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SHORT COMMUNICATION

Factors involved in the response to change of agitation rate during cellulase production from *Penicillium decumbens* JUA10-1

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Abstract Improvement of agitation is a commonly used approach for the optimization of fermentation processes. In this report, the response to improving agitation rate from 150 to 250 rpm on cellulase production from *Penicillium decumbens* JUA10-1 was investigated. It was shown that the production of all the major components of the cellulase mixture increased following improved agitation. Further investigations showed that at least three factors are involved in this improvement: the improved biomass accumulation, proportion of active/mature cellulases, and cellulase transcription level. The transcription levels of the cellulase repressing transcription factor *ace1* and the cellulase activating transcription factor *xlnR*, however, both declined when a higher agitation was applied. These observations, along with our analysis of the carbon catabolite repressor CreA, lead to the suggestion that the molecular mechanism underlying improved cellulase transcription is the competition of two concurrent effects following improved agitation: CreA-mediated derepression induced by the downregulation of *ace1*, and CreA-mediated deactivation induced by the downregulation of *xlnR*.

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Introduction

Energy from the renewable lignocellulosic biomass is considered a promising solution to the possible energy crisis accompanying the depletion of fossil fuels [10]. The key to this technology is improving the production and efficiency of cellulases, which catalyze the most difficult step of lignocellulose degradation: hydrolysis of cellulose [6]. Filamentous fungi are so far the most important cellulase producers due to their incredible protein secretion abilities [4]. Attempts to further improve cellulase production and efficiency from filamentous fungi included constructing mutants or genetically engineered strains that produce or secrete more proteins [12, 25, 30], engineering enzymes for better performance [20], developing better enzyme cocktails to maximize degradation potentials [31] and optimizing fermentation conditions for better cellulase production [3]. Among these approaches, the optimization of fermentation conditions is by far the most readily available approach to scientists and engineers, which is also known for its effectiveness in the improvement of cellulase production.

Penicillium decumbens is a cellulase hyper-producing filamentous fungus that was isolated over 30 years ago and is known for the production of an excellent cellulase system for degrading lignocelluloses [13, 28]. In this study, we showed that improved agitation during fermentative cellulase production is coupled with increased production of all major cellulases. The molecular mechanism behind this phenomenon was further investigated on the transcriptional level, revealing potential factors involved in the increase of cellulase production.

Materials and methods

Strain and chemicals

P. decumbens JUA10-1 is a laboratory-maintained cellulase hyper-producing strain [24]. This industrial mutant received multiple rounds of mutagenesis. As a result, significant improvement of cellulase production and reduction of carbon catabolite repression effect over the wild-type *P. decumbens* 114-2 was achieved. Corncobs and wheat bran were generously provided by Longlive Bio-Technology Co., Ltd., Yucheng, Shandong, China. Solka Floc (80 % pure cellulose; CAS 9004-34-6) was purchased from Fiber Sales and Development (Urbana OH, USA). All other chemicals used in this report were purchased from Sinopharm Chemical Reagents Co., Ltd. (Shanghai, China).

Production of cellulases

Production of cellulases from *P. decumbens* JUA10-1 was carried out in 3-l fermenters (Shanghai Bailun Bio-Technology Co. Ltd., Shanghai, China). Seed cultures were prepared by shaking inoculated media in 1-l flasks in a rotary shaker (Type sky-1112B, Shanghai Sukun Ltd., Shanghai, China) at 200 rpm and 30 °C for 36 h. The media for the seed cultures contain 1 % glucose, 1 % peptone, 1 % wheat bran, 1 % corncob, 0.05 % $MgSO_4$, 0.2 % $(NH_4)_2SO_4$, 0.3 % KH_2PO_4 and 0.5 % $CaCO_3$. The pH of the media was maintained at 6.0. The 200-ml seed cultures were inoculated to 3-l fermenters containing 2 l of media (pH 6.0), which include 3 % wheat bran, 3 % corncob, 0.5 % $CaCO_3$, 1 % Solka floc, 0.5 % peptone, 0.2 % $(NH_4)_2SO_4$, 0.05 % $MgSO_4$, 0.3 % KH_2PO_4, 0.1 % urea, 0.2 % $NaNO_3$ and 0.3 % Tween-80. Fungal growth and cellulase production was subsequently carried out at 30 °C with agitation of 150, 250, or 350 rpm. The air flow level was 2 l/min. The pH value was maintained between 3.5 and 6.0 using KOH and H_2SO_4.

Biochemical assays

The ATP level was assayed using the Checklite 250 plus ATP kit (Kikkoman Biochemifa Company, Minato-ku, Japan). For ATP level determination, an aliquot of the fermentation broth containing mycelium was extracted from the fermenter, and the amount of ATP released following mycelium lysis was measured. The protein content was assayed using the Lowry method according to a previously reported protocol [16]. The filter paperase activities (FPA) and β-glucosidase activities were measured as previously described [29]. Salicin was used as the substrate for β-glucosidase activity determination. Cellobiohydrolase and endoglucanase activities were, respectively, represented by the abilities to hydrolase *p*-nitrophenyl-β-D-cellobioside (*p*NPC) and carboxymethylcellulose (CMC). The *p*NPCase and CMCase activities were assayed as previously reported [8, 18]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as previously described [14].

Real-time PCR and data manipulation

Total RNA was extracted from *P. decumbens* JUA10-1 mycelia 32, 38, 43, and 48 h after inoculation. The quantification of transcriptional abundance of selected genes was carried out by the real-time PCR approach. The PCR reactions were carried out on a LightCycler 480II Real-Time PCR system (Roche Applied Science, Mannheim, Germany) using SYBR Premix EX Taq™ II (Takara Bio Inc., Shiga, Japan) as the dye. Relative transcriptional abundance was calculated with the $2^{-\Delta\Delta Ct}$ method using *actin* as the reference gene [15]. The nucleotide sequences of primers used for these reactions are summarized in Table 1. Each real-time PCR reaction was carried out three times. The average change of transcriptional abundance and standard deviations were calculated from these data.

Results and discussion

Improvement of agitation leads to increased fungal growth and cellulase production

Increasing stirring rate is a conventional approach for the improvement of nutrition and oxygen mass transfer, which

Table 1 Primers used for real-time PCR experiments

Gene	Forward primer	Reverse primer
actin	CTCTCCCTGTTGTCGAAACCT	CTCTCGTCGTACTCCTGCCTG
cbh1	TCCTCTCCTCCCTGCTGGC	CCGTTCTTGTCGTGAACCCA
cbh2	CAACTTCCGTGTCTGCGAAA	AGCCAGCCAGCGTGTCCT
eg1	CGTCTCCCCGTGTCTACCTC	CCACCGCTCCCACTCATCT
eg2	ACGGTCACCACGGGAATTAA	TAAGAAGGGATTTCGAGCAAAG
bgl1	CGGTGATGTGATTCTTGGTAGC	GAAGTTGGGAGGAGTGCGATG
ace1	CAAGCCATGATGATGTCGGG	TTAAAGGCGGGGTCAGTGAG
xlnR	ATCCCATCCCCACGCTCTAA	GACCAGCCAAGTCACCAAGA

has a significant influence on the fermentation process [11, 27]. In this study, the fungal growth and enzyme production were carried out in two 3-l fermenters with different agitation rates: 150 and 250 rpm. Cellulase production with an agitation of 350 rpm was also carried out, but only a low level of biomass accumulation and cellulase production were observed (data not shown), most likely due to the shearing damage when the stirring rate is too high, as was previously reported [1, 7]. When using the level of ATP concentration in the fermentation broth to represent the biomass of *P. decumbens* A10-1, clearly higher biomass accumulation was observed in the fermenter running at 250 rpm than in the fermenter running at 150 rpm (Fig. 1), leading to the suggestion that higher agitation leads to higher biomass production for *P. decumbens* JUA10-1. The production of cellulases was also compared between the two fermenters. The production of extracellular protein, FPA, β-glucosidase activity, *p*NPCase, and CMCase activities were significantly higher in the fermenter running at 250 rpm (Fig. 2), suggesting that improved production of cellulases is coupled with increase of agitation rate.

Both fungal growth and cellulase production are energy-demanding processes, and require high levels of oxygen. It is a common observation that the dissolved oxygen level stays low in the fermentation broth during the fermentation process, which is an indication that the cells are short on oxygen. Improved agitation results in a better oxygen supply to the cells, and partially relieves this problem, leading to a higher level of metabolism, therefore resulting in improved biomass accumulation.

One interesting phenomenon that we observed is the increase of specific activities for every cellulase component in the 250 rpm fermenter (Fig. 3). This cannot be explained by the change of protein ratios in the cellulase preparation, because the proportions of each component of the enzyme mixture cannot increase at the same time, and our SDS-PAGE analysis showed little difference in protein ratios between the two fermenters (Fig. S1). The most reasonable explanation to this observation is higher production of active/mature protein versus inactive/immature proteins when oxygen transfer is improved, which implies a more effective protein folding/modifying mechanism when *P. decumbens* cells are exposed to a higher oxygen level.

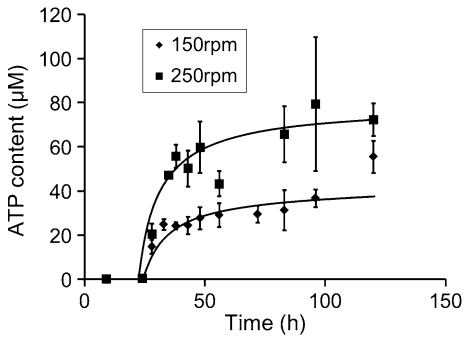

Fig. 1 ATP levels following mycelium lysis in 3-l fermenters with agitation rates of 150 and 250 rpm. The *y*-axis indicates the average ATP content in the fermentation broth

Transcriptional changes of cellulase-coding genes and transcription factors

Total RNA was extracted from *P. decumbens* JUA10-1 cells 32, 38, 43, and 48 h after inoculation to the two fermenters. The expression levels of cellulase-coding genes were quantified using the real-time PCR approach. These genes include *cbh1* and *cbh2* that encode cellobiohydrolases, *eg1* and *eg2* that encode endoglucanases, and *bgl1* that encodes a β-glucosidase. These genes are significantly upregulated in the 250 rpm fermenter at all the time points (Fig. 4), suggesting that improved cellulase expression level also accounts for the overall improved cellulase production in addition to improved biomass.

Examination of the homologue of a known transcription factor *ace1* leads to an interesting finding: the cellulase-repressing gene *ace1* is significantly downregulated (Fig. 4) in the 250-rpm fermenter. It was previously shown that ACEI from *Trichoderma reesei* [2, 22] and other *Trichoderma* species [26] represses cellulase and hemicellulase production. The expression of *ace1* was further shown to be repressed by Cre1, a known carbon catabolite repressor [19]. Previous work done in *T. reesei* showed that Cre1 is a phosphoprotein, and its phosphorylation state is closely linked with its activity [5]. Our analysis of the CreA in *P. decumbens,* a homologue of *T. reesei* Cre1, with a phosphorylation prediction software Kinasephos [9] revealed at least six potential mitogen-activated protein kinase (MapK) phosphorylation sites (T32, S179, S281, T300, T304, T308) and one potential casein kinase II (CKII) phosphorylation site (S263). Both MapK and CKII are known members of the signal transduction cascades in filamentous fungi and are involved in relaying external signals sensed by membrane signal receptors such as the G-protein coupled receptors to the nucleus for transcriptional regulation [21]. These results lead to the suggestion of a mechanism for the induction of cellulase genes under higher agitation: The cells sense the external signal following improved agitation, which is possibly the improved oxygen level, and initiate the signal relay process by phosphorylating downstream kinases, which eventually leads to the phosphorylation of CreA. The activated CreA further represses the expression of *ace1*, which leads to improved cellulase production.

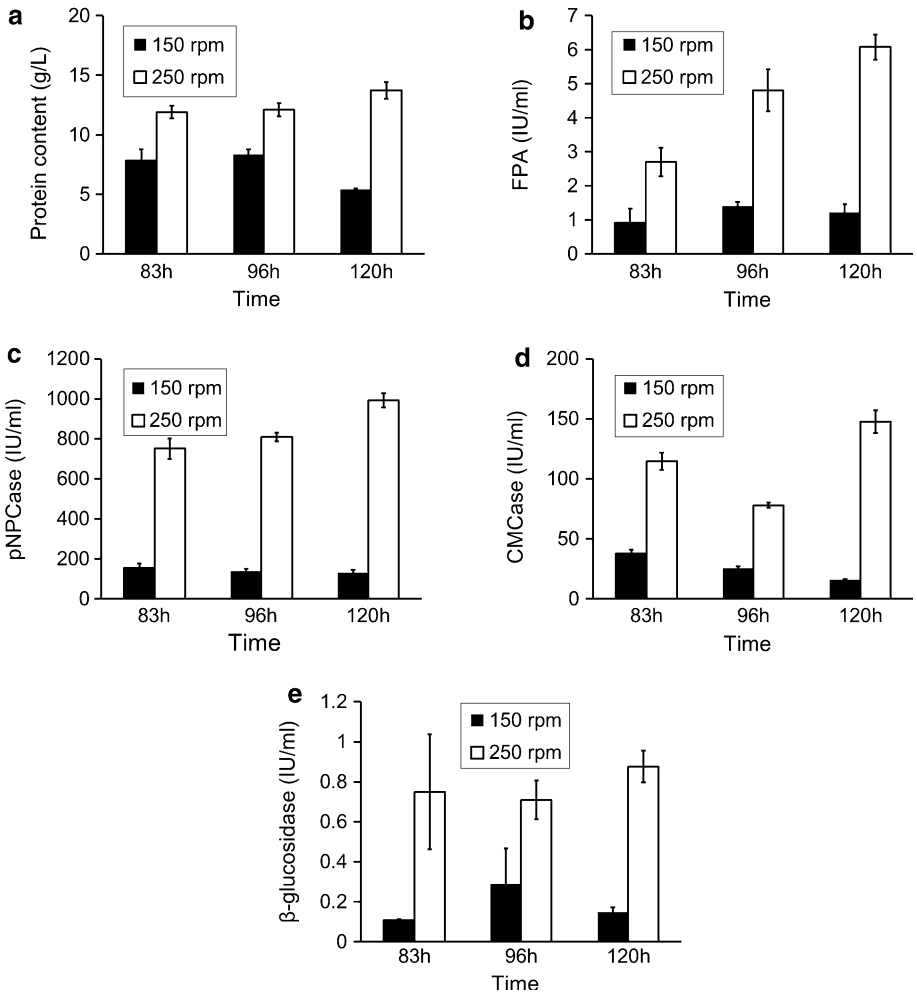

Fig. 2 Cellulase production from *P. decumbens* JUA10-1 grown in 3-l fermenters with agitation of 150 and 250 rpm. **a** extracellular protein levels, **b** FPA levels, **c** *p*NPCase levels, **d** CMCase levels, **e** β-glucosidase levels

It has to be noted that CreA regulates the expression of a series of transcriptional regulators in filamentous fungi, which include the cellulase-activating XlnR [22, 23]. In *P. decumbens*, assuming a similar regulatory mechanism, the activation of CreA will lead to the repression of *xlnR*. Indeed, a downregulation of *xlnR* transcription is observed in the fermenter with an agitation rate of 250 rpm (Fig. 4). CreA would therefore play a role in both the derepression and deactivation of cellulase production, and the relative levels of these two effects determine the final cellulase transcription level. A similar regulatory mechanism is present in *T. reesei*, in which both ACEI and the cellulase-activating transcription factor Xyr1 are subjected to Cre1-mediated carbon catabolite repression [17, 19].

Summarizing factors involved in agitation-mediated improvement of cellulase production

Our analysis of cellulase production by *P. decumbens* JUA10-1 reveals the activities of all major cellulases improved with the increase of agitation rate up to certain level. Further investigations show that at least three factors are involved in this improvement: (1) increase of biomass accumulation; (2) improvement of proportions of active/mature proteins; (3) improved transcription of cellulase-coding genes. The observed downregulation of transcription factors *ace1* and *xlnR* further shed light to the regulatory mechanism of agitation-mediated cellulase upregulation, and a model was suggested that the competition between CreA-mediated derepression and deactivation determines the final change of cellulase transcription. With these results, we identified reasons for the enhancement of cellulase production coupling to improvement of agitation rates, which was rarely reported. In particular, the suggestion that improved proportion of active/mature cellulases accounts for the improvement of secreted cellulase activities is an interesting new finding. Our further analysis of the molecular mechanism underlying improved cellulase synthesis identified new roles of previously identified transcription factors in response to increased agitation rates

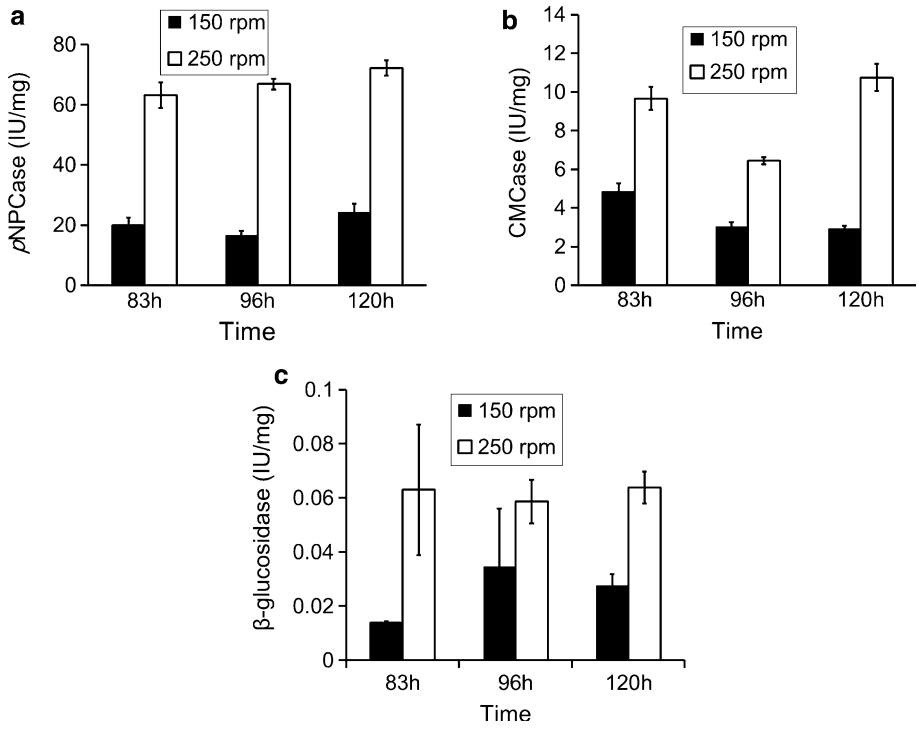

Fig. 3 Specific cellulase activities in fermenters with agitation of 150 and 250 rpm. **a** specific *p*NPCase activities, **b** specific CMCase activities, **c** specific β-glucosidase activities

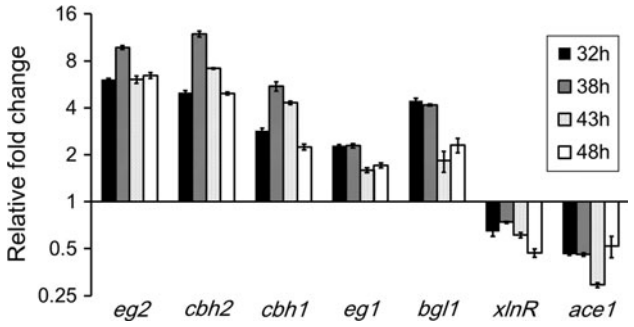

Fig. 4 Transcriptional response of cellulase-coding genes and transcription factors to improved agitation. *y*-axis indicates the relative transcriptional change of the culture stirred at 250 rpm to the culture stirred at 150 rpm

and led to the identification of novel processes these transcription factors are involved in.

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Conflict of interest The authors declare they have no conflicts of interest.

Ethical standards The authors declare all the experiments performed in this study comply fully with the current laws of the country in which it was performed, which is the People's Republic of China.

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