Advanced Research
in Microbial Metabolites & Technology
(ARMMT)
journal homepage: http://armmt.irost.ir

Cellulase production by *Penicillium expansum* MDFS2 by solid-state fermentation of rice straw, rice bran, and wheat straw residues

Farzin Sharifzadeh[1], Golsa Dizajnejad[1], Selma Mohammadhassanpour[1], Nooshin Rahnama [1*]

[1] *Department of Biology, Urmia Branch, Islamic Azad University, Urmia, Iran*

Article Info

Received 29/07/2020
Received in revised form 27/10/2020
Accepted 21/12/2020

Keywords:
Cellulase,
Agricultural waste,
Penicillium expansum MDFS2,
Solid-state fermentation

Abstract

Production of fungal cellulase was performed by the isolate of *Penicillium expansum* MDFS2 on rice straw, rice bran, and wheat straw under solid-state fermentation. The greatest potential growth was detected using rice bran as the carbon source substrate. On the fifth day of fermentation, filter paperase, carboxymethyl cellulase, and β-glucosidase obtained their maximal activities of 4.91 U/g substrate, 36.51 U/g substrate, and 12.21 U/g substrate, respectively. The optimum temperature for filter paperase was reported at 40 °C, whereas carboxymethyl cellulase and β-glucosidase were optimally active at 50 °C. Filter paperase and carboxymethyl cellulase showed maximum activity at pH 5.0. However, β-glucosidase proved to be maximally active at pH 6.0. According to the thermal stability results, all the three components of the cellulolytic enzyme complex proved to be less thermally resistant at 60 °C, as compared to 50 °C. β-glucosidase and carboxymethyl cellulase depicted the highest and the lowest thermal resistance, respectively. β-glucosidase and filter paperase stored for one week at -20 °C proved to be the most and least stable enzymes, respectively. It is hoped that current research findings will help in the cost-effective production of industrially important cellulases using agro-industrial by-products as fermentation substrates.

1. Introduction

Cellulose, hemicellulose, and lignin are the three major components of lignocellulosic biomass. Cellulose contains the highest percentage of biomass (Echreshzadeh et al. 2020). Lignocellulosic biomass, sustainable carbon and energy sources, are currently the substrates most frequently used for the growth of lignocellulosic residues are potential fermentation substrates due to their abundance, low price, and renewability. Cellulase, an essential enzyme in industry, is produced using bacterial and fungal fermentation. Cellulase is also used for the bioconversion of agricultural residues into biofuels and other value-added products (Chang et al. 2011). Fungi are the most important group of cellulase-producing microorganisms. The most widely reported cellulase-producing fungi belong to *Trichoderma* spp., *Penicillium* spp., and

* Corresponding author. Tel: + 984431803000
E-mail address: n.rahnama@iaurmia.ac.ir
DOI: 10.22104/armmt.2020.4326.1045

Aspergillus spp. (Chand et al. 2005; Rahnama et al. 2013). Numerous studies have reported *Penicillium* spp. as potential cellulase producers (Gusakov and Sinitsyn, 2012). *P. decumbens*, *P. expansum*, and *P. digitatum* are among the most widely reported potential strains (Liu et al. 2011; Qui et al. 2020; Cristina dos Santos et al. 2020). Solid substrate cultivation is advantageous over submerged fermentation as conditions are similar to those found in nature, thus making it a more effective technique for cellulase production as compared to liquid fermentation (Kang et al. 2004; Singhania et al. 2009; Vu et al. 2009; Singhania et al. 2010).

The objective of the present study was to evaluate the possibility of the production of cellulases from locally isolated *P. expansum* MDFS2 using the major agricultural residues of rice straw (RS), rice bran (RB), and wheat straw (WS). The cellulases produced using the best potential growth substrate were then characterized in terms of optimum temperature and pH as well as its thermal and storage stability.

2. Materials and methods

2.1. Preparation of agricultural waste

The fermentation substrates were collected at the end of the harvest season in September 2017. The RS was obtained from a paddy field in the Gilan Province, northern Iran. The WS was obtained from a wheat farm in West Azerbaijan, Iran, and the RB was purchased from a local market in Urmia, West Azerbaijan, Iran. The fermentation substrates were sun-dried and ground using a 2 mm sieve. The substrates were then stored in a cold room at 4 °C until use.

2.2. Inoculum preparation

P. expansum isolated from Forest Soil from Mahabad Park, West Azerbaijan, Iran, was used as the cellulase-producing microorganism. The spores of the fungus were preserved in 30% (v/v) glycerol at -80 °C. A Potato Dextrose Agar (PDA) medium was utilized to reactivate the spores. Prior to fermentation, a suspension of the fungal spores was prepared by using sterilized distilled water to wash the PDA agar surface on the seventh day of fungal growth. Quantification of the spores was performed using a hemocytometer. The spores were inoculated at a concentration of 1×10^6 spores mL^{-1}.

2.3. Solid state fermentation

The production and collection of the enzyme was performed in Erlenmeyer flasks (250 mL) with cotton stoppers. Each flask contained one gram of the substrate before being autoclaved. The proper amount of Mandels medium (Mandels et al. 1974) was added to each flask to adjust the moisture content to 70% (w/v). Mandels medium (1 L) contained: 1.4 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 0.63 g urea, 0.3 g $CaCl_2$, 0.3 g $MgSO_4.7H_2O$, 0.75 g peptone, 2 mL Tween 80, and 1 mL of Trace elements: $CoCl_2$ (2.6×10^{-3}), $FeSO_4$ (5×10^{-3}), $MnSO_4.H_2O$ (1.6×10^{-3}), $ZnSO_4.7H_2O$ (1.4×10^{-3}). Incubation of the inoculated flasks was performed at 30 °C, and the production of the enzyme was monitored over a period of eight days. Thirty mL of 50 mM citrate buffer (pH=4.8) was added to each flask in order to extract the crude enzyme. The flask was then agitated for 30 min at 150 rpm at 30 °C. Centrifugation of the mixture was conducted at 4 °C, 1000×g for 10 min. The supernatant was filtered and used immediately for the enzyme activity assay. However, the supernatant was kept at 4 °C unless an immediate cellulase activity assay was possible.

2.4. Cellulase activity assay

The activity of the crude cellulase was assayed according to the protocol described by Wood and Bhat (Wood and Bhat 1988). The activity of the cellulases was calculated and reported in the unit of enzyme activity per gram dry fermented substrate (U/g).

2.5. Characterization of the crude cellulase

The effects of temperature and pH on cellulase activity were investigated. The enzyme thermal stability was evaluated as well. The enzyme was incubated at a temperature range of 40-90 °C, and followed by the assay under the standard assay conditions. McIlvaine buffer solutions at a pH range of 2.5-7.5 were used to study the effect of pH. The crude cellulase enzyme activity was

expressed as relative activity, which is a percentage of the maximum activity. To evaluate the crude cellulase thermal stability, 50 and 60 °C were selected as incubation temperatures and the residual enzyme activity, withdrawn periodically at the time intervals of 30 min, 1 h, 2 h, and 3 h, was assayed under standard assay conditions. Thermal stability of the crude cellulase was expressed as residual activity, which is a percentage of the original activity. The stability of the enzyme was also investigated after being stored for 1 week at room temperature (25 °C), in a refrigerator (4 °C), and in a freezer (-20 °C).

2. 6. Statistical analysis

All experiments were performed in triplicate. The mean and standard deviation were calculated, and a chart was plotted using Microsoft Excel®. The data were analyzed by one-way analysis of variance (ANOVA), and T-Tests (LSD) was used to compare the difference of means among the treatment groups. Differences of $p < 0.05$ were considered significant.

3. Results and Discussion

3.1. Production of cellulase by *P. expansum* MDFS2 from RS, RB, and WS

The substrate is a significant parameter to be considered in the enhancement of microbial cellulase production. A number of researches and various comparative studies have been conducted using various lignocellulosic waste to enhance cellulolytic enzyme production (Deswal et al. 2011; Kumar et al. 2011; Philippoussis et al. 2011; Vincent et al. 2011; Rahnama et al. 2016; Chaoyang et al. 2017; Imran et al. 2019).

In this study, cellulase production experiments were performed using untreated agricultural residues as the sole carbon sources. Along with the growth of *P. expansum* MDFS2, the cellulolytic enzyme was produced on the untreated agricultural-based lignocellulosic residues; RS, RB, and WS. Figure 1 presents the production of filter paperase (FPase) by the fungal isolate investigated over a period of eight days. Maximal FPase activity was observed on day 5 for all substrates. However, exoglucanase activity was determined to be significantly higher when RB was used as the sole carbon source (4.91 U/g substrate) ($p<0.05$).

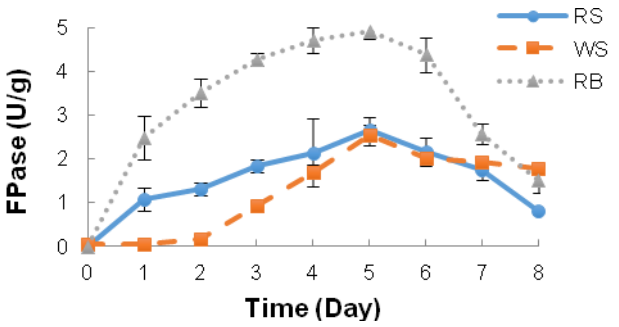

Fig. 1. FPase activity of *P. expansum* MDFS2 from various agricultural residues used as SSF substrate. Data are means of 3 replicates± standard deviation. Error bars smaller than symbols are not shown.

Carboxymethy cellulase (CMCase) production of the isolate was studied over a period of eight days on RS, RB, and WS. The data is presented in Figure 2. A comparison between endoglucanase production from RS, RB, and WS shows that, like the FPase, the maximum production of endoglucanase (36.51 U/g substrate) occurred on day 5 on RB and was significantly higher ($p<0.05$) than that reported as a result of fungal growth on WS. However, the CMCase activity from RS (35.11 U/g substrate) did not prove to be significantly different from that obtained on RB ($p>0.05$).

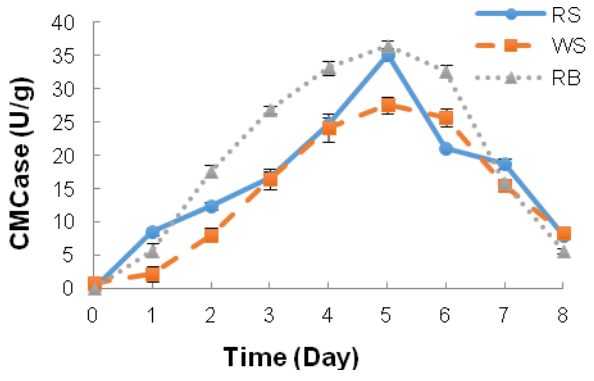

Fig. 2. CMCase activity of *P. expansum* MDFS2 from various agricultural residues used as SSF substrate. Data are means of 3 replicates±

standard deviation. Error bars smaller than symbols are not shown.

A β-glucosidase (BGL) production profile was also plotted over a period of eight days. A comparison between BGL production by *P. expansum* MDFS2 from RS, RB, and WS is illustrated in Figure 3. Maximum production of the fungal cellobiase occurred on day 5 on RB, and the activity obtained (12.21 U/g substrate) was significantly higher ($p<0.05$) than that obtained when RS and WS were used as carbon sources.

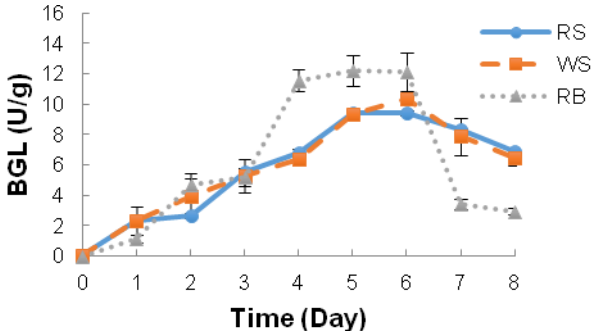

Fig. 3. BGL activity of *P. expansum* MDFS2 from various agricultural residues used as SSF substrate. Data are means of 3 replicates± standard deviation. Error bars smaller than symbols are not shown.

P. expansum MDFS2 grew fast on RB. This is most likely due to the composition of bran. As bran contains a high content of starch and protein, it serves not only as a carbon source but also a nitrogen source. Compared to cellulose and hemicellulose, starch has proven to be easier to degrade into simple carbohydrates for the growth of the filamentous fungus *Aspergillus fumigatus* N2 (Chaoyang et al. 2017). The production of extracellular enzymes by filamentous fungi is shown to be growth-rate associated, i.e., enzyme production and the fungal growth occurs simultaneously (Manning and Wood 1983; Schrickx et al. 1993; Carlsen et al. 1996; Spohr et al. 1998; Withers et al. 1998; Pedersen et al. 2000). In this study, RB was proved to be the significantly most appropriate fermentation substrate for the production of all the three major components of the cellulolytic enzyme complex ($p<0.05$), and resulted in cellulase at maximum activity.

On the other hand, the enzymes' expression throughout the fermentation process is induced by the carbon source compounds (Haltrich et al. 1996). Straw has a high cellulose and hemicellulose content compared to RB, which induces lignocellulolytic enzyme expression. Therefore, the use of straw may be assumed to produce higher lignocellulolytic enzyme activity (Chaoyang et al. 2017). However, a number of research findings have confirmed the suitability of bran, compared to straw, as a fermentation carbon source. According to Sarkar and Aikat (2014), among a large number of cellulase-producing *Aspergillus* species, most *Aspergillus* species grow better on wheat bran than on wheat or rice straw, although there are reports of *A. fumigatus* strains preferring straw to bran (Chaoyang et al. 2017).

Several researchers have utilized *Penicillum* as the source of the enzyme for cellulase production (Picart et al. 2007; Dutta et al. 2008; Karbone et al. 2008; Das and Ghosh 2009; Qui et al. 2020; Cristina dos Santos et al. 2020). *Penicillia* has shown promise as cellulase producers and could compete with *Trichoderma* for biofuel applications. Being rich in BGL, *Penicillium* may even be superior to *Trichoderma*. It is believed that a high level of BGL activity is essential for rapid and complete conversion of cellobiose to glucose. The accumulation of cellobiose, the intermediate product of cellulose hydrolysis, suppresses the overall cellulose hydrolysis process (Gusakov et al. 2012). While RB is generally regarded as a low-cost agricultural waste with limited use, this study indicates that RB can be applied as a cost-effective carbon source in cellulase production by *P. expansum* MDFS2.

3.2. Characterization of the crude cellulase by *P. expansum* MDFS2 on RB

The optimal temperature for FPase was detected at 40 °C, while, CMCase and BGL showed maximal activities at 50 °C. When the enzyme-

substrate mixture was kept for 1 h under standard assay conditions, FPase activity dropped sharply with increased temperature. However, CMCase was maintained above 75% of the maximum activity at 60 °C when the enzyme-substrate mixture was kept for only 30 min. Although, BGL remained 85% and 70% active at 70 °C and 80 °C, respectively (Fig. 4), the cellulases were almost denatured at 90 °C.

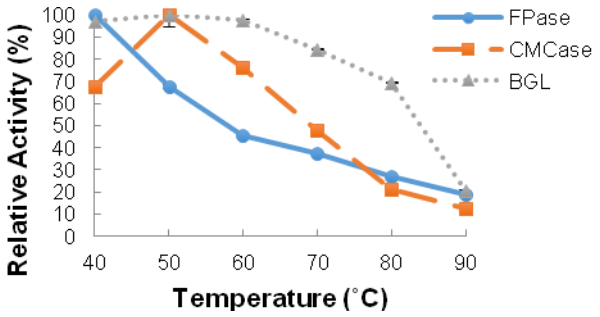

Fig.4. Effect of temperature on the activity of the crude cellulase by *P. expansum* MDFS2 from RB in SSF. Values are means of 3 replicates ±standard deviation. Error bars smaller than symbols are not shown.

The cellulolytic enzymes produced by *P. expansum* MDFS2 were shown to be acidophile and were quite active in a wide range of acidic pH. FPase and CMCase showed optimally active at pH 5.0. It was observed that a pH of 6.0 was the optimum pH for BGL. FPase maintained above 65% maximum activity at pH 4-6. However, CMCase remained 80% active at pH 4.5-5.5, and BGL was above 80% active in a broader pH range of 4.0-6.5 (Fig. 5).

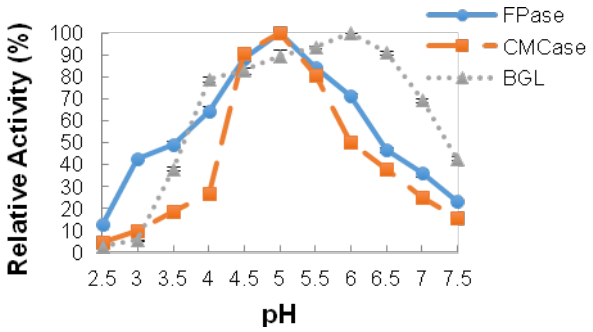

Fig. 5. Effect of pH on the activity of the crude cellulase by *P. expansum* MDFS2 from RB in SSF. Values are means of 3 replicates ±standard deviation. Error bars smaller than symbols are not shown.

The enzyme activity rapidly decreased with an increase in temperature and a rise in enzyme incubation time. However, no significant reduction was observed in the FPase activity at 50 °C when the crude cellulase incubation time was increased from 30 min to 2 h. Exoglucanase lost 90% of its original activity, and the enzyme was almost denatured at 60 °C after 3 h. The CMCase proved less thermally resistant and was almost denatured at both 50 °C and 60 °C after 3 h. BGL remained above 60% active at 50 °C after it was incubated for 3 hours. However, BGL activity dropped rapidly and was almost denatured at 60 °C (Fig. 6 a, b).

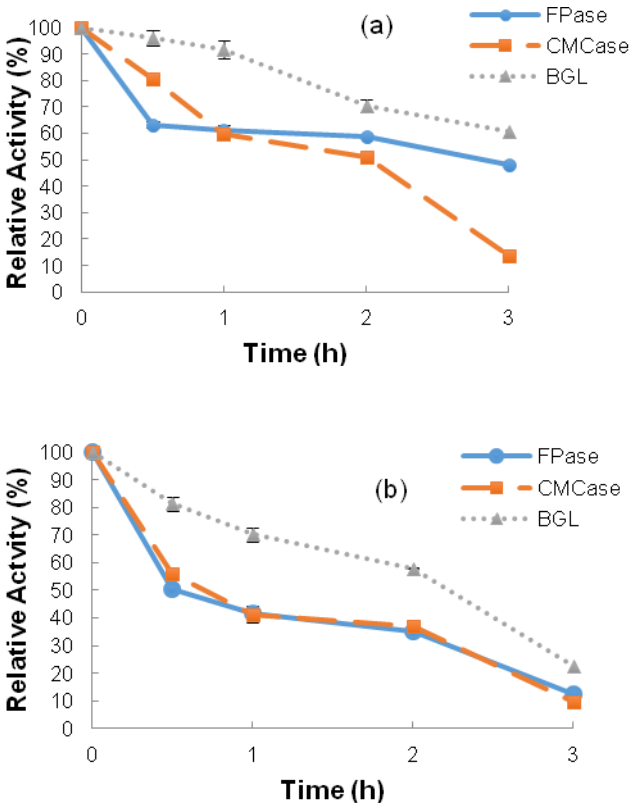

Fig. 6. Thermostability of the crude cellulase by *P. expansum* MDFS2 from RB in SSF at 50 °C (a) and 60 °C (b). Values are means of 3 replicates ± standard deviation. Error bars smaller than symbols are not shown.

The crude enzyme stored for one week at -20 °C proved to be more stable, as compared to that stored at 4 °C and 25 °C. As illustrated in Figure 7, BGL remained almost 100% active at -20 °C. While, a reduction of 20% and 10% was observed in the FPase and CMCase activities, respectively, after one week of storage at -20 °C.

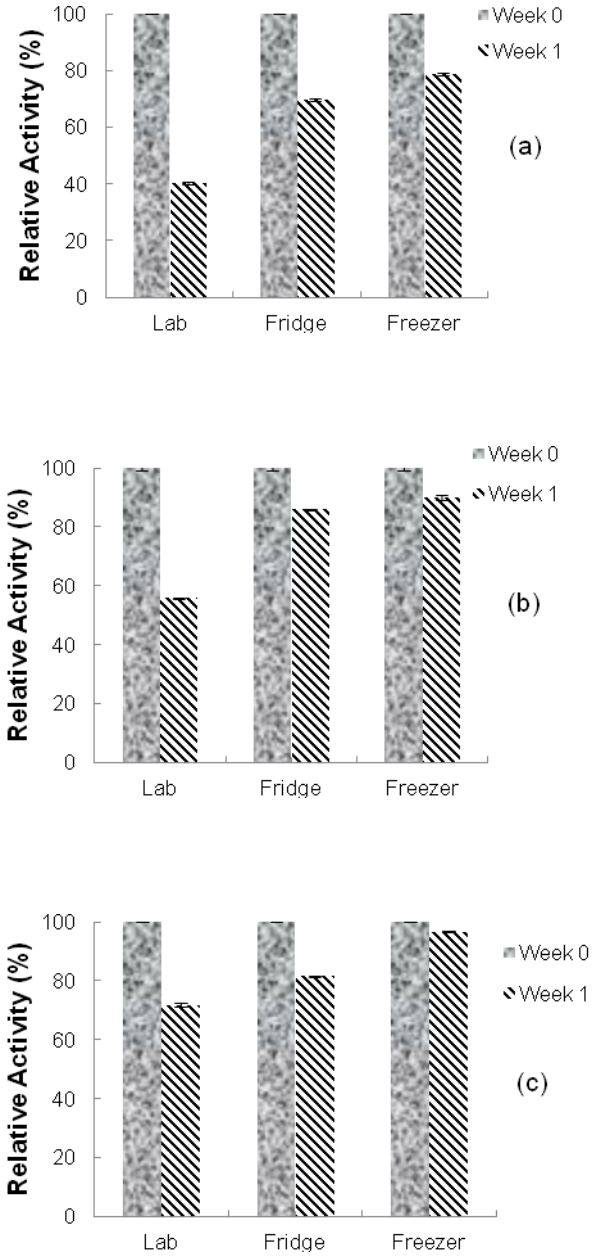

Fig. 7. Effect of the storage temperature on the activity of the crude cellulase by *P. expansum* MDFS2 from RB in SSF. Values are means of 3 replicates ± standard deviation. a: FPase b: CMCase c: BGL. Error bars smaller than symbols are not shown.

4. Conclusion

The aim of the present study was to investigate the effect of the substrate on cellulase production. Therefore, crude cellulase production was carried out by an isolate of *P. expansum* MDFS2 using rice straw, rice bran, and wheat straw, abundantly found agricultural waste, under solid-state fermentation. RB proved to be the most promising growth substrate and a suitable substrate for cellulase production. On day 5 of fermentation, maximum production of the cellulase was observed for FPase, CMCase, and BG with their maximal activities of 4.91 U/g substrate, 36.51 U/g substrate, and 12.21 U/g substrate, respectively. The optimum temperature for FPase was 40 °C, while CMCase and BGL revealed optimal activity at 50 °C. FPase and CMCase revealed maximum activity at pH 5.0. However, BGL proved to be maximally active at pH 6.0. BGL and CMCase showed the highest and the lowest thermal resistance, respectively. BGL and FPase stored for one week at -20 °C proved to be the most and the least stable, respectively. Our results suggest the crude cellulase capability for industrial use. The findings of the current research will hopefully contribute to the cost-effective production of industrially important cellulases using agro-industrial waste.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

The authors would like to thank the Islamic Azad University of Urmia for supporting this research.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

References

[1]. Carlsen, M., Nielsen, J., Villadsen, J. (1996). Growth and α-amylase production by *Aspergillus oryzae* during continuous cultivations. Journal of Biotechnology, 45, 81-93.

[2]. Chand, P., Aruna, A., Maqsood, A.M., Rao, L.V. (2005). Novel mutation method for increased cellulase production. Journal of Applied Microbiology, 98, 318-323.

[3]. Chang, K.L., Thitikorn-amorn, J., Hsieh, J.F. (2011). Enhanced enzymatic conversion with freeze pretreatment of rice straw. Biomass and Bioenergineering, 35, 90-95.

[4]. Chaoyang, L., Zhicheng, S., Wensheng, Q. (2017). Characterization of xylanase and cellulase produced by newly isolated *Aspergillus fumigatus* N2 and its efficient saccharification of barley straw. Applied Biochemistry and Biotechnology, 182, 559–569.

[5]. Cristina dos Santos, F., Aurelio Schuler de Oliveira, M., Augusto Vicente Seixas, F., Parra Barbose-Tessmann, L. (2020). A Novel Cellobiohydrolase I (CBHI) from *Penicillium digitatum*: Production, Purification, and Characterization. Applied Biochemistry and Biotechnology, 192, 257-282.

fermentation of waste cabbage by *Penicillium notatum* NCIM NO-923 for production and characterization of cellulases. Journal of Scientific and Industrial Research, 68(8), 714-718.

[7]. Deswal, D., Khasa, Y.P., Kuhad, R.C. (2011). Optimization of cellulase production by a brown rot fungus *Fomitopsis* sp. RCK2010 under solid state fermentation. Bioresource Technology, 102(10), 6065-72.

[8]. Dutta, T., Sahoo, R., Sengupta Ray S.S. (2008). Novel cellulases from an extremophilic filamentous fungi *Penicillium citrinum*: production and characterization. Journal of Industrial Microbiology and Biotechnology, 35, 275–282.

[9]. Echresh Zadeh, Z., Abdulkhani, A., Aboelazayem, O., Saha, Basudeb. (2020). Recent insights into lignocellulosic biomass pyrolysis: A critical review on pretreatment, characterization, and products upgrading. Processes, 8, 799.

[10]. Gusakov, A.V., Sinitsyn, A.P. (2012). Cellulases from *Penicillium* species for producing fuels from biomass. Biofuels, 3(4), 463–477.

[11]. Haltrich, D., Nidetzky, B., Kulbe, K.D. (1996). Production of fungal xylanases. Bioresource Technology, 58,137–161.

[12]. Imran, M., Hussain, A., Anwar, Z., Irshad, M., Jabeen, F. (2019). Beta-glucosidase production optimization from newly isolated *Aspergillus tubingensis* IMMIS2 using Taguchi statistical design. Iranian Journal of Science and Technology: Transactions A Science, 43: 701–707.

[13]. Karboune, S., Geraert, P.A., Kermasha, S. (2008). Characterization of selected cellulolytic activities of multi enzymatic complex system from *Penicillium funiculosum*. Journal of Agricultural and Food Chemistry, 56(3), 903–909.

[14]. Kang, S.W., Park, Y.S., Lee, J.S. (2004). Production of cellulases and hemicelluloses by *Aspergillus niger* KK2 from lignocellulosic biomass. Bioresource Technology, 91,151- 156.

[15]. Kumar, S., Sharma, H., Sarkar, B. (2011). Effect of substrate and fermentation conditions on pectinase and cellulase production by *Aspergillus niger* NCIM 548 in submerged (SmF) and solid state fermentation (SSF). Food Science and Biotechnology, 20(5), 1289-98.

[16]. Liu, Y-T., Luo, Z-Y., Long, Ch-N., Wang, H-D., Long, M-N., Zhang, H. (2011). Cellulase production in a new mutant strain of *Penicillium decumbens* ML-017 by solid state fermentation with rice bran. New Biotechnology, 28(6), 733-737.

[17]. Mandels, M., Hontz, I., Nystrom, J. (1974). Enzymatic hydrolysis of waste cellulose. Biotechnology and Bioengineering, 16(11), 1471-1493.

[18]. Manning, K., Wood, D.A. (1983). Production and regulation of extracellular endocellulase by *Agaricus bisporus*. Journal of General Microbiology, 129, 1839-184.

[19]. Pedersen, H., Beyer, M., Nielsen, J. (2000). Glucoamylase production in batch, chemostat, and fed-batch cultivations by an industrial strain of *Aspergillus niger*. Applied Microbiology and Biotechnology, 53, 272-277.

[20]. Philippoussis, A., Diamantopoulou, P., Papadopoulou, K., Lakhtar, H., Roussos, S., Parissopoulos, G. (2011) Biomass, laccase and endoglucanase production by *Lentinula edodes* during solid state fermentation of reed grass, bean stalks and wheat straw residues. World Journal of Microbiology and Biotechnology, 27(2), 285-97.

[21]. Picart, P., Diaz, P., Pastor, F.I.J. (2007). Cellulases from two *Penicillium* sp. strains isolated from subtropical forest soil: Production and characterization. Letters in Applied Microbiology, 45(1), 108-113.

[22]. Qui, S.Z., Zeng, F., Zhang, S., Su, S.L., Guo, S., Qian, D.W., Zhao, M.Z., Duan, J.A. (2020). Fermentation of cellulase with multiple types of Salvia miltiorrhiza residues and other solid wastes from Chinese materia medica industrialization. China Journal of Chinese Materia Medica, 45 (4), 890-895.

[23]. Rahnama, N., Mamat, S., Umi Kalsom M.S., Foo, H.L. (2013). Effect of alkali pretreatment of rice straw on cellulase and xylanase production by local *Trichoderma harzianum* SNRS3 under solid state fermentation. BioResources, 8(2), 2881-2896.

[24]. Rahnama, N., MD Shah, U.K., Foo, H.L. (2016). Production and characterization of cellulose from solid state fermentation of rice straw by *Trichoderma harzianum*, SNRS3. Pertanika Journal of Tropical and Agricultural Sciences, 39 (4), 507 – 531.

[25]. Sarkar, N., Aikat, K. (2014). *Aspergillus fumigatus* NITDGPKA3 provides for increased cellulase production. International Journal of Chemical Engineering, 5, 1–9.

[26]. Schrickx, J.M., Krave, A.S., Verdoes, J.C., Van Den Hondel, C.A. (1993). Growth and product formation in chemostat and recycling cultures by *Aspergillus niger* N402 and a glucoamylase overproducing transformant, provided with multiple copies of the glaA gene. Journal of General Microbiology, 139, 2801-2810.

[27]. Singhania, R.R., Patel, A.K., Soccol, C.R., Pandey, A. (2009). Recent advances in solid state fermentation. Biochemical Engineering Journal, 44(1), 13-18.

[28]. Singhania, R.R., Sukumaram, R.K., Patel, A.K. (2010). Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulase. Enzyme and Microbial Technology, 46(7), 541-9.

[29]. Spohr, A., Carlsen, M., Nielsen, J., Villadsen, J. (1998). α-amylase production in recombinant *Aspergillus oryzae* during fed-batch and continuous cultivations. Journal of Fermentation and Bioengineering, 86, 49-56.

[30]. Vincent, M., Pometto, A.L., van Leeuwen, J. (2011). Simultaneous saccharification and fermentation of ground corn stover for the production of fuel ethanol using *Phanerochaete chrysosporium*, *Gloeophyllum trabeum*, *Saccharomyces cerevisiae*, and *Escherichia coli* K011. Journal of Microbiology and Biotechnology, 21(7), 703-10.

[31]. Vu, V.H., Pham, T.A., Kim, K. (2009). Fungal strain improvement for cellulose production using repeated and sequential mutagenesis. Microbiology, 37(4), 267-271.

[32]. Withers, J.M., Swift, R.J., Wiebe, M.G., Robson, G.D. (1998). Optimization and stability of glucoamylase production by recombinant strains of *Aspergillus niger* in chemostat culture. Biotechnology and Bioengineering, 59, 407-418.

[33]. Wood, T.M., Bhat, K.M. (1998). Measuring cellulase activities. Methods in Enzymology, 160, 87-112.