Egypt. J. Chem. **Vol. 66**, No. 10 pp. 163 - 177 (2023)

Egyptian Journal of Chemistry

http://ejchem.journals.ekb.eg/

The Study of the Industrial Aptitude of *Aspergillus fumigatus* Strain for Xylanase Production

Maroua GARES [1], HILIGSMANN Serge [2,3], Mahmoud KASSEM AL SAYED [2], Wiem ALLOUN [1] and Noreddine KACEM CHAOUCHE [1]

[1]*Affiliation 1 Laboratory of Mycology, Biotechnology and Microbial Activity (LaMyBAM). Department of Applied Biology, FSNV, University of Brothers Mentouri, 25017 Constantine, Algeria*

[2]*Affiliation 2 3BIO-BioTech, Université Libre de Bruxelles ULB, Av. F. Roosevelt 50, CP 165/61, Brussels-Belgium*

[3]*Affiliation 3 Biomass Valorisation Platform, Celaborscrl, Avenue du Parc 38, 4650 Herve, Belgium- Belgium*

Abstract

The major microorganisms used in the industry are fungi. The production of fungal metabolites on a large scale needs the use of an industrial-scale efficient strain. Indeed, the microorganisms used in an industrial process must be capable of doing more than simply producing the desired product in high yield. In fact, one of the main bottlenecks in the fermentation procedure is the scaling-up process. Indeed, reproducing the best conditions for microorganism growth and metabolism achieved in small cultures when transferring them to a much larger fermentation scale is nearly impossible. *Aspergillus fumigatus* (ON226990) performance was firstly evaluated in large-scale cultures; batch fermentation using simple sugar (xylose) as substrate in various volumes; 250 mL flasks, 2 L and 20 L bioreactors. The effect of agitation speed on the morphological aspects of this strain was also investigated. Subsequent fermentations were carried out with optimal agitation, using xylan from corncob as substrate in 250 mL flasks and a 2 L bioreactor. Finally, the strain's ability to use low-cost substrate such as Alfa biomass for xylanase production was evaluated as a biotechnological application.

Keywords: Bioreactor, Submerged fermentation, Scale-up fermentation, *Aspergillus fumigatus*, Mycelium morphology.

1. Introduction

Filamentous fungi have been cultivated for thousands of years. They are excellent engineered microbes, also known as cell factories for production of a variety of products. Currently, antibiotics, proteins, organic acid and additional pharmaceutical products are carried out using metabolites derived from the filamentous fungi [1,2]. Indeed, these microorganisms are becoming more important in the industrial synthesis of enzymes, as they create more than half of all industrial enzymes [3].

The use of modern tools of biotechnology and a convenient fermentation physiology are needed to achieve successful large-scale production [4]. The microorganism exploited in the industry must to be (1) capable of growth and produce the product of interest in large-scale culture, (2) produce spores (for an easy inoculation into large bioreactors), (3) grow and produce the desired product in a quite short period of time, (4) grow in a liquid culture medium that can be obtainable and purchased in bulk at a low cost, (5) capable to use a wide range of low-cost carbon sources, (6) suitable for genetic manipulation, increased yields are frequently obtained through genetic engineering; a genetically stable and easily engineered microorganism is clearly advantageous for an industrial process, (7) should not be pathogenic [5–7].

Filamentous fungi are in many industries the preferred production hosts of different proteins, especially enzymes. Xylanases production by filamentous fungi exhibit more intriguing properties

*Corresponding author e-mail: maroua.gares@umc.edu.dz.; (GARES, Maroua).
EJCHEM use only: Received date **14 November 2022**; revised date 21 January 2023; accepted date 04 February 2023
DOI: 10.21608/EJCHEM.2023.174799.7195

for industrial use, these microorganisms have a higher potential for protein production than most other organisms [8]. For this enzyme production, the genus Aspergillus has been extensively studied [9–13].Native strains or engineered strains are used for the production of proteins in highly specialized fungal hosts. Filamentous fungi have distinct advantages over other recombinant protein expression systems [3,14]. Because of their powerful secretory pathways and ability to correctly perform various post-translational processing of eukaryotic proteins, filamentous fungi are preferred over bacterial or yeast hosts [15]. The CRISPR/Cas9 procedure has been successfully applied to metabolic engineering of industrial fermentation strains such as A. nidulans, A. nigerand A. oryzae[16], e.g. the optimization of xylanase production using an Aspergillus nidulans modified by integration of an AFUMN-GH10 gene from A. fumigatus var. niveus, which resulted in high-yield secretion and protein accumulation [17].

A. fumigatus is known as a suitable fungus for the production of heterologous proteins [10,11,18–22]. Despite its pathogenicity, the high enzyme production rates justify the growing interest in this fungus. Indeed, extremely efficient CRISPR-mediated genome editing was studied on A. fumigatus [16,18,23–25]. Furthermore, deletion of genes encoding enzymes of melanin biosynthesis, encoding transcription factors triggering production of secondary metabolites, i.e. gliotoxin, or encoding extracellular proteases and siderophore synthesizing enzymes might result in non-pathogenic strains [21]. Furthermore, the entire genome of A. fumigatus has been sequenced, therefore, this strain is amenable to molecular procedures [26,27].

In this paper, A. fumigatus has been evaluated for its potential as an efficient cell factory. Firstly, the fermentation behavior of A. fumigatus in submerged fermentations (SmF) scale-up was evaluated in order to test its ability to grow in large-scale cultivation using simple and complex sugars, and also to define its logarithmic phase for further exploitation e.g. large production of metabolites of interest as xylanase. Finally, Alfa (Stipa tenacissima) based culture was used as low-cost carbon source for the growth and production of xylanase enzyme since this biomass is considered as quite promising for instance in mediterrean countries [28].

2. Experimental

2.1. Differentcarbonsourcesfermentations

The strain of A. fumigatus (ON226990) was provided by LaMyBAM laboratory. This fungus was newly isolated from soil and screened for its lignocellulolytic enzymes activity, then, identified based on its morphological characteristics and molecular analysis.

To test the ability of this strain to grow and consume various substrates, 250 mL Erlenmeyer flask containing 100 mL of liquid medium 10 g/L of simple and complexes substrate; Glucose, Xylose, Xylan from beechwood (BWX) (SIGMA) and xylan from corncob (XC) (ROTH) were inoculated with 1 mL of a spore suspension (1.106 spore/mL). The tubes were placed in an incubator with agitation at 140 rpm at 30° C for 5 days. The soluble and insoluble molecule concentration was determined by HPLC-RID and expressed in g/L.

2.2. Scale-up processing using xylose substrate; batch submerged fermentation in 250 mL shake flasks, 2 and 20 L bioreactor

Submerged fermentation was conducted using xylose substrate in order to test the ability of this fungus to develop in a submerged medium in different volumes and also to study its fermentation profile.

2.2.1. Biomass Growth and pH

The culture broth was separated from the mycelium by filtration using a vacuum pump and filter paper (Whatman N◦ .1); the mycelium was dried in an oven at 60◦ C until constant weight according to AOAC standards. Mycelial biomass concentration was determined by grams of dry weight per liter of liquid culture (g/L).

During the fermentation period, samples were collected, and subsequently the pH was measured with a pH meter for the flask cultures and a pH sensor for the bioreactor fermentation.

2.2.2. Xylose quantification

Samples of 5 mL collected each day of fermentation were filtrated and centrifuged at 14,000× g for 15 min. The supernatant obtained was separated from the residual biomass at 4◦ C and finally, the soluble molecule concentration (xylose) was determined by HPLC-RID.

2.2.3. Preparation of standard inoculum for A. fumigatus fermentation in shake flask culture

Spore suspension was prepared from five-day-old cultures of *A.fumigatus* on potato dextrose agar (PDA) at 30 °C. Conidia were harvested by adding 10 mL of sterile distilled water containing 1% (v/v) tween 80, then collected in sterile flasks to be used as inoculum for further cultures. The spore concentration was adjusted to 1× 106 spores/mL. Then, the culture flasks with a working volume of 100 mL medium were prepared as the following (gram per liter): 10 xylose, 10 peptone, 10 yeast extract in a 250 mL culture flask. The initial medium culture pH was 6.5 without adjustment. Then, the culture medium was sterilized at 121 °C for 20 minutes before inoculation.

A volume of 1mL of fresh conidia suspension was inoculated into the 250-mL Erlenmeyer flasks and finally incubated in a shaking incubator (200 rpm) (Ecotron, INFORS HT, Switzerland) at 30 °C.

2.3.4. Preparation of standard inoculum for A. fumigatus fermentationin 2 L and 20 L bioreactors

To perform the fermentation scale up in 2 L and 20 L bioreactors, a standard inoculum size of 1×106 spores/mL was inoculated into appropriate corning Erlenmeyer flasks with 150 mL and 1.5L working volume respectively and incubated for 12 h at 120 rpm and 30°C. The flasks transfer caps have two ports. One port ends in a 0.2 µm filter, and the other port is connected with a dip tube that reaches all the way to the bottom of the flask for an easy aseptic transfer of the pre-culture.

The culture medium for both bioreactors was prepared as described in the previous part, the carbon source was prepared and autoclaved separately from other medium compositions. A temperature of 30 °C was adjusted, and the inoculation process started when the adjusted temperature was reached. The incubation period was stopped when the substrate was completely consumed (stationary – decline phase).

Before inoculation, the corresponding vessels and important accessory elements of the fermenter were sterilized. The 2L bioreactor sterilization was conducted in a proper laboratory autoclave, the 20 L is a sterilize-in-place bioreactor. The PO2 and pH sensors were calibrated before inoculation; the pre-cultures were transferred with peristaltic pump intothe 2 L and 20 L (Sartorius Stedim, D) bioreactors respectively. The 2 L bioreactor was agitated at 200 rpm and aerated at 1 vvm. Agitation speed and aeration rate in our study were set in a way to maintain kLa constant during scale-up from 2 to 20 L. Antifoam solution was used when necessary. Samples from pre-culture and main-culture were tested for contamination.

2.4. Agitation speed impact on the fermentation process and morphological changing of *A. fumigatus*

Cultivation was carried out with a 2 L bioreactor with the same submerged medium and pre-culture concentration at an agitation speed of 200 rpm. After 24 hours, the stirring speed was increased. Fungal mycelia samples were harvested from various agitation speeds (350, 500 and 600 rpm). 1 mL of these samples were further inoculated into 100 mL shake flasks containing the same liquid medium but with a xylose concentration of (2g/L), and incubated for 5 days. The substrate consumption was analyzed and the morphological change of samples was viewed under a light microscope.

2.5. Scale-up processing using xylan from corn cob; batch submerged fermentation in 250 mL shake flasks and 2 L bioreactor

Submerged fermentation in 250 mL and 2 L bioreactor were conducted to explore the ability of A. fumigatus strain to consume and develop in a complex substrate using XC. The substrate consumption and xylose liberation were measured. The agitation rate was set based on the previous experience's results. The biomass production and pH measurement were investigated as described before.

Xylanase activity measurement by HPLC

To test the enzymatic activity, the previous bioreactor supernatant is collected and divided into 15mL tubes (8mL in each tube), each containing 1mLof 0.1 M phosphate buffer at pH 5.5 (12.9 g KH_2PO_4; 0.63 g Na_2HPO_4; 0.5 g NaN_3), with 0.1 g of substrate (BWX , XC) in triplicate. Control tubes (supernatant + buffer, and substrate + water) were included in the test. To prevent microbial growth, 1mL of 0.5% sodium azide (NaN_3) (to achieve a concentration of 0.05% sodium azide in the medium) was added to each tube.

The tubes were placed in a 40° water bath for 3 days with stirring at 60 rpm. The tubes were tightly closed and laid down in the direction of the incubator

tray's movement (Grant, OLS 200). Just after the incubation period, the tubes were centrifuged at 8000 rpm for 10 minutes (Universal 32, HETTICH Zentrifugen, Germany), the liberated sugars were analyzed with HPLC. All analyses were carried out in triplicate; the data arepresented as the means of three replicates, along with their standard deviations.

2.6.Xylan from beechwood for the production of xylanase by *A. fumigatus*

Submerged fermentation (SmF) was used to produce xylanase enzyme from XBW substrate. 250 mL flasks with a working volume of 100 mL of liquid media containing 10g/L of XBW were prepared and sterilized at 121 °C for 20 minutes before being inoculated with a spore suspension containing 1×106 spore/mL. The cultures were then incubated at 30° C in a shaking incubator (200 rpm). The flasks were removed on a daily basis to determine substrate consumption and enzymatic activity.

2.7.Low-cost carbon source for the production of xylanase by *A. fumigatus*

Alfa (Stipa tenacissima) was used as a low-cost carbon source substrate to test the capacity of A. fumigatus to develop on low-cost substrate and to produce xylanase enzyme ; 500 mL Erlenmeyer flask containing 10 g of Alfa humidified at a ratio of 89% with a modified minimal medium (in g/L); peptone; 0.1g/l, NaNO3, 1.2; KH2PO4, 3.0; K2HPO4, 6.0; MgSO.7H2O, 0.2; CaCl2, 0.05; MnSO4•7H2O, 0.01; ZnSO4•7H2O, pH 4.5 [29].

After sterilization, the prepared media were inoculated with a spore suspension of 107 spores/g of dry matter and finally incubated at 30 °C. Every 24hours, the flasks content was recovered with 100 mL of sterile distilled water and mixed with a spatula. The filtrate was centrifuged at 8000 rpm for 15 min at 4 °C. Finally, the supernatant was used as a crude extracellular enzyme for xylanase assay [30].

Xylanase activity measurement with DNSA method

Xylanase activity in SSF using Alfa and SmF using XBW substrate was quantified by measuring the amount of reducing sugars released from XBW using the 3,5-dinitrosalicylic acid reagent (DNSA) [31]. 100 µL culture supernatant was mixed with 900 µL Xylan solution (Sigma-Aldrich) (1%, 50 mM sodium phosphate buffer; pH 4) and incubated for 5 minutes at 50 °C. The reaction was then stopped with 1.5 mL DNSA reagent [32]. The treated samples were heated in a boiling bath (100°C) for 5 minutes, the assay also included the appropriate enzyme and substrate controls.

By measuring the absorbance at 540 nm and comparing it to a xylose standard curve (0 to 0.01mol/mL), the amount of reducing sugars released from the reaction was estimated. One enzyme unit was defined as the amount of enzyme required per 1 mL to liberate 1 µmol of reducing sugar from a substrate per minute under the specified assay conditions, expressed as U/g. All experiments were performed in triplicate, the data are expressed as the mean ± standard deviation and presented in the kinetic graph.

3. Results and discussion

3.1. Different carbon sources fermentations

The carbon source is one of the most critical components for industrial fermentations. The carbon sources used in the industries are typically polysaccharides. In nature, Fungi use a diverse set of extracellular carbohydrate enzymes (CAZymes) to degrade plant biomass polysaccharides to monomeric sugars. The subsequent sugars are transported into the cell and converted to energy or biomolecule precursors through a range of metabolic pathways [33].

Several hydrolytic enzymes are required; Endo-1,4-β-xylanase (EC 3.2.1.8) is a key enzyme in xylanolysis, converting polymeric xylan into a mixture of xylo-oligosaccharides that are then hydrolyzed into xylose units by xylan 1,4-βxylosidase[34]. Indeed, for the degradation of BWX (substrate for endo-1,4-β-Xylanase) and MX (Substrate0. for β-1,4- xylosidase), both enzymes are needed.

According to the HPLC analysis and as demonstrated in Figure 1, the strain was able to produce the effective enzyme for xylan degradation and consumed more than 65.20% and 77.23% of BWX and MX, respectively. On the other hand, glucose and xylose monomers are directly consumed by the fungus; indeed, by the end of fermentation, these two substrates had been completely consumed 99.58% and 99.89% for glucose and xylose, respectively.

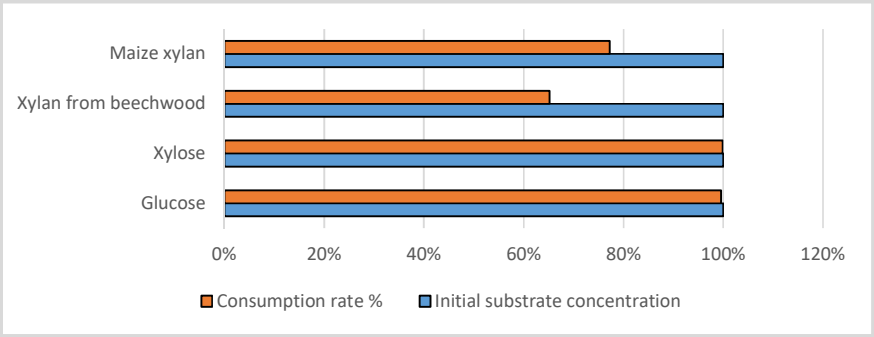

Figure.1: Different substrate consumption by *Aspergillus fumigatus* strain after 5 days of fermentation (100% initial concentration is related to 10 g/L of substrate in the culture medium before inoculation)

3.2. Scale-up processing using xylose substrate; batch submerged fermentation in 250 mL shake flasks, 2 and 20 L bioreactors

3.2.1.250 mL flask culture

Many studies have reported that the best inducers for xylanolytic enzyme production in *Aspergillus spp*., are xylose, xylan and crude xylan-containing substrates [35]. In the current study, xylose was used as the only carbon source to study *A. fumigatus* fermentation profile and to spot its logarithmic phase. The growth profile of dry mycelial cell mass and substrate concentration were presented in (Figure 2 A). The substrate was almost completely consumed after 96 hours of fermentation accompanied by a maximal biomass production (11.81 g/L); the exponential phase was recorded between 24 and 48 hours of fermentation. The stationary phase was observed between 48 h and 96 h after the biomass decreased rapidly due to the negative effect.

Concerning the pH; it dropped from 6.6 to 5.9 when the biomass reached its maximal production and consumed almost all the substrate. This decrease may be due to the production of metabolites derived from xylose consumption. These types of fungal strains produce secondary metabolites such as organic acids and enzymes (e.g., xylanase, cellulases, pectinases, proteases, amylases etc.,) [36,37]. The subsequent increase in pH after 96 hours of fermentation, correlates with the depletion of substrate in the medium. Causing an accumulation of nitrogenous wastes, which lead to an increase in the pH (7.04)[38].

3.2.2. 2 and 20 L bioreactors

Submerged batch cultures are commonly characterized according to the classical growth curve in textbooks (i.e., adaptation, acceleration, exponential, stationary, and declining phase). Although, online respirometry proved to be a powerful tool to distinguish growth phases and revealed more physiological states than expected from the mere biomass curve [39]. In the current research, the monitoring system allows continuous tracking of pH and PO_2 in every 05 and 04 min for 2L and 20L bioreactors respectively, which makes the analysis more efficient.

According to the PO_2 (%) curve figure 2 (B and C), the logarithmic phase of the both 2L and 20L bioreactors was recorded between almost 10 and 24 h. The growth profile of dry mycelial cell mass and substrate concentration were also presented in Figure 2 (B and C). The substrate was completely consumed after 72 hours of fermentation in the 2L bioreactor and only 30 hours in the 20L bioreactor, the maximal biomass production of 16.22 g/L and 12.9 g/L was

reached after 72 and 30 hours of fermentation for the bioreactor of 2L and 20 L respectively.

The pH of the culture medium followed the same trajectory as that of the flask culture; it dropped also to almost 5.7 in the both bioreactor cultures when the biomass reached its maximal production, and then, it started to increase at the end of the fermentation.

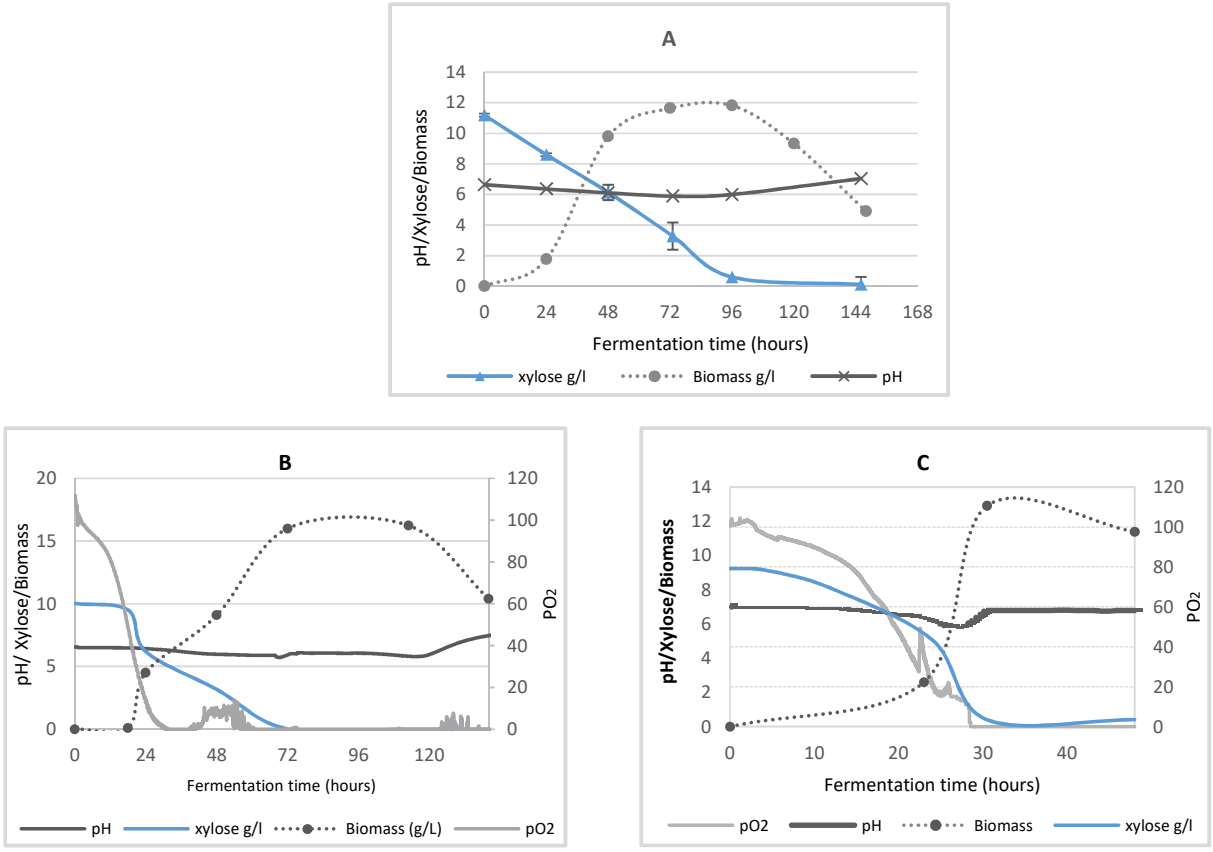

Figure.2: Fermentation profile of *Aspergillus fumigatus* under submerged fermentation using xylose substrate in 250 mL flasks (A) (xylose consumption points were the mean values, and the standard deviations were represented as error bars), 2 L bioreactor (B) and 20 L bioreactor (C)

3.3. Agitation speed impact on the fermentation process and morphological changing of *A. fumigatus*

The morphology of the filamentous fungi in SmF is mainly influenced by the culture environment and the strain employed. There have been numerous studies looking into how the hyphae's morphology affects the production of biomass and secretion of proteins [40–42]. Since, these microorganisms are not single-celled but filamentous, and fungal hyphae grow heterogeneously in liquid broth, the filamentous fungi show diverse macro-morphology such as hypha pellets (Figure 4). These mycelial aggregates interfere with the substrate uptake, causing metabolic limitations and decreasing production efficiency. This research study prevented these macro-pellets' formation by three steps: (1) the inoculation of the pre-culture with concentrated conidia and decreasing its incubation time, (2) short term culture and (3) high agitation velocity.

Achieving a homogeneous medium in SmF can result in efficient mass transfer and a high quality and

quantity of production. Many studies have sought optimal conditions for agitation,because mycelial damage at high stirrer speeds or power inputs can limit the capability and volumetric productivity of a bioreactor [43]. According to Ghobadi et al., [43], high agitation can cause a huge effect on microorganisms such as cell damage, morphological changes, and variations in growth rate and product formation. For each submerged culture, the optimal conditions for agitation depend partly on the resistance of hyphae to the mechanical forces and also on its physiological state [44]
.

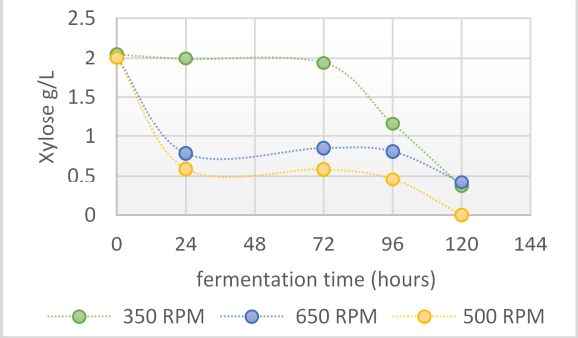

Figure.3: Agitation speed impact on the fermentation profile of *Aspergillus fumigates*

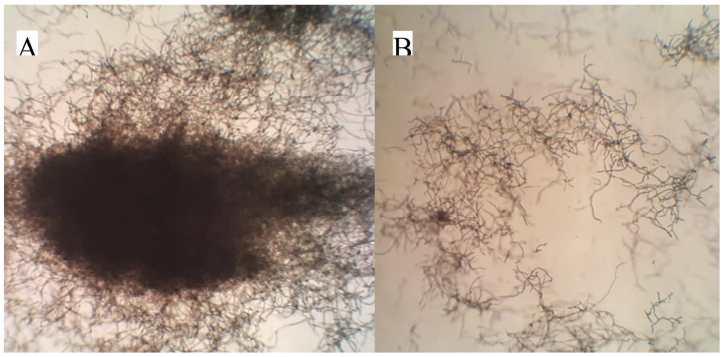

Figure. 4: microscopic morphology of *Aspergillusfumigatus* flocs (pellets) in stirred vessel cultivation at 200 rpm (A) and "shear effect" at 650 rpm (B)

In the present study, agitation speed impact on *A.fumigatus* morphology was found to accelerate substrate consumption. However, the fermentation process is slightly reduced with 650 rpm samples (figure 3). Macroscopic morphology at the end of the incubation period showed that hyphae were not attached to the fermenter as in (Figure 5 A), but forming free micro-pellets (Figure 5 C). Indeed, in fine-dispersed mycelia, each hyphal filament is reached by the convection of the stirred medium [21].However, in macro-pellets, gas exchange as well as transport of substrates and products is hindered by pseudo-tissue. This may be the cause of the exponential phase delay for 350 rpm samples.

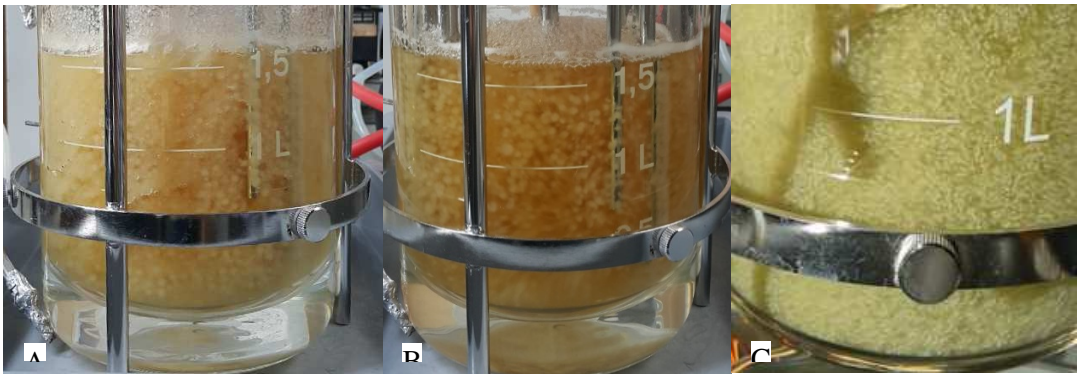

Figure.5: Macroscopic morphology of *A. fumigatus* flocs (pellets) in stirred 2L bioreactor at the end of cultivation process, at 200 rpm (A), 350 rpm (B) and 500 rpm (C).

3.4. Scale-up processing using xylan from corncob; batch submerged fermentation in 250 mL shake flasks and 2 L bioreactor

3.4.1 250mL flask culture

The dry mycelial cell mass growth, complex substrate concentration (XC), and monomer release (Xylose) were all presented in (Figure 6 A). Unlike xylose substrate which was completely consumed after 96 hours, the strain *A. fumigatus* was unable to degrade and consume all the substrate even after 240 hours (data not shown). The maximal biomass production (9.47 g/L) was recorded after 96 hours of fermentation. The fungus began to degrade the oligosaccharides in XC via the action of xylan 1,4-β xylosidase to monomer of xylose as shown in (Figure 6 A). The medium saturation let to the pH increase (almost 8) which affected the growth of the mold.

3.4.2. 2L bioreactor culture

In batch cultures, all concentrations change minute by minute. In order to minimize diffusion barriers, high conidia concentrations were used as inoculum for the pre-culture (1×10^8). The short fermentation time avoided macro-pellet formation. Furthermore, in this kind of cultures, oxygen limitation is a very important aspect to be taken into account due to the high viscosity of the fungal broth that results in a much lower gas-liquid mass transfer coefficient (kLa) in comparison to unicellular microorganism fermentation [45].

The agitation speed in this fermentation was set at 200 rpm for the first 15 hours, then increased to 600 rpm to prevent macro pellet formation. After only 45 hours of fermentation, the substrate was completely consumed, and the maximum biomass production of 9.86 g/L was recorded. The pH of the culture medium dropped to almost 5.1 after 24 hours and started to increase at the end of the fermentation reaching 7.3.

Despite its complexity, XC consumption was faster comparing to xylose substrate consumption in the previous fermentation, which was most likely due to optimal agitation and an adequate dissolved O_2 level in the medium (figure 6 B). Dead biomass increases along with biomass production; higher viscosity leads to poorer oxygen transfer, which may result in a decrease in productivity. Indeed, oxygen limitation is a common cause of low level production of aerobic products like enzymes. To circumvent this limitation, Fratebianchi et al., [46] controlled the effects of airflow and agitation speed to enhance polygalacturonase production in SmF by *Aspergillus*

sojae. And found that agitation of 600 rpm and cascading airflow from 1 up to 1.7 vvm, fixed the oxygen limitation problem, therefore, maximum enzyme production was achieved. Same study reported that higher agitation speed causes microorganisms stress leading to a decrease in the enzymatic production. A corresponding reaction was previously reported regarding recombinant protein production by *A. niger* in SmF bioreactors [47,48]. Bandaipeth and Prasertsan[49] reported that increasing the airflow is a better choice than increasing agitation speed in order to get high kLa values and avoid microorganisms stress. Another study conducted by Bakri et al., [50] recorded a maximum xylanase activity at agitation speed of 200 rpm and aeration rate of 1.0 vvm. Although, in this agitation speed, macro-pellets formation is inevitable. In summary, the fermentation carried out at higher agitation speeds (more than 500 RPM) and higher conidia concentrations for the pre-culture (1×10^8) were more effective, and we cite: accelerated development, reduced biomass formation, medium homogeneity, improved aeration, smaller pellet formation, and shorter fermentation times.

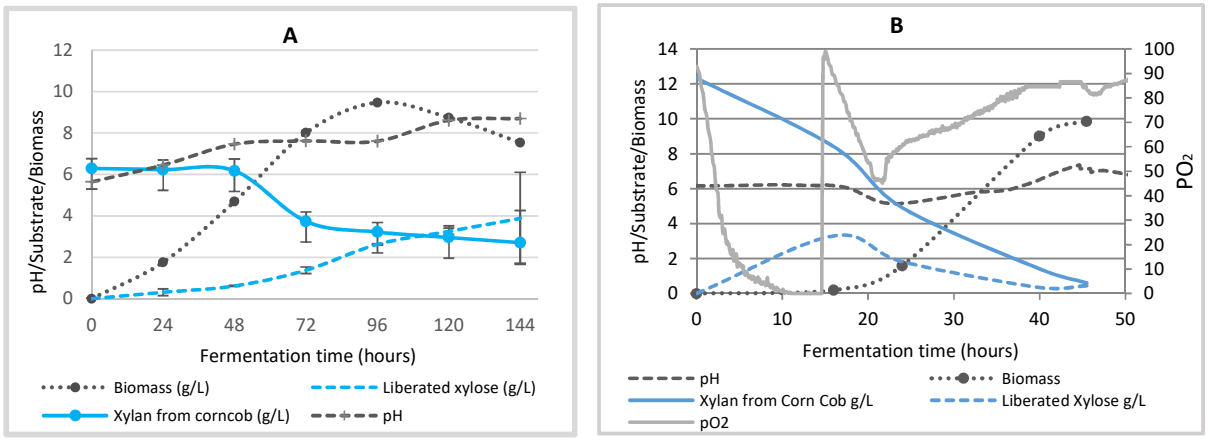

Figure. 6: Fermentation profile of *Aspergillus fumigatus* under submerged fermentation using Xylan from corncob substrate in 250 mL flasks (A) and 2 L bioreactor (B) (the corncob xylan consumption and the liberated xylose points were the mean values, and the standard deviations were represented as error bars)

3.4.3 Xylanase activity in the culture broth

A. fumigatus hydrolytic potential was tested using the previous bioreactor aseptic supernatant for the hydrolysis of BWX and XC. Products formed during the hydrolysis of the both substrate and the culture supernatant containing the enzyme were analysed by HPLC (Figure 7). After the incubation period, 5.88 ± 0.34 g and 6.20 ± 0.08 g of xylose was liberated from 10g of BWX and XC respectively (Figure 8).

ßxylosidase for the liberation of D-xylose fromBWX and MX.

Several industries require a cocktail of enzymes rather than a single enzyme, as the latter will not perform as well in many applications, such as biomass hydrolysis or detergent development [51]. One of the most difficult challenges remains the creation of a low-cost cocktail. Single microorganisms capable of producing multiple enzymes are required, as are protocols for the concurrent production of multiple enzymes [52]

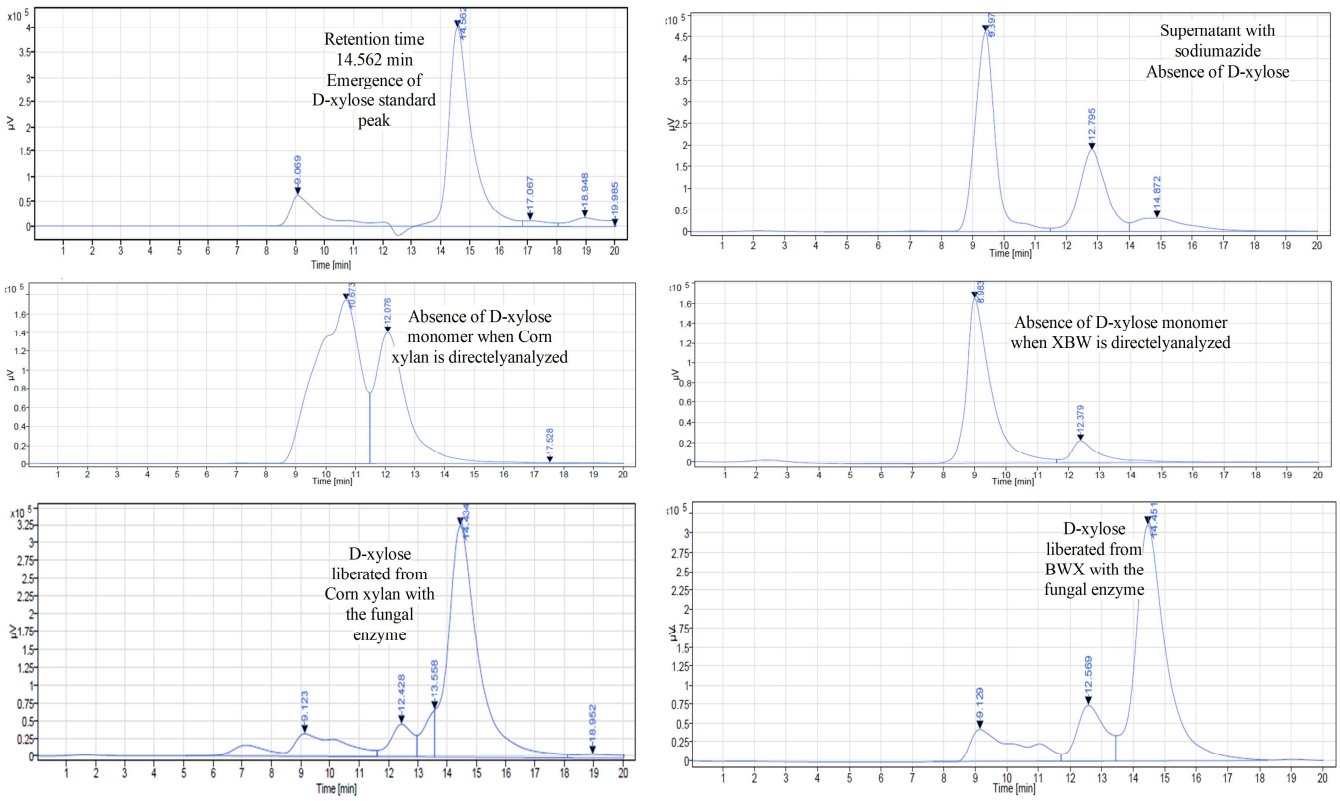

Figure. 7: HPLC analysis of D-xylose released from Xylan Beachwood and Corn Xylan by thebioreactor supernanant

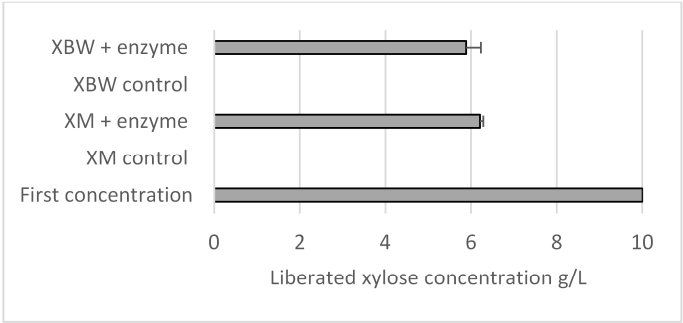

Figure. 8: Enzymatic hydrolysis of BeechWood Xylan and Xylan from Corncob with the bioreactor supernatant

3.5. Xylanase production under SmF and SSF

The logarithmic phase of xylanase production under SmF and SSF is an important step for further large scale exploitation. (Figure 9) shows the xylanase production by *A. fumigatus* kinetic under SmF using XBW and Alfa as substrate for SSF. In SmF, the xylanase activity was detectable after 48h of fermentation and increased to attain a maximum production of 88.49 IU/mL after 4 days. Coinciding with the exponential phase of biomass production, which was recorded between 48 and 72 hours of fermentation. On the other hand. The xylanase

under SSF activity was detectable after only 24h of incubation and increased rapidly to attaint maximum activity of 85. 24 ± 1.13 IU/mL after five days of fermentation and decreased thereafter. This decrease could be related to the fermentation medium saturation with metabolite and the reduction of nutrients along time, which could impact fungal physiology [53]. Abdel-Sater and El-Said., Thomas et al., and Singh et al., reported a maximum production of xylanase in 8, 4, and 7 days with *Trichoderma harzianum,* and *Aspergillus sp*. SH-1 and SH-2 strains respectively [30,53,54]. Other studies conducted by Ang et al., and Desai et al., achieved maximum xylanase production after only two days of incubation with *A. fumigatus* F-993 and *Aspergillus flavus* under SSF respectively [10,55].

In several studies, the performance of SSF and SmF has been compared. The xylanase production by *A. foetidus*and *A. niger* were lower in SSF than SmF[56,57]. A similar result has been recorded for different organisms used for xylanase production on different substrates [58,59], which is in agreement with our results.

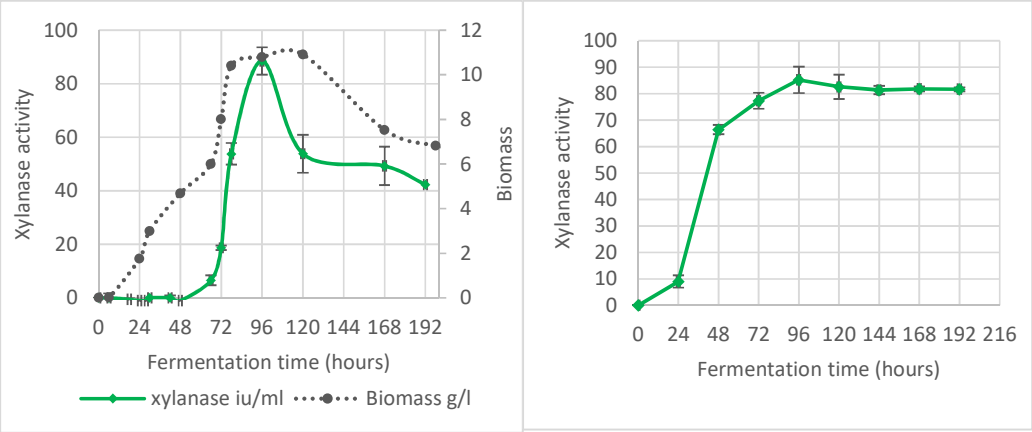

Figure.9: Kinetic of xylanase production by *Aspergillus fumigatus* using beechwood xylan and Alfa as substrate under liquid and solid state fermentation

4. Conclusion

Aspergillus fumigatus has the potential to be a cell factory for future production of industrial enzymes. This strain, proved its ability to use a various carbon sources and low-cost sources for the production of xylanase. The morphology of filamentous fungi has a significant impact on productivity and the supply of substrate for the culture. Though, cultivation parameters that result in the desired morphology are frequently chosen empirically as the mechanisms leading the processes involved are frequently unknown. High conidia concentrations were used as inoculum and the influence of shear forces on the process, caused by agitation, has been studied. For microorganisms like *A. fumigatus*, the morphological growth is thought to start from spores and progress to aggregations right after inoculation. This research study prevented the macro-pellets formation ensuring medium homogeneity.

5. Conflicts of interest

We have no conflicts of interest to disclose.

6. Formatting of funding sources

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The research received a financial support of Wallonie-Bruxelles International (WBI, Belgium)

7. References

[1] Kumar, V., Singh, S., Singh, J. Fungal Biotechnology: Role and Aspects Current Perspectives. pp. 91–103 (2018).

[2] Lübeck, M., Lübeck, P. S. Fungal Cell Factories for Efficient and Sustainable Production of Proteins and Peptides. Microorganisms 10, 753 (2022). https://doi.org/10.3390/microorganisms10040753

[3] Nevalainen, H. *Grand challenges in fungal biotechnology*. (Springer, Springer Nature, 2020). https://doi.org/10.1007/978-3-030-29541-7

[4] Goyal, S., Ramawat, K. G., Mérillon, J.-M. Different Shades of Fungal Metabolites: An Overview. Fungal Metabolites (eds. Mérillon, J.-M., Ramawat, K. G.) pp. 1–29 (Springer International Publishing, 2017). https://doi.org/10.1007/978-3-319-25001-4_34

[5] Osho, D. A. INDUSTRIAL MICROBIOLOGY MCB 406.

[6] Waites, M. J., Morgan, N. L., Rockey, J. S., Higton, G. *Industrial Microbiology: An Introduction*. (Wiley, 2001).

[7] Casida, L. E. J. R. *Industrial Microbiology*. (NEW AGE INTERNATIONAL, 2016).

[8] Okafor, U. A., Okochi, V. I., Onyegeme-okerenta, B. M., Nwodo-Chinedu, S. Xylanase production by *Aspergillusniger* ANL 301 using agro - wastes. African Journal of Biotechnology 6, (2007). https://doi.org/10.4314/ajb.v6i14.57761

[9] Robledo, A., Aguilar, C. N., Belmares-Cerda, R. E., Flores-Gallegos, A. C., Contreras-Esquivel, J. C., Montañez, J. C., et al. Production of thermostable xylanase by thermophilic fungal strains isolated from maize silage. CyTA - Journal of Food 14, 302–308 (2016). https://doi.org/10.1080/19476337.2015.1105298

[10] Ang, S. K. Cellulases and xylanase production by '*Aspergillusfumigatus'* SK1 through solid state fermentation for ethanol fermentation. (Universiti Teknologi Malaysia, Faculty of Biosciences and Medical Engineering, 2015).

[11] Ang, S. K., E.M., S., Y., A., A.A, S., M.S, M. Production of cellulases and xylanase by *Aspergillus fumigatus* SK1 using untreated oil palm trunk through solid state fermentation. Process Biochemistry 48, 1293–1302 (2013). https://doi.org/10.1016/j.procbio.2013.06.019

[12] Azzouz, Z., Bettache, A., Boucherba, N., Prieto, A., Martinez, M. J., Benallaoua, S., et al. Optimization of β-1,4-Endoxylanase Production by an *Aspergillusniger* Strain Growing on Wheat Straw and Application in Xylooligosaccharides Production. Molecules 26, 2527 (2021). https://doi.org/10.3390/molecules26092527

[13] Azzouz, Z., Bettache, A., Boucherba, N. Biotechnological production and statistical optimization of fungal xylanase by bioconversion of the lignocellulosic biomass residues in solid-state fermentation. Biomass Conversion and Biorefinery (2020). https://doi.org/10.1007/s13399-020-01018-z

[14] Nevalainen, H., Peterson, R. Making recombinant proteins in filamentous fungi- are we expecting too much? Frontiers in Microbiology 5, (2014).

[15] Bergquist, P. L., Te'o, V. S. J., Gibbs, M. D., Cziferszky, A. C. E., De Faria, F. P., Azevedo, M. O., et al. Production of recombinant bleaching enzymes from thermophilic microorganisms in fungal hosts. Appl Biochem Biotechnol 98, 165–176 (2002). https://doi.org/10.1385/ABAB:98-100:1-9:165

[16] Jin, F.-J., Wang, B.-T., Wang, Z.-D., Jin, L., Han, P. CRISPR/Cas9-Based Genome Editing and Its Application in *Aspergillus* Species. Journal of Fungi 8, 467 (2022). https://doi.org/10.3390/jof8050467

[17] Abdella, A., Segato, F., Wilkins, M. R. Optimization of nutrient medium components for production of a client endo-β-1,4-xylanase from *Aspergillus fumigatus* var. niveus using a recombinant *Aspergillus nidulans* strain. Biocatalysis and Agricultural Biotechnology 20, 101267 (2019). https://doi.org/10.1016/j.bcab.2019.101267

[18] Zhang, C., Meng, X., Wei, X., Lu, L. Highly efficient CRISPR mutagenesis by microhomology-mediated end joining in *Aspergillus fumigatus*. Fungal Genet Biol 86, 47–57 (2016). https://doi.org/10.1016/j.fgb.2015.12.007

[19] Abdulmajeed, A. T. A., Şahin, S., Ozmen, I. Production and Purification of the Endoglucanase

Enzyme from Local Isolate *Aspergillus fumigatus* HBF356. Biointerface Res Appl Chem 12, 4337–4347 (2021). https://doi.org/10.33263/BRIAC124.43374347

[20] Das, A., Paul, T., Halder, S. K., Jana, A., Maity, C., Das Mohapatra, P. K., et al. Production of cellulolytic enzymes by *Aspergillus fumigatus* ABK9 in wheat bran-rice straw mixed substrate and use of cocktail enzymes for deinking of waste office paper pulp. Bioresource Technology 128, 290–296 (2013). https://doi.org/10.1016/j.biortech.2012.10.080

[21] Nieland, S., Barig, S., Salzmann, J., Gehrau, F., Zamani, A. I., Richter, A., et al. *Aspergillus fumigatu*s AR04 obeys Arrhenius' rule in cultivation temperature shifts from 30 to 40°C. Microbial Biotechnology 14, 1422–1432 (2021). https://doi.org/10.1111/1751-7915.13739

[22] Mondal, S., Soren, J. P., Mondal, J., Rakshit, S., Kumar Halder, S., Mondal, K. C. Contemporaneous synthesis of multiple carbohydrate debranching enzymes from newly isolated *Aspergillus fumigatu*s SKF-2 under solid state fermentation: A unique enzyme mixture for proficient saccharification of plant bioresources. Industrial Crops and Products 150, 112409 (2020). https://doi.org/10.1016/j.indcrop.2020.112409

[23] Al Abdallah, Q., Ge, W., Fortwendel, J. R. A Simple and Universal System for Gene Manipulation in *Aspergillus fumigatus:* In Vitro-Assembled Cas9-Guide RNA Ribonucleoproteins Coupled with Microhomology Repair Templates. MSphere 2, e00446-17 (2017). https://doi.org/10.1128/mSphere.00446-17

[24] van Rhijn, N., Furukawa, T., Zhao, C., McCann, B. L., Bignell, E., Bromley, M. J. Development of a marker-free mutagenesis system using CRISPR-Cas9 in the pathogenic mould *Aspergillus fumigatus.* Fungal Genet Biol 145, 103479 (2020). https://doi.org/10.1016/j.fgb.2020.103479

[25] Fuller, K. K., Chen, S., Loros, J. J., Dunlap, J. C. Development of the CRISPR/Cas9 System for Targeted Gene Disruption in *Aspergillus fumigatus*. Eukaryotic Cell 14, 1073–1080 (2015). https://doi.org/10.1128/EC.00107-15

[26] Kjærbølling, I., Vesth, T., Frisvad, J. C., Nybo, J. L., Theobald, S., Kildgaard, S., et al. A comparative genomics study of 23 *Aspergillus* species from section Flavi. Nat Commun 11, 1106 (2020). https://doi.org/10.1038/s41467-019-14051-y

[27] Puértolas-Balint, F., Rossen, J. W. A., Oliveira dos Santos, C., Chlebowicz, M. M. A., Raangs, E. C., van Putten, M. L., et al. Revealing the Virulence Potential of Clinical and Environmental *Aspergillus fumigatus* Isolates Using Whole-Genome Sequencing. Frontiers in Microbiology 10, (2019).

[28] Gares, M., Hiligsmann, S., Kacem Chaouche, N. Lignocellulosic biomass and industrial bioprocesses for the production of second generation bio-ethanol, does it have a future in Algeria? SN Appl. Sci. 2, 1680 (2020). https://doi.org/10.1007/s42452-020-03442-2

[29] Gares, M. New approach for the valorization of Stipa tenacissima as a potential raw material for the production and the optimization of fungal hemicellulase and cellulase using Box–Behnken design. [Manuscript submitted for publication] (2022).

[30] Singh, S., Tyagi, C. H., Dutt, D., Upadhyaya, J. S. Production of high level of cellulase-poor xylanases by wild strains of white-rot fungus Coprinellus disseminatus in solid-state fermentation. N Biotechnol 26, 165–170 (2009). https://doi.org/10.1016/j.nbt.2009.09.004

[31] Miller, G. L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar | Analytical Chemistry. Analytical Chemistry (1959).

[32] Bailey, M. J., Biely, P., Poutanen, K. Interlaboratory testing of methods for assay of xylanase activity. Journal of Biotechnology 23, 257–270 (1992). https://doi.org/10.1016/0168-1656(92)90074-J

[33] Mäkelä, M. R., Aguilar-Pontes, M. V., van Rossen-Uffink, D., Peng, M., de Vries, R. P. The fungus *Aspergillus niger* consumes sugars in a sequential manner that is not mediated by the carbon catabolite repressor CreA. Sci Rep 8, 6655 (2018). https://doi.org/10.1038/s41598-018-25152-x

[34] Li, Y., Zhang, X., Lu, C., Lu, P., Yin, C., Ye, Z., et al. Identification and Characterization of a Novel Endo-β-1,4-Xylanase from *Streptomyces* sp. T7 and Its Application in Xylo-Oligosaccharide Production. Molecules 27, 2516 (2022). https://doi.org/10.3390/molecules27082516

[35] Amore, A., Giacobbe, S., Faraco, V. Regulation of Cellulase and Hemicellulase Gene Expression in Fungi. Curr Genomics 14, 230–249 (2013). https://doi.org/10.2174/1389202911314040002

[36] Ghosh, P., Ghosh, U. Statistical optimization of laccase production by Aspergillus flavus PUF5 through submerged fermentation using agro-waste as cheap substrate. 10 (2017).

[37] Gupta, V. K., ed. *New and future developments in microbial biotechnology and bioengineering. Aspergillus system properties and applications*. (Elsevier, 2016).

[38] Lin, T.-C., Chen, C. Enhanced mannanase production by submerged culture of Aspergillus niger NCH-189 using defatted copra based media. Process Biochemistry 39, 1103–1109 (2004). https://doi.org/10.1016/S0032-9592(03)00218-8

[39] Vrabl, P., Schinagl, C. W., Artmann, D. J., Heiss, B., Burgstaller, W. Fungal Growth in Batch Culture – What We Could Benefit If We Start Looking Closer. Frontiers in Microbiology 10, (2019).

[40] Riquelme, M., Aguirre, J., Bartnicki-García, S., Braus, G. H., Feldbrügge, M., Fleig, U., et al. Fungal Morphogenesis, from the Polarized Growth of Hyphae to Complex Reproduction and Infection Structures. Microbiol Mol Biol Rev 82, e00068-17 (2018). https://doi.org/10.1128/MMBR.00068-17

[41] He, R., Li, C., Ma, L., Zhang, D., Chen, S. Effect of highly branched hyphal morphology on the enhanced production of cellulase in Trichoderma reesei DES-15. 3 Biotech 6, 214 (2016). https://doi.org/10.1007/s13205-016-0516-5

[42] Cairns, T. C., Zheng, X., Zheng, P., Sun, J., Meyer, V. Moulding the mould: understanding and reprogramming filamentous fungal growth and morphogenesis for next generation cell factories. Biotechnology for Biofuels 12, 77 (2019). https://doi.org/10.1186/s13068-019-1400-4

[43] Ghobadi, N., Ogino, C., Ohmura, N. Intensifying the Fermentation of in a Stirred Bioreactor Using Maxblend Impeller. The Open Chemical Engineering Journal 10, (2016). https://doi.org/10.2174/1874123101610010088

[44] Papagianni, M., Mattey, M., Kristiansen, B. Design of a tubular loop bioreactor for scale-up and scale-down of fermentation processes. Biotechnol Prog 19, 1498–1504 (2003). https://doi.org/10.1021/bp030002y

[45] Cui, Y. Q., van der Lans, R. G., Luyben, K. C. Effects of dissolved oxygen tension and mechanical forces on fungal morphology in submerged fermentation. Biotechnol Bioeng 57, 409–419 (1998).

[46] Fratebianchi, D., Crespo, J. M., Tari, C., Cavalitto, S. Control of agitation rate and aeration for enhanced polygalacturonase production in submerged fermentation by *Aspergillus sojae* using agro-industrial wastes: Enhanced polygalacturonase production by controlled agitation rate and aeration. J. Chem. Technol. Biotechnol. 92, 305–310 (2017). https://doi.org/10.1002/jctb.5006

[47] Kelly, S., Grimm, L. H., Hengstler, J., Schultheis, E., Krull, R., Hempel, D. C. Agitation effects on submerged growth and product formation of *Aspergillusniger*. Bioprocess Biosyst Eng 26, 315–323 (2004). https://doi.org/10.1007/s00449-004-0368-y

[48] Wang, L., Ridgway, D., Gu, T., Moo-Young, M. Effects of process parameters on heterologous protein production in *Aspergilusniger* fermentation. Journal of Chemical Technology and Biotechnology 78, 1259–1266 (2003). https://doi.org/10.1002/jctb.898

[49] Bandaiphet, C., Prasertsan, P. Effect of aeration and agitation rates and scale-up on oxygen transfer coefficient, kLa in exopolysaccharide production from *Enterobactercloacae* WD7. Carbohydrate Polymers 66, 216–228 (2006). https://doi.org/10.1016/j.carbpol.2006.03.004

[50] Bakri, Y., Mekaeel, A., Koreih, A. Influence of agitation speeds and aeration rates on the Xylanase activity of *Aspergillus niger* SS7. Brazilian Archives of Biology and Technology (2011). https://doi.org/10.1590/S1516-89132011000400003

[51] Naganthran, A., Masomian, M., Rahman, R. N. Z. R. A., Ali, M. S. M., Nooh, H. M. Improving the Efficiency of New Automatic Dishwashing Detergent Formulation by Addition of Thermostable Lipase, Protease and Amylase. Molecules 22, 1577 (2017). https://doi.org/10.3390/molecules22091577

[52] Savino, S., Bulgari, D., Monti, E., Gobbi, E. Agro-Industrial Wastes: A Substrate for Multi-Enzymes Production by Cryphonectria parasitica. Fermentation 7, 279 (2021). https://doi.org/10.3390/fermentation7040279

[53] Fadel, M., Keera, A. A., Abdel-Aziz, S., Kahil, T. Clean Production of Xylanase from White Corn Flour by *Aspergillusfumigates* F-993 under Solid State Fermentation. (2014).

[54] Thomas, L., Parameswaran, B., Pandey, A. Hydrolysis of pretreated rice straw by an enzyme cocktail comprising acidic xylanase from *Aspergillus* sp. for bioethanol production. Renewable Energy 98, 9–15 (2016).

[55] Desai, D. I., Iyer, B. D. Optimization of medium composition for cellulase-free xylanase

production by solid-state fermentation on corn cob waste by *Aspergillusniger* DX-23. Biomass Conv. Bioref. (2020). https://doi.org/10.1007/s13399-020-00749-3

[56] Shah, A. R., Madamwar, D. Xylanase production under solid-state fermentation and its characterization by an isolated strain of *Aspergillusfoetidus* in India. World J Microbiol Biotechnol 21, 233–243 (2005). https://doi.org/10.1007/s11274-004-3622-1

[57] Deschamps, F., Huet, M. C. Xylanase production in solid-state fermentation: a study of its properties. Appl Microbiol Biotechnol 22, 177–180 (1985). https://doi.org/10.1007/BF00253605

[58] Purkarthofer, H., Sinner, M., Steiner, W. Cellulase-free xylanase from *Thermomyceslanuginosus*: Optimization of production in submerged and solid-state culture. (1993). https://doi.org/10.1016/0141-0229(93)90068-D

[59] Archana, A., Satyanarayana, T. Xylanase production by thermophilic *Bacilluslicheniformis* A99 in solid-state fermentation. Enzyme and Microbial Technology 21, 12–17 (1997). https://doi.org/10.1016/S0141-0229(96)00207-4