Bioprocess optimisation for high cell density endoinulinase production from recombinant Aspergillus niger

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Abstract

Endoinulinases gene was expressed in recombinant Aspergillus niger for selective and high-level expression using an exponential fed-batch fermentation. The effects of the growth rate (μ), glucose feed concentration, nitrogen concentration and fungal morphology, on enzyme production were evaluated. A recombinant endoinulinases with a molecular weight of 66 KDa was secreted. Endoinulinases production was growth associated at μ> 0.04 h -1 , which is characteristic of the constitutive gpd promoter used for the enzyme production. The highest volumetric activity (670 U/ml) was achieved at a growth rate of 93% of μ max (0.07 h -1), while enzyme activity (506 U/ml) and biomass substrate yield (0.043 g biomassDW /g glucose) significantly decreased at low μ (0.04 h -1). Increasing the feed concentration resulted in high biomass concentrations and viscosity, which necessitated high agitation for improved mixing and oxygen. However, the high agitation and low DO levels (ca. 8% of saturation) led to pellet disruption and growth in mycelial morphology. Enzyme production profiles, product (Y p/s) and biomass (Y x/s) yield coefficients were not affected by feed concentration and morphological change. The gradual increase in the concentration of nitrogen sources showed that, a nitrogen limited culture was not suitable for endoinulinases production in recombinant A. niger. Moreover, the increase in enzyme volumetric activity was still directly related to an increase in biomass concentration. An increase in nitrogen concentration, from 3.8 to 12 g/L, resulted in volumetric activity increase from 393 to 670 U/ml, but the Y p/s (10053 U/g glucose) and Y x/s (0.049 g biomasDWs /g glucose) did not significantly change. The data demonstrated the potential of recombinant A. niger and high cell density fermentation for the development of largescale endoinulinases production system.

1. Introduction

Endoinulinases is an important polyfructose hydrolysing enzyme used for the production of fructooligosaccharides from inulin, and thus has a wide range of application in the food and pharmaceutical industries [1, 2]. Endoinulinases is also important in the fermentation of inulin into ethanol, single cell protein, biodiesel and platform chemicals through enzymatic conversion into simple sugars [3, 4, 5].

Endoinulinases naturally exist in plants, fungi, yeast and, bacteria and produced as an inulinases cocktail composed of endo- and exoinulinases [2, 6, 7]. Microorganisms are the best sources for commercial endoinulinases production [8], because of their ease for large scale production; however, the low enzyme productivity from native strains is a major disadvantage. Moreover, the production of heterogeneous enzymes from native host is a major challenge due to co-expression and secretion of other native enzymes [9], and this necessitates costly downstream processing in commercial production [10].

High-level and homogenous endoinulinases expression can be achieved through heterologous protein expression using recombinant DNA technology with [18, 11]. The filamentous fungi, *Aspergillus* sp., is widely used as host for industrial production of recombinant protein. *Aspergillus* sp. has rapid growth, high production and secretion capacity, as well the ability to perform post translational modifications [9,

12]. The use of inducible promoters is also important to control the expression of native enzymes in *A. niger* [11]. Moreover, *Aspergillus* sp. has the GRAS (Generally Regarded As Safe) status and thereby has a potential for application in recombinant enzyme production in the food and pharmaceutical industries [12].

Fed batch fermentation is an industrially relevant production method, used to achieve high enzyme volumetric activity [13, 14]. However, the high viscosity associated with high cell density fermentation severely affects productivity [15, 16]. The high broth viscosity impacts the mixing efficiency, oxygen and nutrients diffusion [17, 18]. Therefore, overcoming viscosity challenges in high density fermentation of *Aspergillus* is crucial to achieve high biomass yield and volumetric concentration of the recombinant protein [15, 18]. The feed concentration is also an important factor in reducing broth viscosity through controlling the biomass concentration during fed batch fermentation [15, 17]. The use of highly metabolizable nutrients results in rapid growth in filamentous morphology which results in oxygen transfer limitations and reduced protein synthesis [19].

Currently, most of the available literature studies on recombinant endoinulinases production have been in bacterial or yeast hosts, and not filamentous fungi, despite their application of the latter for industrial enzyme production. Therefore, there is a need for the development and optimisation of bioprocess parameters for the use of *A. niger* in the production of endoinulinases.

The aim of the study was to develop and assess the potential of a recombinant *A. niger* strain for endoinulinases production with a glucose limited fed batch exponential fermentation strategy. *A. niger* D15 (uridine auxotrophic (pyrG), protease-deficient (prtT), nonacidifying (phmA) mutant was transformed with pGT (bla gpd_p-gla_r) vector containing Inu A gene encoding endoinulinases. The transcription was under the control of the glyceraldehyde-3-phosphate dehydrogenase promoter (gpd_P) of A. niger and glucoamylase terminator ($glaA_T$) of *A. awamori*. Enzyme secretion was directed by the native gene's secretion signal. The effects of the bioprocess parameters, glucose feed concentration, nitrogen sources concentration and growth rate, on biomass growth and enzyme production in fed-batch culture were investigated. The study aimed to gain insight on the factors influencing and challenges related to recombinant endoinulinases production from *A. niger* during high cell density fermentation.

2. Materials And Methods

2.1 Media and cultivation conditions

All chemicals were of analytical grade and unless stated otherwise, sourced from Merck (Darmstadt, Germany). The E. coli DH5α strains were cultivated at 37°C in Terrific Broth and on Luria Bertani agar containing 100 µg ampicillin/ml for selective pressure (20).

The A. niger D15 parental strain was cultivated at 30°C in minimal media (5 g/L yeast extract, 0.4 g/L $MgSO_4 \cdot 7H_2O$, 2 g/L casamino acids, 20 ml 50×AspA (300 g/l $NaNO_3$, 26 g/L KCl, 76 g/L KH_2PO_4, pH 6), 0.01 M uridine and 1 ml/L 1000×trace elements) (21). Transformants were selected for on minimal

medium lacking uridine. Media were inoculated to a concentration of 1×10^6 spores per ml unless stated otherwise. The *A. niger* D15 transformants were initially cultivated in 20 ml double-strength minimal media (2×MM) containing 10% glucose for screening purposes (enzyme activity determination). Cultivation took place in 125 ml Erlenmeyer flasks on a rotary shaker at 200 rpm at 30°C for three days. Supernatants were obtained by centrifugation at 12 000 g for 10 min at room temperature and stored at 4°C for further analysis.

2.2 DNA manipulations and gene amplification by PCR

Standard protocols were followed for all DNA manipulations and *E. coli* transformations [20]. The *A. niger* ATCC10864 strain was cultivated in minimal media for 72 h. Mycelia were harvested, frozen under liquid nitrogen and the DNA isolated [21]. The InuA was amplified from the genome using the polymerase chain reaction (PCR) with oligonucleotide primers listed in Table 1. TaKaRa Extaq Polymerase (TaKaRa Bio Inc. Otso Japan) was used for amplification of the genes with the reaction set up in accordance with the supplier's specifications in the Perkin Elmer GeneAmp® PCR system 2400 (Perkin Elmer, USA). The InuA was cloned into the NotI site of plasmid pGT (21) to obtain pGT-InuA under the transcriptional control of the glyceraldehyde-3-phosphate dehydrogenase promoter (gpd_P) of A. niger and glucoamylase terminator ($glaA_T$) of A. awamori. The pyrG marker gene had been retrieved from pBS-pyrGamdS [22] via PCR and was cloned into the EcoRI site on plasmid pUC18, generating pUC-pyrG. Spheroplasts were prepared from the *A. niger* D15 (cspA1, pyrG1, prtT13, phmA, a non-acidifying mutant of AB1.13, ATCC 9029) strain using lyzing enzymes (Sigma-Aldrich, Steinheim, Germany) in accordance with [23]. The pGT and pGT-InuA vectors (Fig. 1) were respectively co-transformed with pUC-pyrG to *A. niger* D15 to generate the A. *niger* D15[pGT-control] and D15[InuA] strains.

2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

The proteins in the supernatant samples (20 µL) were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (12% SDS-PAGE) as described by Sambrook et al. (1989). Electrophoresis was carried out at 100 V at ambient temperature and the proteins visualised using the silver staining method [24]. The broad-range Page Ruler Prestained SM0671 Protein Ladder (Fermentas, Shenzhen, China) was used as a molecular mass marker.

2.4 Pre-inoculum preparation and cultivation medium for the fermentations

Stock cultures of the strains were stored at -80^{o}C in 30% (v/v) glycerol as cryoprotectant. A. niger spore production was performed in spore plates containing 18 g/L agar, 2 g/L peptone, 1 g/L yeast extract, 10 g/L glucose and 2 g/L casamino acids with nitrates, at 30^{o}C. The densely conidiated culture was harvested with saline solution (9 g/L NaCl) after 5 days. The minimal medium (MM) without uridine [22] was used for the batch phase and was composed of 20 g/L glucose, 1 g/L casamino acids, 1 g/L peptone, yeast extract and 1.8 g/L MgSO4•7H2O. A pre-inoculum was prepared in an 1L Erlenmeyer flask containing 400 ml of the medium by inoculating with a spore concentration of *Aspergillus* of 1 X 10^6

spores mL^{-1}. The flask was incubated at 30^{o}C on a rotary shaker (Innova 2300, New Brunswick Scientific, Edison, USA) at 180 rpm for 12 hours.

2.5 Bioreactor operating conditions and growth medium

Enzyme production was carried out in batch cultures in 14 L BioFlo 110 bioreactors (New Brunswick Scientific company, Inc, USA) with a 10 L working volume and equipped with a polarographic DOT probe and a glass pH electrode (Mettler, Toledo, Sandton, South Africa). A batch culture with a total volume of 4 L of minimal medium (MM) without uridine [22] was used for the enzyme production. Batch culture fermentation were carried out with an initial total nitrogen concentration in the range of 3.8-18 g/L. The pre-inoculum was subsequently transferred directly from the flask into the bioreactor. The cultivation temperature and pH were 30^{o}C and pH 5.5, respectively. The pH was maintained through the cultivation period using 25 % w/v NH_4OH. Furthermore, a constant aeration rate of 0.8 v/v/m was maintained in the bioreactor during the cultivation. The dissolved oxygen was maintained above 30% saturation through a control loop that linked the agitation to the dissolve oxygen. The agitation speed was cascaded between 250 and 400 rpm, with 400 rpm set as a maximum to limit biomass degradation [15]. Foaming was controlled by addition of 0.1% (v/v) of 30% antifoam (Sigma-Aldrich, Kempton Park, South Africa). At the end of the cultivation, the fermentation broth was vacuum filtered with a Buchner funnel and the enzyme activity in the broth determined before storage at 4^{o}C.

2.6 Exponential feeding

An exponential feeding strategy was employed to evaluate the effects of growth rate on biomass and enzyme concentrations. Feeding was initiated at the end of the batch phase following depletion of the carbon source (glucose). Residual glucose was tested with glucose test strips (Accu-Chek®). The prediction of biomass (X_t) produced at a time t, using an exponential growth equation 1 [25], enabled estimation of the mass of glucose (S_t), the primary growth-limiting factor, fed during the fed-batch phase. This subsequently enabled the regulation of a pre-determined growth rate, where the glucose concentration in the reactor is assumed to be zero during the feeding phase [10]. Furthermore, the amount of glucose (S_t) to maintain a specific cell biomass (X_t) was determined according to equation 2 [26, 27]:

$$X_t = X_0 e^{\mu t} \quad (1)$$

where, X_t, X_0 and μ is biomass is time t, biomass at the end of the batch culture and growth rate.

$$S_t = \frac{V_0 X_0 (e^{\mu set t} - 1)}{Y_{X/S}} + S_0 \quad (2)$$

where, S_t, V_0, X_0, μ_{set}, S_0 and $Y_{x/s}$ is mass of glucose at time t, volume of broth at the end of the batch culture, biomass at the end of batch culture, the desired growth rate, mass of glucose at time 0 and biomass substrate yield coefficient, respectively.

2.7 Enzyme assay

Endoinulinases activity was determined based on the method of [14]. The broth culture from the fermentation was filter sterilised for use in the enzyme assays. A 100 µL solution of 25% w/v inulin (Novozyme), 750 µL of 0.1M sodium acetate buffer (pH 5) and 100 µL of the crude enzyme were mixed and incubated at 50 ^{o}C for an hour. The reaction was terminated by placing in boiling water for 5 min. The solution was centrifuged and analysed for reducing sugars with the DNS assay. One unit of endoinulinases activity was defined as the amount of enzyme required to produce 1µmol of reducing sugar per min under assay conditions. Positive control assay was performed with a commercial inulinase (Megazyme). The negative control was performed with broth culture from the *A. niger* host strain that had not been transformed with inulinase genes.

3. Results And Discussion

3.1 Strain development

Fig. 2 is an illustration of an SDS PAGE for the selection of the positive transformant strains used in the fed-batch fermentation for endoinulinases (InuA) production. Endoinulinases with a molecular weight of ca. 66 kDa (28) was identified relative to the molecular weight marker. The presence of the enzyme was further confirmed by the absence of a corresponding band of 66 kDa from the negative control lane 4 (Fig. 2). Therefore, the recombinant *A. niger* overexpressed and secreted the endoinulinases extracellularly.

3.2 Effects of glucose feed concentration on DO levels and mixing as well as subsequent impact on biomass and enzyme production

Biomass production, enzyme activity, DO and agitation speed profiles, during endoinulinases production at four different glucose feed concentrations are demonstrated in Fig. 3. The maximum specific growth rate (μ_{max}) and biomass yield on substrate ($Y_{x/s}$) estimated in the batch culture prior to exponential feeding were 0.075 h^{-1} and 0.49 $g_{biomassDW}/g_{glucose}$ (data not shown), respectively. Fed batch fermentations were carried out at a constant exponential growth rate (µ) of 0.06 h^{-1} for the glucose feed concentration of 15 g/L and 100 g/L and 0.07 h^{-1} for the glucose feed concentration of 200 and 300 g/L.

Biomass concentration and volumetric enzyme concentrations increased significantly ($p<0.05$) when the feed concentration was increased from 15 to 100, 200 and 300 g/L (Fig. 3a). Biomass concentration (Fig. 3a) and enzyme volumetric activity (Figure 3b) increased by almost 2-fold when the glucose feed concentration was increased from 15 to 200 and 300 g/L. The morphology of the recombinant *A. niger* strain used in this study remained in pellet form during culture (Fig. 4) and this coincided with DO levels of ca. 30% of saturation (Fig. 3c) maintained in the culture broth during fed batch fermentation with a dilute glucose feed (15 g/L).

In contrast, fed batch fermentation with a concentrated feed (100-300 g/L) resulted in a drastic change in the fungal morphology from pellet to mycelia form. The DO levels and agitation remained constant at ca. 30% of saturation (Fig. 3c) and 250 rpm (Fig. 3d), respectively, during the first 12 hours in the stationary phase of the culture. However, these changed drastically (Fig. 3c and d, respectively) when the culture entered the exponential growth phase. The DO levels dropped rapidly reaching a low of 8% of saturation and remained at this level throughout the fermentation period (Fig. 3c), due to the exponential increase in biomass concentration (Fig. 3a) and apparent increase in oxygen demand from the increased biomass concentration [29, 30, 31]. Accordingly, the agitation speed increased, reaching the maximum set-speed limit of 400 rpm, because of the system attempting to maintain the DO setpoint of 30% of saturation (Fig. 3d). Consequently, gradual pellet fragmentation (Fig. 3e), to mycelial form, was observed following the continued low DO levels of 8% of saturation (Fig. 3c) and agitation at 400 rpm (Fig. 3d). The experimental data illustrated the importance of bioprocess parameters on fungal morphology and the complex interdependence of the former in controlling the morphology [32]. Therefore, the culture conditions can be controlled to induce fungal growth in pellet morphology and subsequently minimise the viscosity limitations associated with fungal growth in mycelial [13, 15, 33]. High cell densities are responsible for both increased broth viscosity [13, 18] and low DO levels in the culture, due to rapid oxygen uptake [16]. The internal resistance, because of the former, results in inefficient mixing, oxygen as well as nutrient diffusion and subsequent pellet disintegration [31, 32]. Change in the culture conditions, associated with inefficient mixing, such as pH has been hypothesised to contribute to pellet disintegration due the impact on electrostatic forces that contribute to the pellet integrity [34]. Broth viscosity, at high cell densities, also increases the probability of collision and friction between the pellets which in turn weaken the hydrophobic and electrostatic interactions that keep the pellet structure intact [34-35]. Although, increasing agitation is necessary to improve mixing efficient, oxygen and nutrient diffusion, at high biomass concentrations [29-30], high agitation speeds cause shear forces that cause pellet disintegration and growth in mycelial which further affect broth viscosity [13, 31]. Sporh and co-workers [36] illustrated that agitation speeds of at least 400 rpm resulted in fungal pellet degradation.

The change in fungal morphology, however, did not affect both biomass growth and enzyme production which contrasts with what has been reported for recombinant A. niger. In addition, the feed concentration did not have a significant impact on the yields, biomass yield on substrate ($Y_{x/s}$) or enzyme yield on substrate ($Y_{p/s}$), and the specific enzyme productivity ($Y_{p/s}$) (Table 5.2). However, increasing feed concentration did significantly increase the volumetric productivity (Q_p) of the system. For instance, an increase from 15 to 100 g/L resulted in a Q_p increase from 7980 to 9831 U/L/h. The control of bioprocess conditions to support a specific fungal morphology, in recombinant enzyme production using recombinant *Aspergillus* sp., may also contribute to productivity improvements [15, 29]. However, the experimental data for the recombinant endoinulinases production in A. niger is contrary to what has been reported previously, regarding enzyme production under critical dissolved oxygen levels and different fungal morphology.

Lopez and co-workers [15] reported that *A. terruus* grew in large fluffy pellet morphology at DO levels of 80% saturation and agitation speeds less than 300 rpm and that agitation speeds > 300 resulted in growth in small pellet and a significant reduction in lovastatin productivity. Haack and co-workers [29] reported that *A. oryzae* growth and lipase production were inhibited by low oxygen availability as result of increased biomass concentration, morphology change from pellets to mycelium, during the feeding phase of an exponential fed batch culture. The insignificant impact of the fungal morphology on the endoinulinases productivity in recombinant A. niger could be attributed to the point of enzyme synthesis. Haack and co-workers reported that lipase production was localised from the hyphal tips of *A. oryzae*, and morphology change from pellets to mycelial reduced the density of active hyphal tips thereby reducing lipase productivity.

In contrast, phytase production from *A. ficuum* [33] and fructofuranosidase production from *A. niger* [13] were enhanced by fungal growth in small pellets. Driouch and co-workers [18] further illustrated that the total biomass obtained when the fungi was growing in large pellets was not significantly different to the biomass when fungi was growing in small pellets. High DO levels were not a necessity for recombinant endoinulinases production which was growth-associated [13, 18], and therefore productivity was not significantly different between pellet and hyphal growth despite the poor aeration and oxygen transfer of the latter.

3.3 Effects of nutrient concentration of biomass yield and enzyme production

Nitrogen sources are vital nutritional components for biomass growth and biosynthesis pathways [37-38]. Moreover, nutrient composition is an important factor in bioprocess optimisation and plays a critical role in maintaining optimal bioprocess conditions for the maximum productivity [32]. Therefore, fed batch fermentation were performed to evaluate the effect of nitrogen concentration, on fungal morphology, biomass yields, and enzyme productivity. The nutrients were comprised of a cocktail of three organic nitrogen sources, which are yeast extract, peptone and casamino acids in proportions of 50, 25 and 25 %, respectively [22]. Exponential fed batch cultures were carried out at a μ of 0.07 h^{-1}, glucose feed concentration of 300 g/L and organic nitrogen cocktail concentrations of 3.8, 12 and 18 g/L in the batch culture medium. An increase of approximately 3-fold in the nitrogen cocktail, from 3.8-12 g/L, resulted in significant ($p<0.05$) increase in the final biomass concentration (Figure 5a) and volumetric enzyme activity (Fig. 5b), from 18.36 to 34.4 g/L and 393 U/L to 670 U/L, respectively. A further increase of the nutrients to 18 g/L did not result in a further increase in the biomass concentration and enzyme activity (Fig. 5a and b, respectively). The data thereby, demonstrated that an increase in the feed concentration should be supplemented with a corresponding amount of nitrogen to ensure the culture has excess nitrogen and carbon limited. Although, the increment in the nutrient concentration resulted in an increase in the final biomass concentration and enzyme volumetric activity, the $Y_{x/s}$ $Y_{p/x}$ and $Y_{p/s}$ did not significantly ($p>0.05$) differ (Table 3) at the different nutrient concentrations, demonstrating that the enzyme yield on biomass and carbon source (glucose) did not change.

Therefore, the organic nitrogen sources did not have a direct induction effect on recombinant endoinulinases production, confirming that enzyme production was growth related. The data demonstrated that at a feed concentration of at least 100 g/L glucose, biomass growth and enzyme production were nitrogen limited at a concentration of 3.8 g/L nitrogen cocktail and conversely carbon limited at an excess nitrogen concentration of 12 g/L. The supplementation of the culture media with organic nitrogen sources of up to 12 g/L was sufficient to prevent nitrogen limitation without impacting the fungal morphology of A. niger in a negative way. Fu and co-workers [39] reported that peptone had a positive impact on pellet formation. However, excess nitrogen components in the medium can result in fungal growth in mycelial form and consequently cause viscosity limitations.

3.4 Effect of growth rate on biomass growth and enzyme production as well as yields and productivities

Exponential fed batch fermentation at 93% and 53% of μ_{max}, μ of 0.07 h^{-1} and 0.04 h^{-1}, respectively, were performed to determine the effects of the growth rate on biomass growth and enzyme production, with a high glucose concentration feed (300 g/L) and excess nitrogen (18 g/L) supplementation. The agitation was cascaded between 250 and 400 rpm to minimize biomass degradation. Exponential feeding resumed after depletion of 20 g/L glucose in the batch culture and was terminated when residual glucose started to accumulate and DO levels spiked from the 8%, maintained during the exponential growth phase, to ca. 20% level of saturation (Fig. 5d). The highest biomass concentration was obtained at the higher growth rate of 0.07 h^{-1} and was equivalent to 33.94 g/L, compared to 29.03 g/L obtained at the lower growth rate of 0.04 h^{-1} (Fig. 6a). The highest volumetric activity (680 U/ml) reported in this study was significantly lower than reported in a *Pichia pastoris*, a yeast host, which reported a volumetric activity of 4000 U/ml (Chen et al., 2014). The enzyme yield on this host was 36 $U/g_{biomass}$ in comparison to 20 $U/g_{biomass}$ reported for *A. niger*. The plots for the natural log of the total biomass (Fig. 6c) show that an exponential growth rate was maintained during the fed batch and that the measured growth rate deviated by approximately 5% and 10% from μ_{set} for μ of 0.07 h^{-1} and 0.04 h^{-1}, respectively. Moreover, the plots had R^2 values of 0.99, further showing that a constant exponential growth rate was maintained during the fed batch fermentations. The end of the fermentation was accompanied by glucose accumulation and subsequent biomass growth cessation as well as enzyme production (Fig. 6a) and an increase in the DO levels to ca. 30% of saturation (Fig. 6d). In addition, visual inspection indicated that the culture was characterised by highly fragmented fungal mycelia components (Fig. 4). Fungal fragmentation (Fig. 4) was preceded by drastic drop in DO level to 8% (Fig. 6d), as result of an exponential increase in biomass concentration (Fig. 6a), and a subsequent increase in agitation speed, to maximum speed of 400 rpm (data not shown), to improve the mixing efficiency. Fragmentation started (Fig. 4) with gradual pellet disruption into mycelia and eventually degradation of the mycelia, at the end of fermentation (T 54 and 66 h for μ of 0.07 h^{-1} and 0.04 h^{-1}, respectively), and this coincided with growth cessation. Furthermore, the transition from pellet morphology to mycelial, during the exponential growth phase, did not impact endoinulinases production (Fig. 6b). Comparisons of the yields and productivities (Table 4) between enzyme production at high growth rates (0.07 h^{-1}) and low growth rates (0.04 h-1) showed that the biomass and enzyme yield coefficients ($Y_{x/s}$ and $Y_{p/s}$ was 0.049 and 10068, respectively)

were significantly ($p<0.05$) higher at the high growth rates (0.07 h^{-1}). The substrate specific consumption (Q_s = 0.101) was also significantly higher at high growth rates (0.07 h^{-1}) compared to a Q_s of 0.072 at μ of 0.07 h^{-1} and 0.04 h^{-1}. The lower enzyme productivities and biomass yield at low specific growth rate could be attributed to a high maintenance energy requirement at low growth rates [40-41]. Slow growing biomass has been reported to have high metabolic energy requirements for maintenance of cellular integrity and viability [42-42] at the expense of biomass growth. The growth associated nature of endoinulinases production at higher growth rates, where maintenance energy did not have a significant impact, was attributed to the constitutive gpd promoter controlling endoinulinases expression from recombinant *A. niger* [9, 43].

4. Conclusions

Viscosity challenges, associated with high cell densities during the fed batch phase, limited the ability of system to achieve enzyme volumetric activity similar to yeast systems. Therefore, strategies that improve the specific productivity of the *A. niger* system will enable attainment of improved volumetric activity, considering the culture has limitations in the maximum attainable biomass concentration. In summary, recombinant endoinulinases production from *A. niger* was not strictly growth associated and only growth associated at high growth rates. Moreover, the high maintenance requirements at low specific growth rate could have resulted in reduced enzyme production. High biomass concentrations combined with increased agitation rates during the fed-batch feeding, resulted in lower DO levels, and a combination of these factors triggered a change from pellet to hyphae morphology. A. niger continued to grow and produce the growth-associated enzyme at high growth rates, with low maintenance requirements, even when the DO levels reached critical level of 8% of saturation. Moreover, enzyme production continued with no changes in productivity at this critical level which was characterised by high viscosity and pellet disintegration.

Declarations

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Authors' contributions

Author	Contribution	Contribution (%)
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	Execution experiments	80
	Data interpretation	80
	Manuscript compilation	90
Shaunita H. Rose	Experimental planning	5
	Executing experiments	20
	Data interpretation	10
	Strain development & manuscript compilation	10
Eugéne van Rensburg	Experimental planning	10
	Data interpretation	5
	Manuscript revision	25
Annie F. A. Chimphango	Experimental planning	5
	Data interpretation	5
	Manuscript revision	10
Johann F. Görgens	Experimental planning	20
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Tables

Tables 1-4 not available with this version:

Table 1: The DNA sequences of the oligodeoxyribonucleotide primers used in this study.

Table 2: Biomass growth and enzyme production productivities at glucose feed concentrations for exponential fed batch cultures of A niger D15 (InuA) strain at a nitrogen concentration of 3.8 g/L. The data is reported as a mean of three runs with the standard deviations.

Table 3: Biomass growth and enzyme production productivities at different nutrient concentrations for exponential fed batch cultures of the A niger D15 (InuA) strain. The data is reported as a mean of three runs with the standard deviations.

Table 4: Biomass growth and enzyme production productivities at two specific growth rates for exponential fed batch cultures of A niger D15 (InuA) strain. The data is reported as a mean of three runs with the standard deviations.

Figures

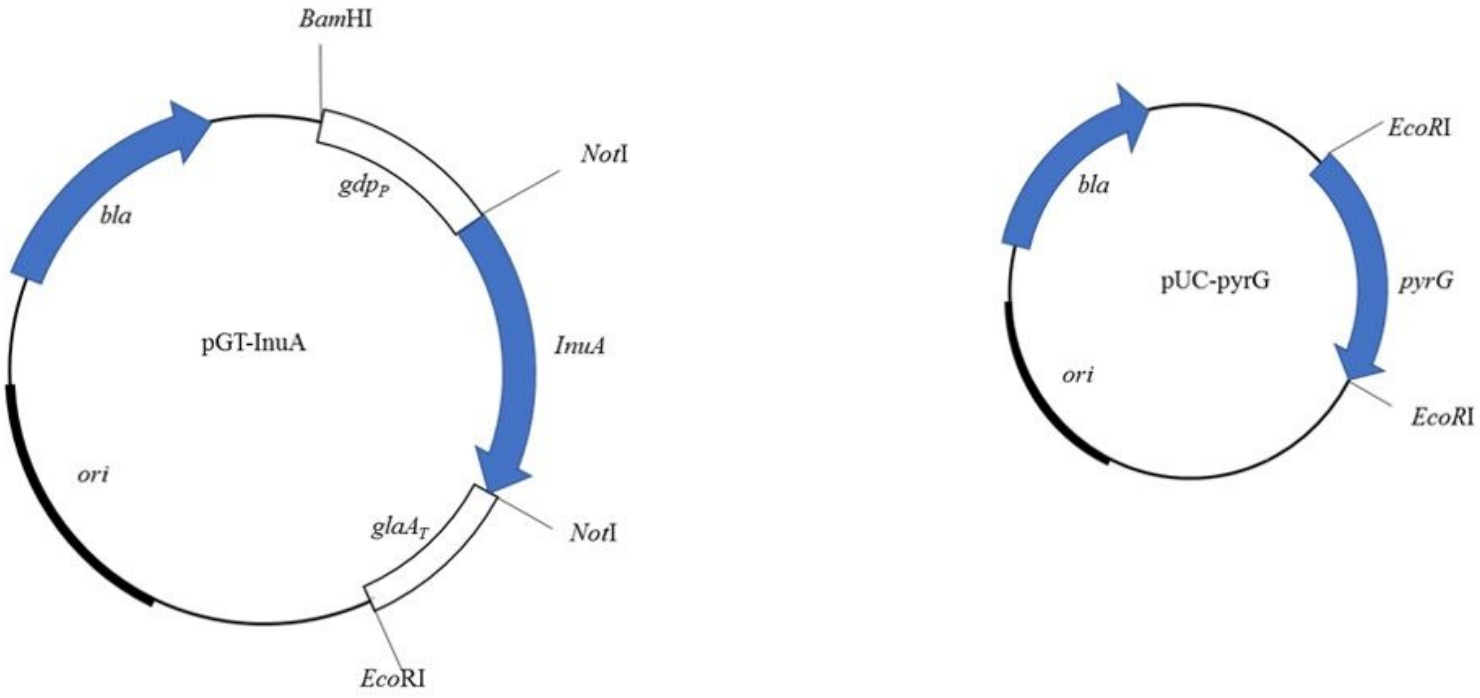


Figure 1

A schematic representation of the vector used in this study to construct the recombinant A. niger D15[InuA].

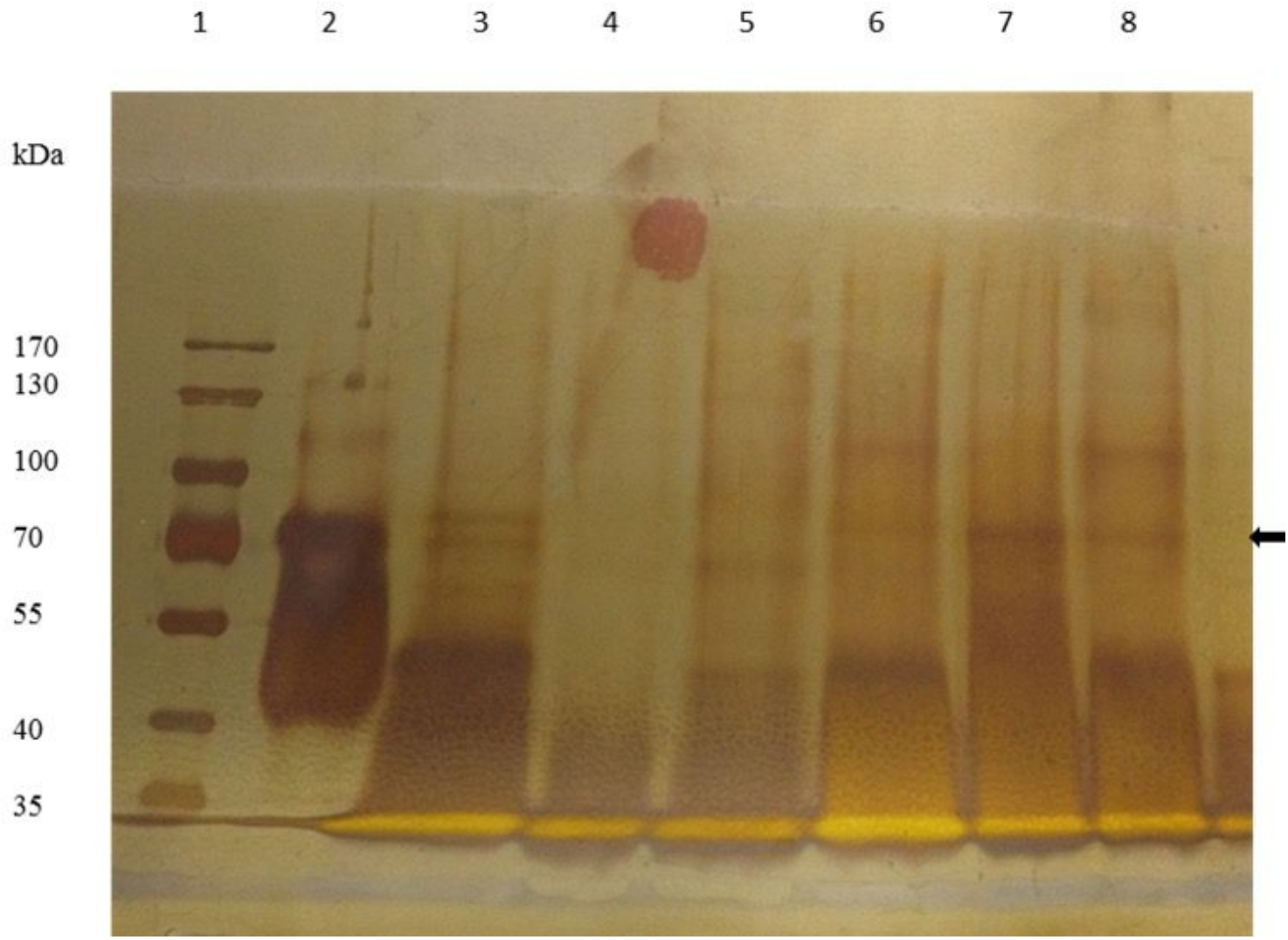


Figure 2

Silver-stained SDS-PAGE for crude supernatant from recombinant A. niger strain grown in shake flasks in MM media at 30 oC for 60 hours. Lane 1 is the molecular marker, lane 2-3 and 5-8 was obtained from A. niger D15 (InuA) strains and lane 4 is the A niger D15 (pGT control). The black arrow indicates the band representing endoinulinases (InuA)

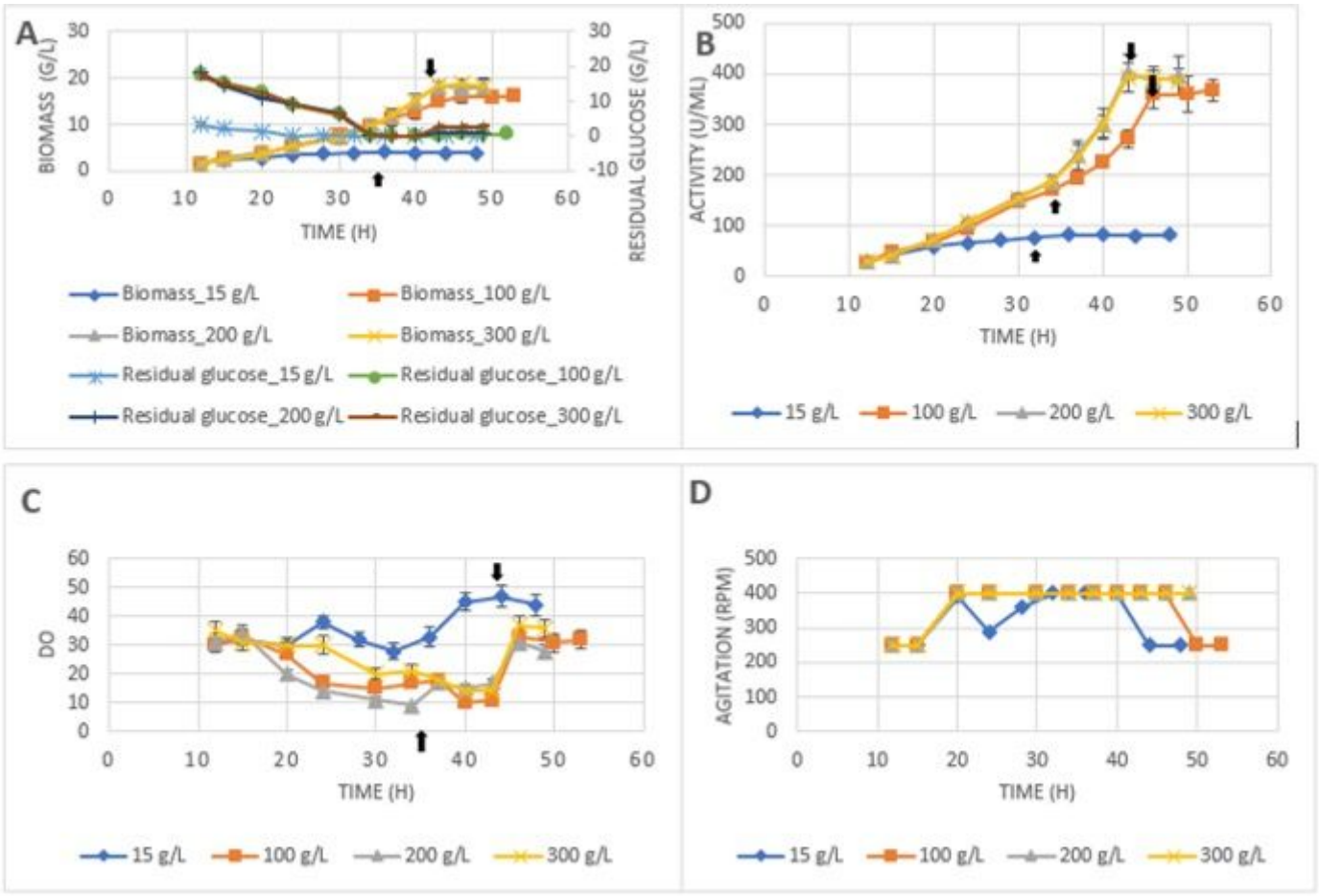
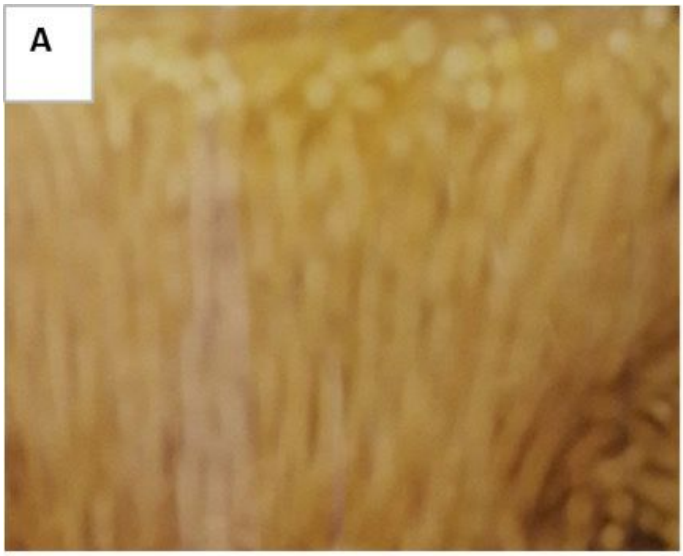


Figure 3

Effects of feed concentration on biomass production (A), enzyme activity (B) DO (C) and agitation speed (D) from recombinant A. niger D15 (InuA) strain. Exponential feeding was used and four feed concentrations (15, 100, 200 and 300 g/L) were evaluated at a growth rates close to μmax. The nitrogen-nutrients concentration was 3.8 g/L. Agitation speed was cascaded between 250-400 rpm. The feeding start and end point (s) are indicated by an arrow pointing upwards and downwards, respectively.



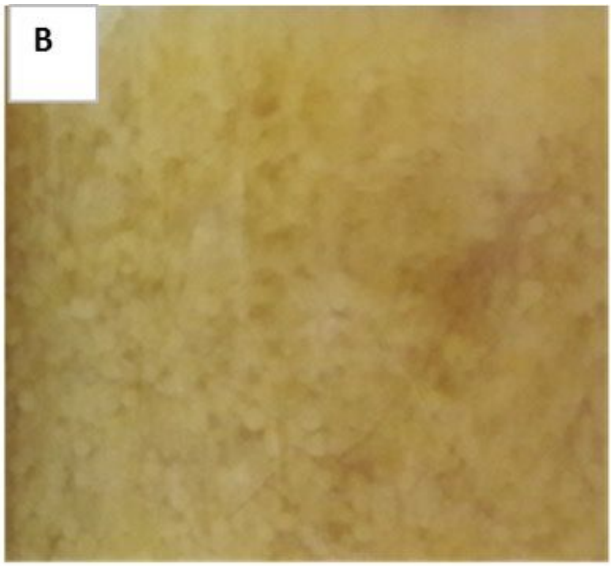


Figure 4

Fungal growth morphology during endoinulinases production from recombinant A. niger D15 (InuA) strain in high-cell density fed-batch culture. The pellet morphology (A) was predominant during the batch phase of the culture while mycelial morphology (B) was predominant during the feeding phase

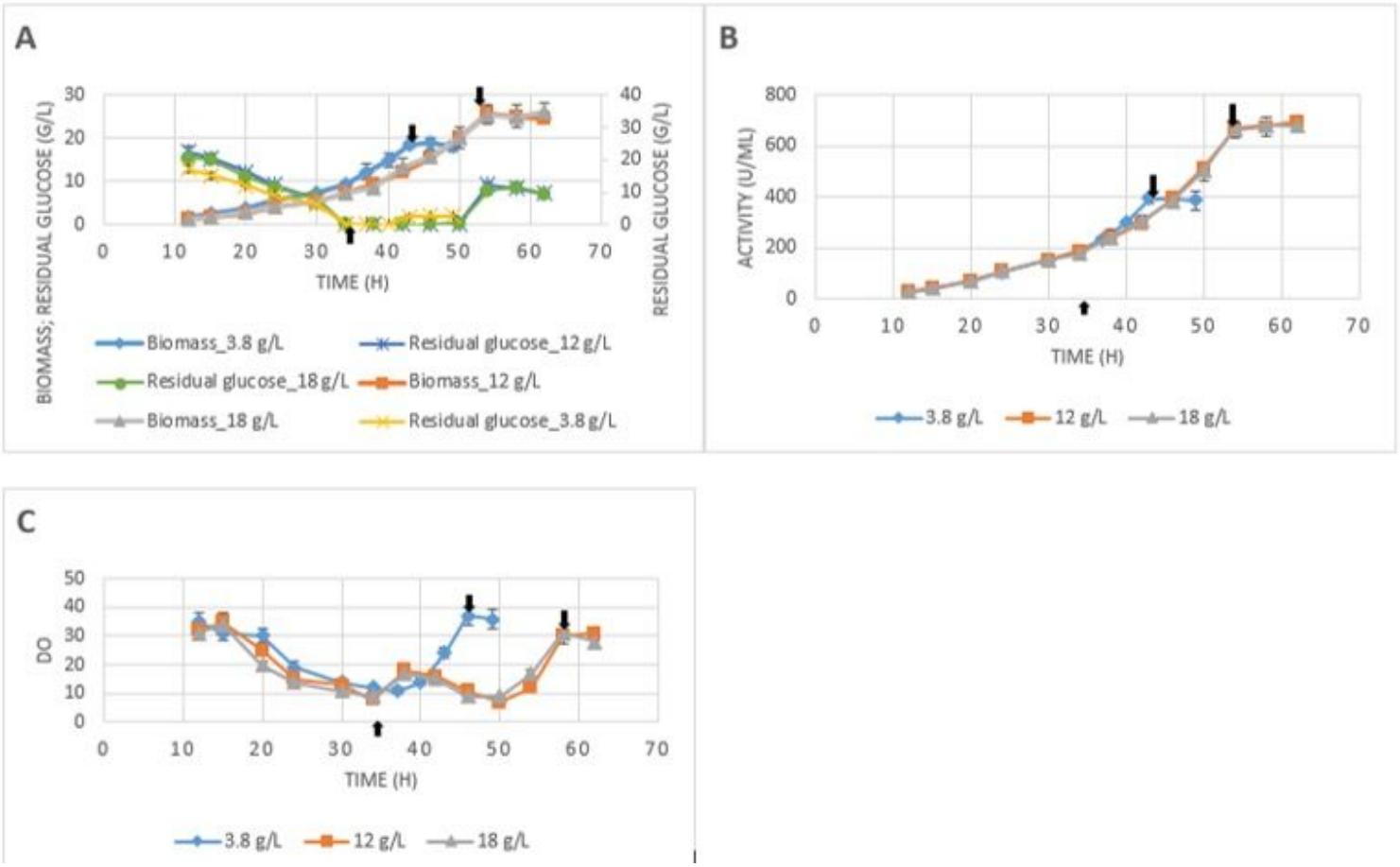


Figure 5

Biomass concentration (A), enzyme activity (B) and DO (C) using different concentration of the nitrogen sources. A cocktail of three nitrogen sources which are yeast extract, peptone and casamino acids in proportions of 50, 25, and 25 %, respectively. An exponential feeding rate of μ = 0.07 was used with a fixed glucose feed of 300 g/L. Agitation speed was cascaded between 250-400 rpm. The feeding start and end point (s) are indicated by an arrow pointing upwards and downwards, respectively.

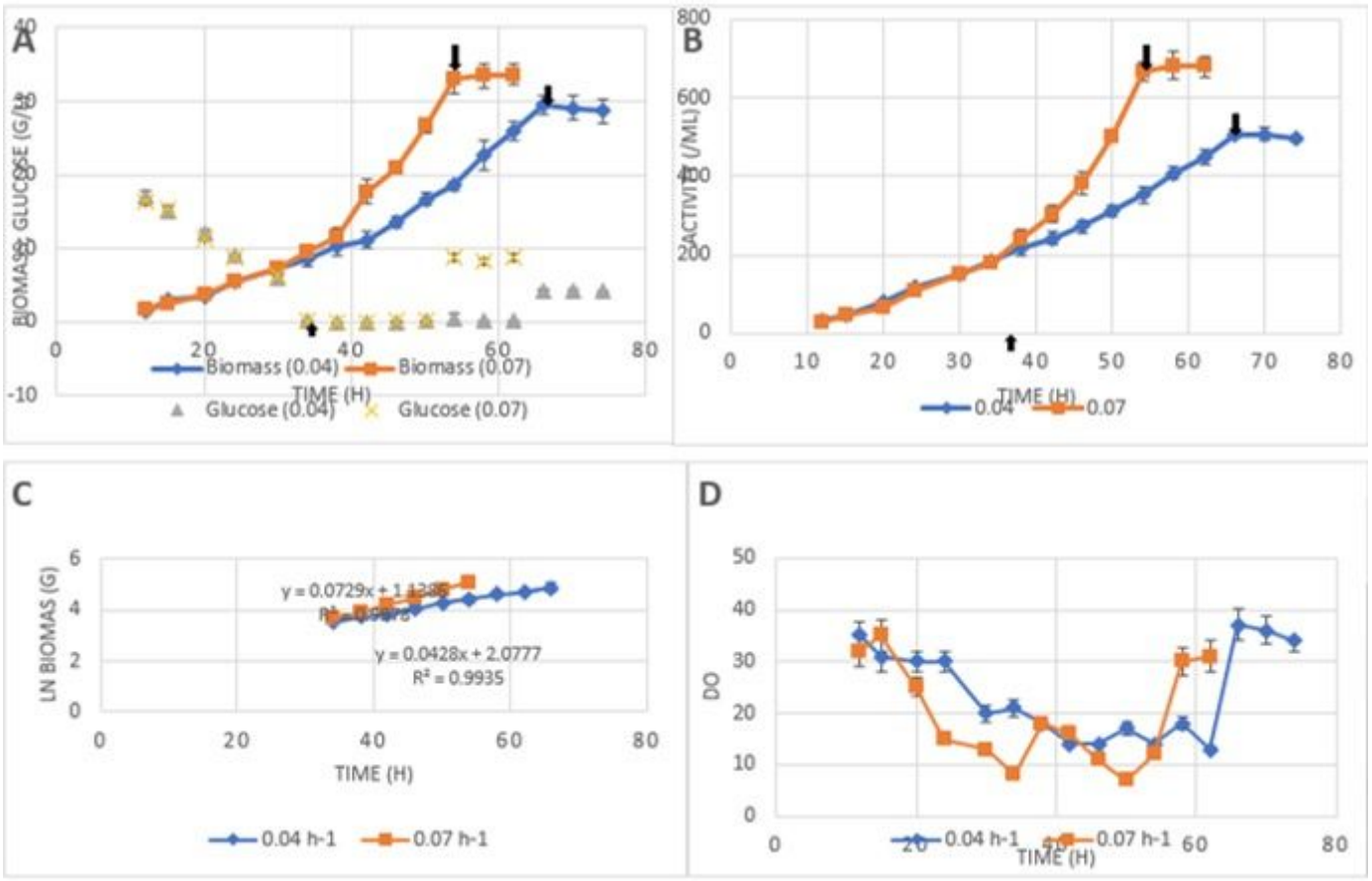


Figure 6

Biomass concentration (A), volumetric enzyme activity (B), specific growth rate profiles (C), DO and (D) during fed batch fermentation at different growth rates. Agitation speed was cascaded between 250-400 rpm. The feeding start and end point (s) are indicated by an arrow pointing upwards and downwards, respectively

Supplementary Files

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