. Absorbency measurements for yeast cultures were performed at 600 nm.

Metabolite and substrate concentrations

Glucose, glycerol and ethanol concentrations in culture supernatant were measured by HPLC, using a Biorad Aminex HPX-87H column at a temperature of 45 °C, and a mobile phase of 0.005 M sulphuric acid pumped at 0.6 ml/min. For detection, a Waters refractive index detector and Waters PDA UV detector were used (Waters Corp., USA). Starch was measured by the phenol–sulphuric acid assay, as described by Dubois *et al.* (1956).

Cytoplasmic and membrane protein extraction

Cytoplasmic and membrane protein fractions were extracted from mycelia. Mycelial samples were filtered through Miracloth filters, the residual mycelia was washed twice with RO water, squeezed to remove excess water and stored at −20 °C. The cells were ground under liquid nitrogen using a mortar and pestle. 0.3 g ground cell mass was placed in an Eppendorf tube, 1 ml extraction buffer (50 mM sodium phosphate buffer, pH 7, 1 mM EDTA, 20 mM phenylmethylsulphonylfluoride (PMSF), 0.1% Triton X-100) was added with vigorous mixing, incubated on ice for 10 min and centrifuged at 14 000 rpm for 15 min at 4 °C. The supernatant (cell-free extract) was used for protein and enzyme assays.

Enzyme assays

Glucose oxidase concentration was estimated using a coupled-enzyme assay with *O*-dianisidine (Larsen *et al.*, 2004). Amylase activity in liquid samples was determined by the 3,5-dinitrosalicylic acid (DNS) assay (Miller, 1959). The absorbance of samples was read at 540 nm (Bogar *et al.*, 2003).

Protein determination

The protein concentration was determined by using a BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as the standard (Smith *et al.*, 1985).

Cell morphology

Wet mounts of yeast and mycelia were prepared and observed under the microscope at ×40 magnification.

Results

The production of heterologous GOX and native amylase by the dimorphic recombinant *M. circinelloides* KFA199 strain in morphology-controlled bioreactor cultivation was studied. GOX and amylase production by *Mucor* KFA199 was compared with its parental strain ATCC 1216b and a wild-type strain CBS 232.29, both producing amylase under similar morphology-controlled conditions.

Cultivation medium

During shake-flask cultivation of the KFA199 strain, the highest extracellular GOX concentration of 0.34 ± 0.02 U/ml$_{culture}$ was obtained in Vogel's medium, followed by Hansson's medium (0.25 ± 0.02 U/ml$_{culture}$), YNB (0.10 ± 0.02 U/ml$_{culture}$) and YPG (0.10 ± 0.02 U/ml$_{culture}$). Varying the glucose concentration in the medium between 10 and 20 g/l did not affect GOX production. The maximum biomass yield of the KFA199 strain in Vogel's medium containing 20 g/l glucose was 0.37 g/g glucose (data not shown). Replacement of glucose in Vogel's medium with 10 g/l and 20 g/l lactone resulted in increased GOX production of up to 0.44 U/ml$_{culture}$ and 0.9 U/ml$_{culture}$ respectively (data not shown). Glucose in Vogel's medium was replaced with 10 g/l starch for the induction of amylase production during the cultivation of wild-type CBS 232.39 strain in shake flasks. The highest amylase activity of 10 U/ml$_{culture}$ was obtained in Vogel's medium with a corresponding biomass concentration of 5.8 g/l (data not shown).

Morphological control during anaerobic cultivation on glucose

The growth and enzyme production of the dimorphic recombinant strain of *Mucor circinelloides* KFA199 was studied under controlled conditions

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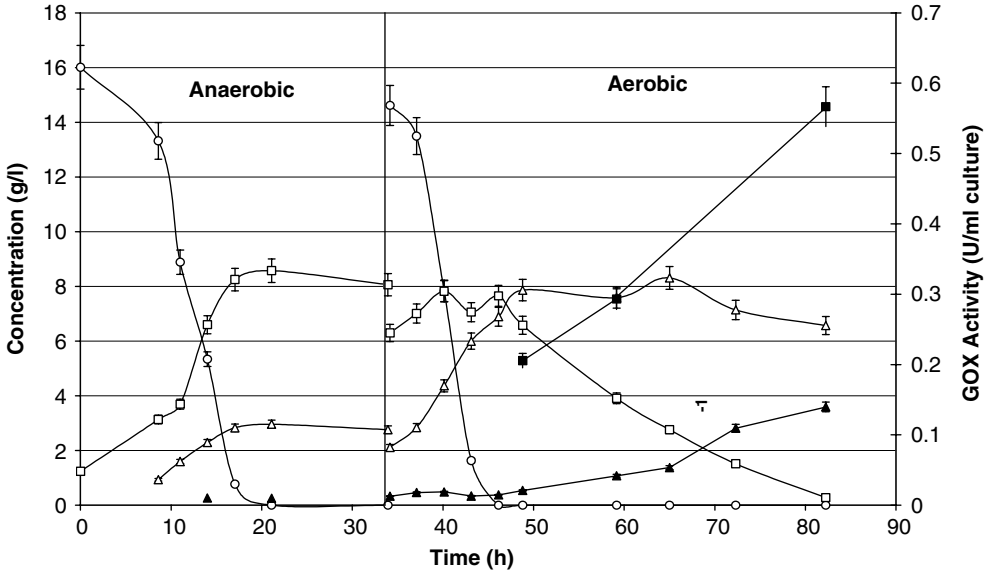

Figure 1. Growth and glucose oxidase production by recombinant strain KFA199 during fermentation with glucose as the carbon source; ○, glucose concentration; △, biomass concentration; □, ethanol concentration; ▲, extracellular GOX concentration, ■, intracellular GOX concentration

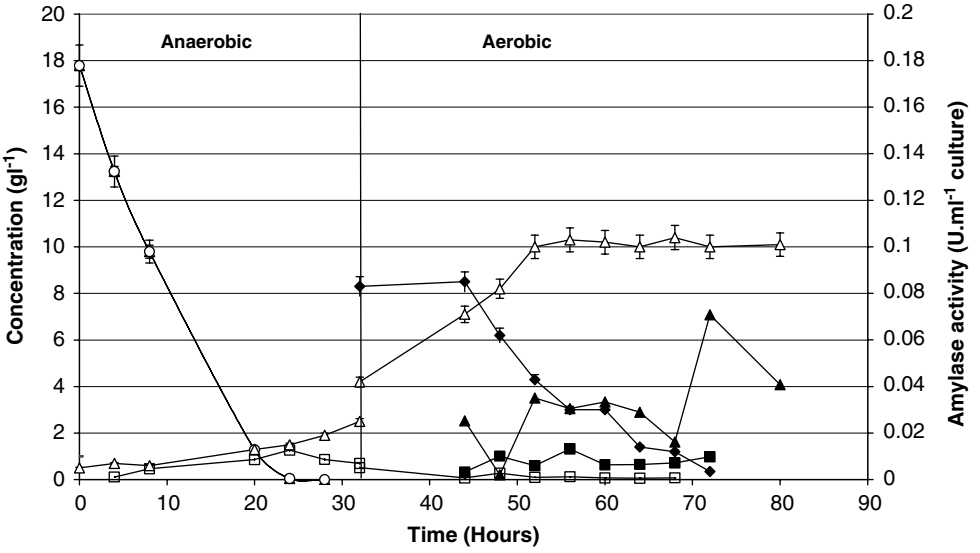

Figure 2. Growth and amylase production by recombinant strain KFA199 during fermentation with starch as the carbon source during aerobic growth; ○, glucose concentration; △, biomass concentration; □, ethanol concentration; ♦, starch concentration; ▲, extracellular amylase concentration; ■, intracellular amylase concentration

in a 1 l fermenter and compared to the parental and wild-type strains. The fermentations were operated under anaerobic conditions to obtain yeast-like growth on glucose, followed by an aerobic phase for mycelial growth on glucose or starch (Figures 1–4). Fermentation was conducted under anaerobiosis for 32–34 h, followed by the switch to aerobic conditions. During aerobic growth the medium was supplemented with either glucose for recombinant GOX production by the KFA199 strain (Figure 1) or starch for amylase production by the KFA199 (Figure 2), ATCC

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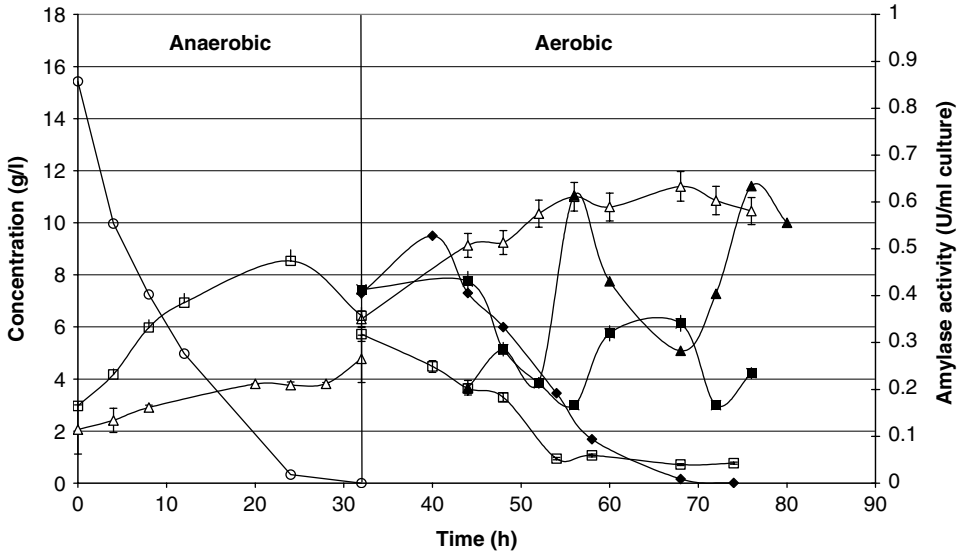

Figure 3. Growth and amylase production by the parental strain ATCC 1216b during fermentation with starch as the carbon source during aerobic growth; ○, glucose concentration; △, biomass concentration; □, ethanol concentration; ♦, starch concentration, ▲, extracellular amylase concentration; ■, intracellular amylase concentration

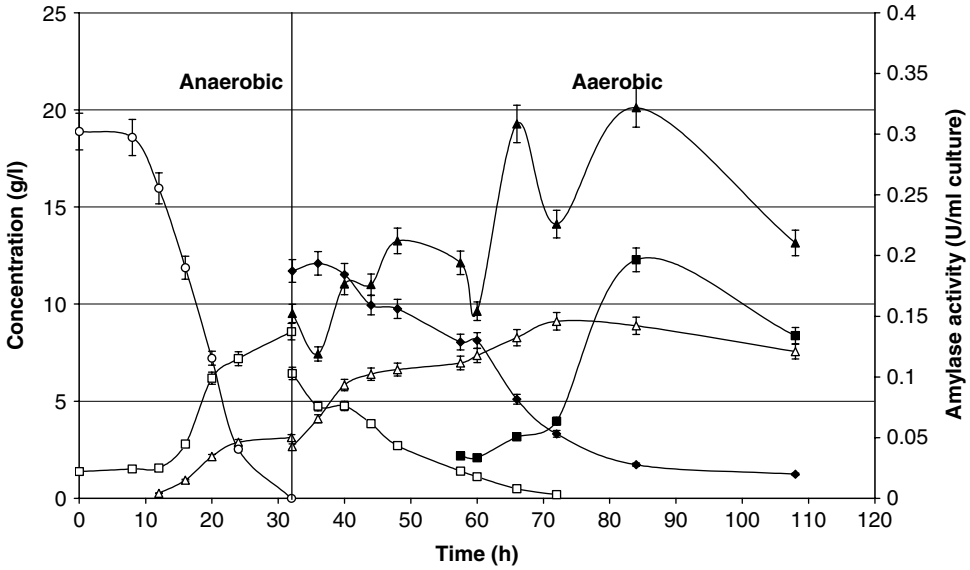

Figure 4. Growth and amylase production by wild-type strain CBS 232.29 during fermentation with starch as the carbon source during aerobic growth; ○, glucose concentration; △, biomass concentration; □, ethanol concentration, ♦, starch concentration; ▲, extracellular amylase concentration; ■, intracellular amylase concentration

1216b (Figure 3) and CBS 232.29 (Figure 4) strains. Fermentations were continued until 80–110 h of incubation (Figures 1–4).

The suitability of using a gas atmosphere, consisting of N_2, a N_2/CO_2 mixture, N_2 gas with an O_2 trap or N_2 with ergosterol/Tween 80 (E/T80) medium supplements for the maintenance of *Mucor* yeast-like growth under anaerobiosis was compared for the recombinant and the wild-type strains. The yeast-like morphology, necessary for obtaining higher biomass concentrations, could be maintained by using N_2 gas passed

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Table 1. Effect of various gas atmospheres and medium supplements on growth rates (μ_{max}, h^{-1}) and biomass yields ($Y_{X/S}$) of the recombinant (KFA 199) and the wild-type (CBS 232.29) strains

Strain	Gas	Supplement	μ_{max}, h^{-1}	$Y_{X/S}$	Morphology
KFA199	N_2	E/T80	0.140 ± 0.020	0.16	Yeast-like
KFA199	N_2 (trap)	—	0.0414	0.10	Yeast-like
KFA199	CO_2/N_2	—	0.0340 ± 0.004	0.09	Yeast-like
CBS 232.29	N_2	—	0.0750 ± 0.004	0.14	Yeast-like/filamentous
CBS 232.29	N_2 (trap)	—	0.0863	0.10	Yeast-like
CBS 232.29	CO_2/N_2	—	0.090 ± 0.016	0.07	Yeast-like
CBS 232.29	N_2	E/T80	0.226 ± 0.015	0.19	Yeast-like

Table 2. Growth rates (μ_{max}, h^{-1}), biomass yields ($Y_{X/S}$, g/g) and ethanol yields ($Y_{E/S}$, g/g) of recombinant and wild-type strains during anaerobic and aerobic growth in shift fermentations

	Anaerobic growth on glucose			Aerobic growth on glucose or starch		
Strain	Logarithmic growth rate (μ_{max}, h^{-1})	Biomass yield ($Y_{X/S}$, g/g)	Ethanol yield ($Y_{E/S}$, g/g)	Logarithmic growth rate (μ_{max}, h^{-1})	Biomass yield ($Y_{X/S}$, g/g)	Ethanol yield ($Y_{E/S}$, g/g)
KFA 199 (glucose–glucose)	0.14 ± 0.02	0.16 ± 0.02	0.42 ± 0.02	0.12 ± 0.01	0.31 ± 0.01	0.15 ± 0.04
		0.019 ± 0.02*			0.33 ± 0.05*	0.33 ± 0.18*
KFA 199 (glucose–starch)	0.14 ± 0.02	0.16 ± 0.02	0.42 ± 0.02	0.16 ± 0.01	0.23 ± 0.03	Not produced
		0.019 ± 0.02*			0.26 ± 0.04*	
ATCC 1216b (glucose–starch)	0.05 ± 0.03	0.22 ± 0.05	0.39 ± 0.1	0.28 ± 0.07	0.47 ± 0.03	Not produced
		0.026 ± 0.01*			0.39 ± 0.03*	
CBS 232.29 (glucose–starch)	0.24 ± 0.02	0.19 ± 0.06	0.48 ± 0.19	0.11 ± 0.03	0.40 ± 0.1	Not produced
		0.022 ± 0.07*			0.17 ± 0.05*	

* Cmol yield for the recombinant, parental and the wild-type strains, calculated as cell dry weight produced (in Cmol) over substrate consumed, where substrate was the sum of sugar or starch and ethanol (in Cmol) used.

through an O_2 trap, which removed traces of O_2 from N_2 gas. As reported by Lübbehüsen *et al*. (2003b), the use of a N_2/CO_2 mixture or N_2 gas supplemented with E/T80 also maintained the *Mucor* strains in the yeast-like morphology (Table 1). However, the highest growth rate during yeast-like growth on glucose under anaerobic conditions by KFA199 strain (0.14 ± 0.02/h) was obtained during cultivation of in Vogel's medium supplemented with E/T80 and sparged with N_2. This was higher than the anaerobic growth rates of 0.041/h and 0.034 ± 0.004/h, obtained with the KFA199 strain during the use of N_2 passed through the O_2 trap or the N_2/CO_2 gas mixture, respectively (Tables 1, 2). The maximum yeast-like cell concentration attained during anaerobic cultivations on glucose of the KFA199 recombinant strain was 2.75–2.96 g/l (Figures 1 and 2). Anaerobic cultivations of the wild-type strain CBS 232.29 on glucose, sparged with N_2 and supplemented with E/T80, attained higher growth rates (0.226 ± 0.015/h) than cultivation using N_2, the N_2/CO_2 mixture or N_2 gas passed through an O_2 trap (0.0750 ± 0.004/h, 0.0863/h and 0.090 ± 0.016/h, respectively; Tables 1, 2). N_2 gas that was not cleared of traces of O_2 or not supplemented with E/T80 resulted in filament formation by the wild-type *Mucor* during anaerobiosis (Table 1). The maximum yeast-like cell concentration attained with the CBS 232.29 strain during anaerobic growth on glucose was 3.1 g/l (Figure 2). Anaerobic cultivation of the parental ATCC 1216b strain on glucose, sparged with N_2 and supplemented with E/T80, resulted in a low growth rate of 0.05 ± 0.03/h, and a maximum yeast-like cell concentration of 3.81 g/l (Table 2, Figure 2). The anaerobic growth rate

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Table 3. Intracellular and extracellular glucose oxidase production during various fungal fermentations

Species	GOX concentrations (U/ml$_{culture}$)		Reference
	Intracellular	Extracellular	
M. circinelloides KFA 199^{++}	0.56	0.14	Present data
Aspergillus niger^{+}	1.5	0.8	Traeger *et al.* (1991)
Hansenula polymorpha^{++}	76*	445*	Hodgkins *et al.* (1993)
Penicillium variable (P16)$^{+}$	—	19	Petruccioli *et al.* (1995)
Aspergillus niger^{+}	850	—	Kona *et al.* (2001)
Saccharomyces cerevisiae^{++}	5	100	Malherbe *et al.* (2003)
Penicillium variable (NRRL 1048)$^{++}$	0.96	—	Pulci *et al.* (2004)

* Concentrations (IU/ml).
$^{+}$ Wild-type strains.
$^{++}$ Recombinant strains.

Table 4. Extracellular amylase production during different fungal fermentations

Species	Extracellular amylase concentrations		Reference
	U/ml$_{culture}$	U/g$_{biomass}$	
M. circinelloides KFA 199^{++}	0.07	6.19	Present data
M. circinelloides ATCC 1216b^{+}	0.63	48.46	Present data
M. circinelloides CBS 232.29^{+}	0.33	36.66	Present data
Thermomyces lanuginosus^{+}	39.1	2522	Arnesen *et al.* (1998)
Aspergillus niger^{++}	28	1120	Wallis *et al.* (1999)
Aspergillus oryzae^{++}	80	2285	Bhargava *et al.* (2003)

$^{+}$ Wild-type strains.
$^{++}$ Recombinant strains.

of the wild-type CBS 232.29 strain was therefore higher than the growth rates of the recombinant KFA199 and parental ATCC 1216b strains (Table 2).

The biomass yield of 0.22 ± 0.05 g/g, obtained during anaerobic cultivation on glucose of the ATCC 1216b strain was higher than the biomass yields of the wild-type CBS 232.29 (0.19 ± 0.06 g/g) and the recombinant KFA199 (0.16 g/g $\pm$ 0.02) strains (Figure 1, Table 2). Comparable yields of ethanol were obtained during anaerobic cultivations of the recombinant, parental and the wild-type *Mucor* strains. The highest ethanol yield of 0.48 ± 0.19 g/g was obtained with the CBS 232.29 strain, followed by 0.42 ± 0.02 g/g for the recombinant KFA199 strain and 0.39 ± 0.1 g/g for the parental CBS 232.29 strain (Table 2).

Aerobic cultivation on glucose for GOX production

Introduction of air into the cultivation medium transformed the yeast-like cells of *Mucor* into mycelial forms. The recombinant KFA199 mycelia grew exponentially for 50 h to reach the stationary phase and maximum mycelial concentration of 8.3 ± 0.01 g/l. At 65 h the stationary phase was followed by the cell lysis phase (Figure 1). Glucose, replenished at the start of the aerobic phase, was rapidly consumed during the exponential growth of mycelia and its depletion caused the mycelia to enter into the stationary phase of growth (Figure 1). The growth rate and biomass yield of the recombinant strain during aerobic cultivations were 0.12 ± 0.01/h and 0.31 ± 0.01 g/g, respectively (Table 2). The glucose consumption rate by the recombinant KFA199 strain during aerobic growth on glucose (1.35 g/g/h) was substantially higher than during anaerobic growth on glucose (0.60 g/g/h) (Table 2, Figure 1). Ethanol formation by KFA199 was observed during the initial 6 h of aerobic cultivation on glucose, at a yield of 0.15 g/g. Ethanol produced during GOX fermentations was depleted after 40 h of incubation (Figure 1).

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The recombinant strain KFA199 showed intra- and extracellular formation of GOX during aerobic cultivations on glucose, while GOX production during anaerobic cultivation was very low (Figure 1). The mycelial forms of the recombinant strain produced low levels of extracellular GOX from the start of aerobic cultivations, whereas the substantial intracellular build-up of GOX occurred during the 50–80 h of mycelial growth (Figure 1). The maximum concentrations of intracellular and extracellular GOX obtained at the end of fermentation (80 h) were 0.56 $U/ml_{culture}$ and 0.14 $U/ml_{culture}$, respectively (Figure 1). The recombinant GOX production by *Mucor* was lower than other established GOX-producing systems (Table 3). No GOX production was detected during the parental ATCC 1216b and wild-type strain cultivations (data not shown).

Amylase production and *Mucor* physiology during aerobic growth on starch

The recombinant KFA199 strain, its parental strain and a wild-type *Mucor* strain transformed into mycelial forms at the initiation of aerobic cultivations on starch. Exponential growth by the KFA199 mycelia during aerobic cultivation on starch was observed from the start of the aerobic phase (32 h) until 52 h of incubation, when stationary phase and a maximum mycelial concentration of 10.4 g/l was reached (Figure 2). The parental ATCC 1216b strain reached stationary phase at 55 h, corresponding to a maximum mycelial concentration of 11.3 g/l (Figure 3). The wild-type CBS 232.29 strain entered stationary phase after 40 h of fermentation, and reached a maximum mycelial concentration of 9.1 g/l (Figure 4). The highest growth rate and biomass yield during aerobic cultivation on starch was obtained with the parental ATCC 1216b strain (0.28 ± 0.07/h and 0.47 ± 0.03 g/g; Table 2). The aerobic growth rates of the recombinant KFA199 strain on starch (0.16 ± 0.04/h) was higher than for the CBS 232.29 strain (0.11 ± 0.03/h), although the biomass yield of wild-type strain (0.40 ± 0.1 g/g) was higher than the recombinant strain (0.23 ± 0.03 g/g) (Table 2). Ethanol produced during anaerobic cultivations of the recombinant, parental and wild-type strains was depleted under aerobic conditions (Figures 2–4).

The recombinant KFA199 strain showed intra and extracellular production of amylase from 44 h of incubation during growth on starch (Figure 2). Extracellular amylase reached a maximum concentration of 0.07 $U/ml_{culture}$, whereas a maximum intracellular amylase level of 0.013 $U/ml_{culture}$ was obtained (Figure 2). During the parental ATCC 1216b and wild-type strain cultivations, the extracellular production of amylase started at the initiation of aerobic growth and was continued until the end of fermentations (Figures 3, 4). The maximum extracellular concentrations of amylase obtained with the ATCC 1216b and CBS 232.29 strains were 0.63 $U/ml_{culture}$ and 0.33 $U/ml_{culture}$, respectively (Figures 3, 4). The corresponding maximum intracellular amylase levels were 0.34 $U/ml_{culture}$ and 0.22 $U/ml_{culture}$, respectively (Figures 3, 4).

Discussion

In the present study, the potential of recombinant *M. circinelloides* to produce heterologous glucose oxidase (GOX) during dimorphic growth under well-controlled bioreactor conditions was determined. The physiology and enzyme production of the recombinant KFA199 strain was compared to its parental ATCC 1216b strain as well as a wild-type CBS 232.29 strain. The recombinant KFA199 strain resulted from work by Larsen *et al*. (2004), where intracellular GOX expression during shake flask cultivations was determined. In the present study, dimorphic cultivation of the recombinant and wild-type strains in a bioreactor comprised a biomass production phase, where yeast-like growth was maintained under anaerobic conditions, followed by mycelial growth in the aerobic phase. GOX production was determined during growth on glucose during both the aerobic and anaerobic phases, whereas amylase formation was determined during aerobic growth on starch.

Morphology and physiology during dimorphic growth

During anaerobiosis, Vogel's medium supplemented with the E/T80 combination and sparged with nitrogen gas provided the most suitable conditions for biomass production under yeast-like morphology for the recombinant and wild-type *Mucor* strains. Lübbehüsen *et al*. (2003b) reported that ergosterol supplementation during anaerobic cultivation of *M. circinelloides* resulted in the

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formation of spherical multipolar budding yeast. Higher anaerobic growth rates were observed in the presence of E/T80 supplements (Table 2), while the formation of filaments during the absence of E/T80 indicated a need for O_2 scavenging to enable sterol and fatty acid synthesis (Lübbehüsen *et al.*, 2003b). During cultivation in complex YPG medium no supplementation of E/T80 was necessary, since small peptides from medium components can aid in yeast-like growth (Elmer and Nickerson, 1970). Supplementation of ergosterol to the medium apparently fulfilled the growth-related functions necessary for the attainment of yeast-like morphology, previously observed for *Saccharomyces cerevisiae* (Rodriguez *et al.*, 1985).

This study confirmed that *M. circinelloides* was able to grow and produce ethanol under complete anaerobiosis, as previously reported (Lübbehüsen *et al.*, 2004). The correlation of anaerobic growth rates to ethanol yields during anaerobic cultivation (Table 2) indicates a requirement for high rates of ethanol production during anaerobic conditions to maintain cell growth. During anaerobic cultivation on glucose the highest growth rate, obtained with the wild-type CBS 232.29 (0.24 ± 0.02/h; Table 2), was similar to the lowest growth rates typically obtained with other yeasts. The anaerobic growth rate of the ATCC 1216b (0.05 ± 0.03/h; Table 2) was substantially lower than previously reported value of 0.1/h (Lübbehüsen *et al.*, 2004) and possibly reflects the experimental difficulties in measuring low growth rates. The change in environmental conditions from anaerobic to aerobic cultivation, and the resulting increase in respirative metabolism, substantially increased the biomass yields of *Mucor* strains, due to a decrease in the ethanol formation (Table 2). Ethanol production under anaerobic cultivation conditions therefore occurred at the expense of biomass production (Lübbehüsen *et al.*, 2004). The absence of ethanol production during aerobic growth on starch could be related to the instantaneous consumption of glucose released during starch hydrolysis and its subsequent unavailability for ethanol production, as was reported earlier during growth of *Mucor* ATCC 1216b on cellulose (Lübbehüsen *et al.*, 2004).

Both the anaerobic and aerobic stages of cultivation resulted in a stress response by the *Mucor* strains, although neither dominated in the present study. During anaerobic growth, both the wild-type and recombinant *Mucor* strains exhibited almost linear growth rather than typical Monod (exponential) growth patterns (Shuler and Kargi, 2002), indicating a limitation in biomass formation. Similarly, the increased broth viscosity during the aerobic mycelial growth of *Mucor* could increase the level of shear damage that may limit biomass formation. The morphological change from yeast-like (anaerobic) to mycelial (aerobic) growth was not dominated by either of these, considering the varying effects on growth rates of strains. The KFA199 and CBS 232.29 strains grew more slowly under aerobic conditions compared to anaerobic conditions, while the ATCC 1216b strain grew more quickly.

GOX and amylase production

The recombinant KFA199 strain of *Mucor* mostly produced GOX during mycelial aerobic growth and not during yeast-like growth under anaerobic conditions (Figure 1), despite the use of the glucose-induced *gpd1P* to drive expression of the *gox* gene (Larsen *et al.*, 2004). The mechanism by which GOX production was repressed during anaerobic growth was not clear, although possibly related to the increase in the specific rate of glucose consumption from anaerobic (0.60 g/g/h) to aerobic cultivation (1.35 g/g/h) on glucose. Recombinant GOX produced by recombinant *Mucor* in the aerobic phase was accumulated intracellularly in the mycelia, after attaining maximum biomass upon glucose depletion in the medium (Figure 1). The heterologous protein production was therefore not growth-related, since GOX production mostly occurred during the stationary phase of mycelial growth. Production of GOX during the stationary phase of mycelial growth was contrary to the growth-related GOX production observed in native fungal systems (Kona *et al.*, 2001; Petruccioli *et al.*, 1995), and the glucose-induced characteristics of the *gpd1* promoter (Larsson *et al.*, 2004). The secretion of GOX by recombinant *Mucor* was particularly poor. The slight increase in the extracellular GOX level towards the end of cultivations (Figure 1) was probably due to the release of enzyme during cell autolysis in the post-stationary death phase. The poor secretion of recombinant GOX could be related to ineffective functioning of the *A. niger* secretion signals in the *M. circinelloides* host (Larsen *et al.*, 2004). The absence of GOX formation during ATCC 1216b fermentations

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confirmed the absence of the *gox* gene (Larsen *et al*., 2004).

Extracellular amylase production during aerobic growth on starch (Table 4) and was correlated to the growth rates of the different strains (Table 2). The maximum amylase concentrations produced by the ATCC 1216b and wild-type CBS 232.29 strains were similar, although an order of magnitude higher than amylase production by the recombinant KFA199 strain during aerobic growth of starch (Table 2). The improved amylase production levels by the ATCC 1216b strain resulted in the highest aerobic growth rate, which was higher than the growth rate of the KFA199 strain and previous literature reports on the particular strain (0.17/h; Lübbehüsen *et al*., 2004). Starch hydrolysis by *Mucor* strains was rapidly induced during the shift to aerobic cultivations (Figures 2–4), indicating that the low levels of amylase production did not limit growth on starch. The low levels of amylase production by the KFA199 strain may be related to the leucine auxotrophy of the R7B (ATCC 90 680) strain (derived from the parental ATCC 1216b strain) or genetic changes induced during transformation of the R7B strain with the *gpd1p–gox* expression cassette, which may have a negative effect of GOX production.

Mucor as host for heterologous proteins

The production levels of both recombinant GOX and native amylase by *Mucor* strains during morphology-controlled cultivation were substantially lower than with established fungal systems (Tables 3, 4). The low levels of recombinant GOX and native amylase production by mycelial *Mucor* during bioreactor cultivations may be attributed to the damage inflicted to the mycelia by agitation in stirred tank bioreactors. The activity of the native *gpd1* promoter resulted in recombinant GOX production during the mycelial growth phase, with incumbent difficulties due to broth viscosity and cell breakage. Fungal filaments tend to get damaged due to shear forces in the fermenter leading to lower product formation (Vahidi *et al*., 2005). Fungal strains also tend to produce abundant quantities of endogenous proteases during cell lysis that may cause extracellular degradation of heterologous proteins (Punt *et al*., 2002). The low-shear cultivation conditions in aerobic shake flasks enabled higher recombinant GOX (0.37 vs. 0.14 $U/ml_{culture}$) and amylase (10 vs. 0.33 $U/ml_{culture}$) extracellular enzyme production, in comparison with bioreactor cultivations (Tables 3, 4). GOX production during shake-flask cultivations improved significantly when the glucose in Vogel's medium was replaced with lactone (data not shown), indicating the potential for medium optimization using alternative hexose sources. Production levels may be further improved by cultivation in a larger fermenter, to reduce the shear stress on the mycelial morphology, and by medium optimization. The lower production levels of recombinant GOX may be related to the limited post-translational capacity of the KFA199 strain, as evident from the lower secretion levels of native amylases.

The low production levels of recombinant and native fungal enzymes indicated that *Mucor* is unlikely to replace the mainstream fungal expression systems as a preferred expression host. Although the dimorphic behaviour of *Mucor* benefited biomass production by some of the strains during anaerobic yeast-like growth, it did not assist in avoiding the typical cultivation problems associated with the mycelial morphology of filamentous fungi during aerobic enzyme production. The inherent morphological advantages of *Mucor* could therefore not be utilized to improve recombinant protein production with the present expression tools. *Mucor* may instead find application as a host for the production of zygomycete-specific enzymes, such as lipases, that may be problematic for the established fungal systems.

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