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Isolation and Identification Studies on Potential Xylanase Producing Strain *Trichoderma* sp. WICC F46 Isolated from Tropical Soil

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Trichoderma is one of few fungal species of high potential application as bio factory for xylanase production. During last few years increased demand have been observed on the need to isolate high efficient strains from soil sample and to use different microbiological and bioprocess engineering approaches to increase the production yield. In this work, efficient xylanase producer strain was isolated from soil and fully identified using phylogeny analysis. In order to develop an efficient submerged medium for xylanase production, media screening was carried out followed by optimization using one factor at a time (OFAT) method. After optimization of medium composition, the maximal xylanase volumetric production reached 311.71 UmL^{-1}. This medium was composed of corn cob, peptone, magnesium sulphate, calcium chloride, and ammonium sulphate.

Keywords: *Trichoderma*, Strain isolation, Xylanase, Medium optimization, Submerged culture

Introduction

Nowadays, fungal biofactories gain high attention in white biotechnology based on their capacity to produce and excrete a wide range of enzymes for wide application range[1–3]. *Trichoderma* is a fungus which can be isolated from soil and characterized by the high capacity of degrading soil organic matter. Besides its high hydrolytic enzyme production, this fungus was also efficiently used as biocontrol agent. This fungus has many intraspecific groups where some of the biotypes enable them to antagonize against phytopathogenic fungi by using the substrate colonization process[4]. Apart from being used as an effective bio control agent many phytopathogenic fungi and microorganisms, *Trichoderma* is also well known for its effectiveness for the production of many useful hydrolytic industrial enzymes using different substrates[5,6]. Among different hydrolase enzymes, xylanases are considered as one of the key enzyme group based on their wide range of industrial applications[7–9]. For the efficient enzyme production submerged fermentation have been carried out as it allows for better control in terms of the correct condition during the fermentation process[10]. The present work focuses on isolation of highly efficient xyalanase producers followed by full medium optimization to improve growth and enzyme production in submerged cultivation system.

Materials and method

Sample Collection

For each sample, soil samples were taken from a depth of 5–10 cm and were transferred into a sterile plastic bags. Then, the soil was mixed and all the foreign materials like the roots, and stones were removed. One hundred (100) grams of the sample was placed into the sterile zip lock bag, and stored at 4 ºC until further strain isolation (usually within 2–3 days).

Fungal Isolation

Malt Extract Agar (MEA) media was used for isolation of the fungal strain from soil. Ten grams of the freshly taken soil was mixed with ninety ml of sterile distilled water and stirred in a vigorous mode. Then, a serial dilution was made and 0.1ml of the inoculum was transfered to each of the plate and the MEA agar was supplemented with 0.01% chlorotetracycline to prevent the growth of bacteria. All the plates were incubated at 30° for 5 days. The

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suspected colonies from the first MEA plate were transferred to fresh MEA repeatedly until the pure culture was obtained. Slide culture technique was used to observe the culture for the physical characteristics observation. The sample was sub-cultured on Potato Dextrose Agar (PDA) slant for short term preservation[11].

Phylogenetic Analysis

For the identification of the isolates, the nucleotide sequences were compared with the one which already deposited in the data bank of the National Centre for Biotechnology and Information (NCBI), using the BLAST search tool. In order to do the comparison with the currently available sequences, the 20 sequences were retrieved with having over 98% similarity to 20 different genera from NCBI. Following this, multiple alignment was performed by using MEGA version 7.0 and the identified sequences were submitted in NCBI Genbank by suing the BLAST tool. The Bootstrap Test of Phylogeny was performed by using the same MEGA version 7.0 software and by determining the distance matrix, a neighbour-joining tree was constructed for the identification of the fungi strains[12]. The most potential candidate from the phylogenetic tree identified was selected for further studies.

Inoculum Preparation

Ten Petri dishes that contains the fully grown fungal cultures were washed out by adding 100 ml of sterile distilled water. Then, using a sterile inoculating loop, all the grown mycelium was gently rubbed and scratched out. Then, 2.5ml of the mixture from the plates were distributed into each of the flasks that contained the production medium.

Production Medium and Cultivation Condition

The scratched out mycelium was inoculated into 250 ml shake flask which contain 50ml of production medium. The pH of the production medium was adjusted to 5.5 for all the experiments. The media then incubated on the rotary shaker at 200 rpm and at 30° for 96 hours.

Medium Screening

Six types of media from the previous literature studies were selected based on the enzyme production level to carry out the media screening procedure in order to choose the best medium that shows the highest xylanase enzyme production (Table 1). The formation of fungal mycelium either in forms of pellets or filamentous was recorded and the enzyme assays were calculated.

Table 1 — General composition of 6 different media for xylanase enzyme production from *Trichoderma* sp.

Component	Concentration $g.L^{-1}$					
Different Medium	1	2	3	4	5	6
Birchwood Xylan	1.5	2.0	2.5	1.0	1.5	1.5
Bacteriological Peptone	1	2.0	2.0		1.5	1.5
Urea	1		3.0			
Yeast extract		2.0		2.0	2.0	2.0
Magnesium Sulphate	0.3	0.5	0.8	0.4	0.5	0.5
Monopotassium Phosphate	0.2	1.0	2.7	2.0	0.5	0.5
Calcium Chloride	0.3		0.5			
Ammonium Sulfate	0.4		1.0	1.4	0.4	0.4
Zinc Sulfate				0.22		
Iron (III) Sulfate				0.05		
Trisodium Citrate						0.5

Shake Flask Cultivation Using OFAT Method

One-factor-at-time (OFAT) method was carried out to determine the best carbon, nitrogen and mineral source that enhances the xylanase enzyme production. The most important carbon source for the cell growth was replaced with agricultural-waste corn cob, which tested at different concentrations to determine the best concentration for high enzyme production. Afterward, a nitrogen source from the original medium was replaced with another nitrogen sources like yeast extract, bacteriological peptone, peptone water and casein hydrolysate. Lastly, the mineral sources was replaced by Potassium chloride, Sodium chloride, Iron (III) sulphate and Manganese (II) sulphate.

Determination of Enzyme activity

Xylanase production was measured by using xylan as the substrate. Three ml of reaction mixture that contain 1ml of crude enzyme, 1ml of 1% of xylan which dissolved in 0.05 M Na-citrate buffer at pH of 5.3, and 1ml of 0.05 M citrate buffer prepared. Then, the mixture was incubated at 55 ºC for 10 minutes. Following that, the reaction was stopped by the addition of 3.0 ml of 3,5-dinitrosalicyclic acid (DNS) and the contents was boiled for 15 minutes. After cooling in ice bath, the absorbance was read at 540nm by using spectrophotometer[13].

Growth Kinetic Study

The growth kinetics for *Trichoderma* sp. was carried out in a shake flask through both un-optimized and OFAT-optimized media. Samples were taken in

the form of triplicates (3 flasks) every 4 hours and analysis was done subsequently table 2.

Results and Discussion

Phylogenetic Analysis

The phylogenetic analysis was carried out to determine the history of the evolutionary relationship between the strains being studied and the group that shared the common ancestor. For this study, the strain which displayed good performance in previous assays and also its capability of producing xylanase enzyme. The 16S rDNA sequencing method was carried out to identify the molecular phylogenetic classification for the targeted fungal strain. The, the evolutionary history was inferred using Maximum Likelihood method. All the percentage of the trees which associated taxa clustered together shown above the branches and the branch lengths was measured in the number of substitutions per site. In particular, this analysis involved 17 nucleotide sequences and the gaps and missing data in the positions were eliminated. Figure 1 below shows that the targeted strain WICC F46 (Gen Bank Accesion no MK328504.1) fell within the clade consists of *Trichoderma* species where the closest was *Trichoderma Asperellum* (Gen Bank Accesion no AY531610.1) and it was later named as *Trichoderma sp.* WICC F46.

Table 2 — Comparison between the values of the total biomass and xylanase enzyme production during the cultivation's in shake flask before and after medium optimization.

Attributes	Un-Optimized Medium	Optimized Medium
X_{max} (gL^{-1})	45.41	70.57
P_{max}(Uml^{-1})	151.34	311.71
P_{max}/ X_{max} (Ug^{-1})	3,332	4,417

Medium Screening

This experiment was carried out to study the effect of different media (previously published in literature) on cell growth and xylanse production by the newly isolated strain. *Trichoderma*sp WICC F46. To further understand this relationship, this fungus was grown in six media of different composition. The effect of the different medium composition on the growth and production of xylanase enzyme is shown in figure 1. As shown, medium number 3 shows the highest biomass production reaching about 6 g L^{-1}. This followed by medium number 6, which supported biomass production up to 4.5 gL^{-1}, medium 2 (3.45 gL^{-1}), medium 5 (3.10 gL^{-1}), medium 1 (2.5 gL^{-1}) and medium 4 (2 gL^{-1}). The higher biomass obtained in medium 3 may be attributed to the presence of

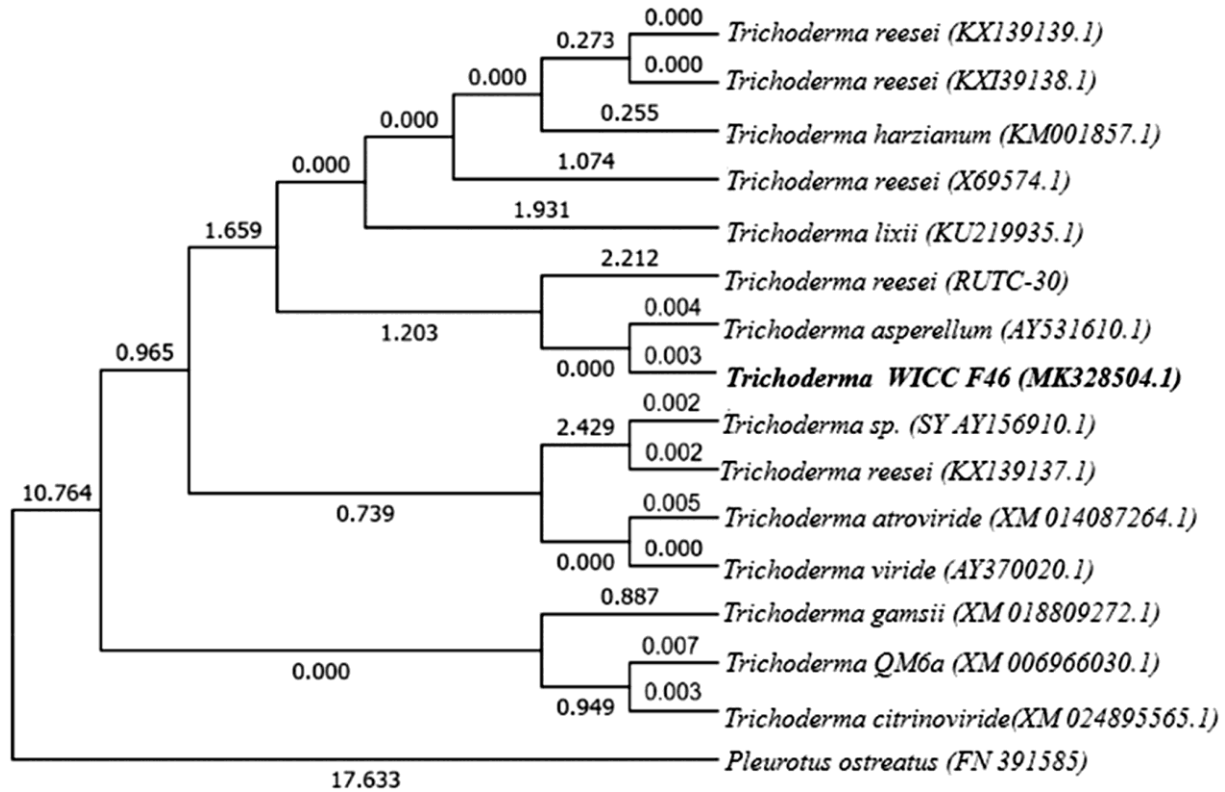

Fig. 1 — Phylogenetic relationships of *Trichoderma*sp WICC F46 and related species which is based on 18S rDNA sequences.

2 different organic nitrogen sources which are urea and bacteriological peptone at 3.0 gL^{-1} and 2.0 gL^{-1}, respectively. On the other hand, one of the important factors that needed to be onsidered during the xylanase enzyme production is pH as it greatly affects the enzyme production level. Fungi that have been grown at unfavourable pH conditions tend to produce enzymes at limited growth rate. When it comes to the pH of the submerged media, medium number 6 shows the highest pH among the rest which is up to 6 while the medium number 3 shows pH around 6. According to[14] most of the fungal xylanase enzyme will be produced in pH range from 4.5 to 6.5. Other medium showed lower pH at the end of the cultivation time. As for the xylanase enzyme production, medium 3 showed significantly higher level which is up Uml^{-1}. Medium 1 showed an activity level up to 50 Uml^{-1}, medium 2 shows around 140 µml, medium 5 around 130 Uml^{-1} and last medium 6 around 95.40 Uml^{-1}. The differences in enzyme production for each medium type mainly caused by the different compositions. As for medium 3 consists of two different types of nitrogen sources which are urea and bacteriological peptone around 3 gL^{-1} and 2 gL^{-1}. Moreover, the specific usage of Birchwood xylan around 2.5 gL^{-1} as sole carbon source also enhances the production of xylanase enzyme. The high yield of xylanase obtained from this study by *Trichoderma*sp WICC F46 was aligned with the literature study done by other research group who reported that the genera of *Aspergillus* and *Trichoderma*are dominant in xylanase production when comparing with other mesophilic fungi[14].

Medium optimization using One Factor at Time (OFAT)

If there is a single change in any factor, there will be a drastic effect and changes on cell growth as well as on enzyme production. Therefore, it is important to understand the mechanisms underlying the growth and the enzyme production by individual factor. OFAT normally involves changing one factor at a time by fixing the other factors. The carbon source which is the most potent in the medium for efficient cell growth was replaced with agricultural waste corn cob. The usage of the corn cob as a carbon source is much more economical and environmentally friendly as it reduces the decomposition of the agricultural waste to the environment and it can be purchased in large quantity with cheap price. In addition, corn cob composed of organic and inorganic nutrients, vitamins, carbohydrates as well as cellulose, hemicellulose and lignin that give huge contribution for efficient enzyme production. According to figure 2, the xylanase enzyme activity was the highest at the concentration of 50 $gL^{-1}$with enzyme activity of 230.1 Uml^{-1}. The enzyme activity level starts to decrease slowly, when the concentration of corn cob increased from beyond 50 gL^{-1}. From this, it can be concluded that, high concentration of carbon source will inhibit the enzyme activity which was also supported by other authors[15]. Nitrogen source have significant effect on the cell growth and enzyme production in the growth medium. Therefore, the effect of the nitrogen source on cell growth as well as on xylanase enzyme production by replacing the urea which used in the original medium with other different nitrogen sources such as yeast extract, bacteriological peptone, peptone water and casein hydrolysate. From the figure 2, it can be concluded that, bacteriological peptone enhances the cell growth and enzyme activity level up to 241.17 Uml^{-1}. This followed by yeast extract, peptone water and casein hydrolysate. The superiority of peptone compared to other nitrogen sources to support xylanse production have been also reported by other authors[16]. In addition,5 gL^{-1} peptone showed the highest enzyme activity of 245.61 Uml^{-1}. Furthermore, effect of inorganic mineral sources were evaluated andit was found that, magnesium sulphate supports high enzyme production up to 238.45 Uml^{-1}. This obtained result was supported bysoliman *et al*., who reported also on the importance of mineral source supplementation for xylanse production when using several agriculture waste based medium[17]. However, magnesium sulphate was added in different concentrations ranges from 0.2 gL^{-1} to 1 gL^{-1} where 0.5 gL^{-1} supported the maximal enzyme production.

Kinetics of cell growth and xylanase production in un-optimized and optimized medium

The growth kinetics of *Trichoderma*sp WICC F46 in un-optimized and optimized (OFAT) in shake flask culture were carried out. These growths explain in detail about the correlation between the total biomass, pH as well as the xylanase enzyme production. The cultivation was conducted in shaking incubator at 30 ℃ and 200 rpm for 72 hours for un-optimized and 104 hours for optimized OFAT. Figure 3. below shows the result of the both un-optimized and optimized (OFAT) growth kinetics of *Trichoderma*sp WICC F46.

For the un-optimized growth kinetics, the total biomass start to increase after 30 hour, where after

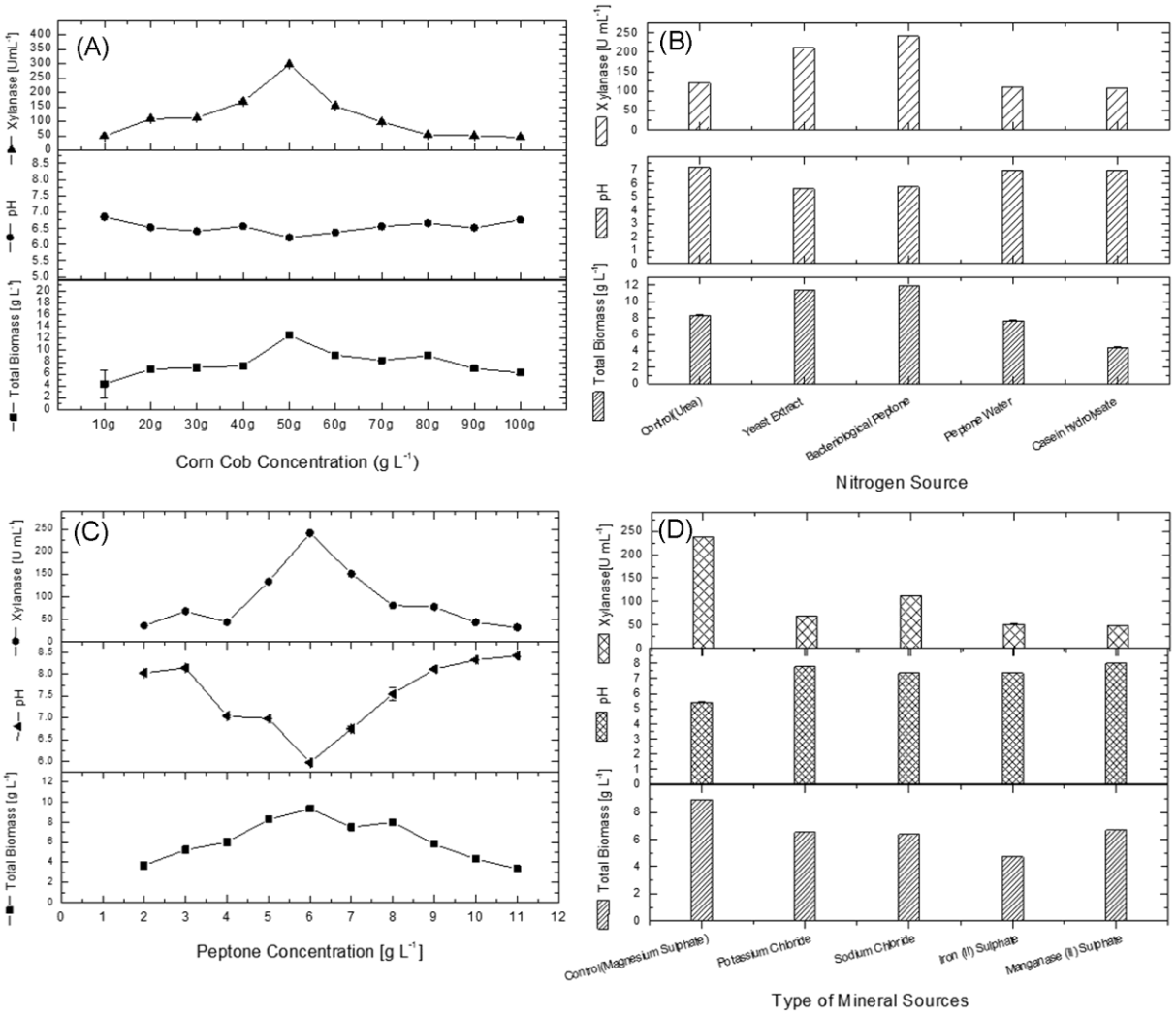

Fig. 2 — OFAT studies on A: Different corn cob concentrations. B: Different type of organic nitrogen source. C: Different concentrations of peptone. D: Different type of mineral sources.

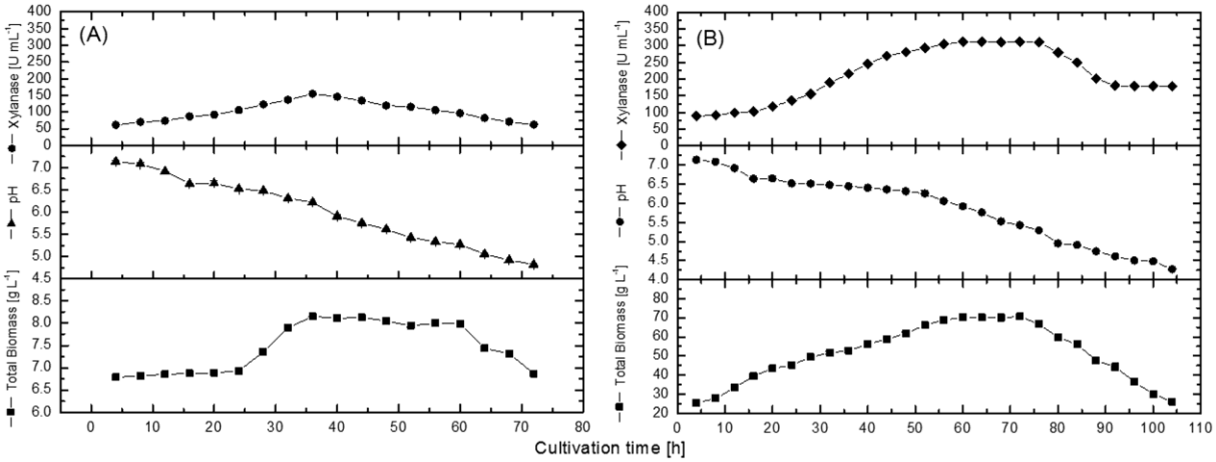

Fig. 3 — A. Kinetics of cell growth and enzyme production by *Trichoderma*sp. WICC F46 during cell cultivation in Un-optimized (A) and optimized media (B).

35 hour, it showed the highest biomass which is around 8.35 $gL^{-1}$and it was at its stationary phase between 40–60 hours where the total biomass production was under static level. After 60h cultivation, the total biomass starts to decrease which indicates that the fungal cells start to enter the death phase due to factors like limited nutrient availability and increase in number of cells. The factor pH always plays a pivotal role and give significant effects towards any kind of enzymes production. For xylanase enzyme production, according to[18,12] the best pH condition for xylanase enzyme production is from the range of 4.5 to 5.5. When the pH condition is around 5, the higher level of xylanase enzyme production was observed which is around 151.34 Uml^{-1} for un-optimized growth kinetics and 311.71 Uml^{-1} for OFAT growth kinetics. As the pH starts to drop below 4 and when it starts to increase more than 6 the enzyme production as well as the total biomass also starts to decrease gradually which indicates that, too much of acidic and alkaline condition its not suitable for both the xylanase enzyme production as well for the biomass production. The pH condition always correlates with the enzyme production. Similar literature studies also reveal that the optimum pH for fungal xylanases in general slightly lowers than the pH optima of bacterial xylanase. The growth rate of total biomass was achieved in optimized media with significant high xylanase enzyme activity in the terms of product specificity (P_{max}/X_{max}) which is 4,417 (Ug^{-1}). The total biomass for the optimized media was significantly higher reaching about 70 (gL^{-1}) with enzyme activity reached a maximal value of 311.71 Uml^{-1}.

Conclusion

As a conclusion the optimized medium not lead to significant increase in the fungal growth but also increased xylanase production by about 200%. In this study, the optimal medium composition was: 50 gL^{-1}corncob, 5 gL^{-1} bacteriological peptone, 0.5 gL^{-1} magnesium sulphate, 0.5 gL^{-1} calcium chloride, and 1.0 gL^{-1} ammonium sulphate. This optimized medium can be used for large scale and it will be cost effective compared to other medium currently used for xylanse production. In addition, this medium replaced the expensive carbon sources (such as mono- and disaccharides fermentable sugars) by corncob which is considered as agricultural waste. However, further bioprocess improvement in terms of biochemical engineering parameters for *Trichoderma* sp WICC F46 growth and enzyme production in large scale need to be further studied for industrialization of this process.

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