Bioprocessing of *Anogeissus leiocarpus* Sawdust for Optimum Production of Total Soluble Protein by *Aspergillus niger* and T*richoderma harzianum*

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ABSTRACT

Sawdust of different types adorn our cities' landscape as lignocellulosic wastes of the wood industry; whose disposal often involves burning a process that pollutes the atmosphere. The availability of sawdust as alternative substrate, and the wood-degrading ability of fungi make it cost-effective for bioproduction of value-added products of industrial importance. In this study, fungi isolated from sawdust were morphologically identified. *Anogeissus leiocarpus* sawdust moistened with cellulase medium was thermally processed for 1 h. Duplicate flasks were optimized by varying environmental factors to produce Total Soluble Protein (TSP) in Solid State Bioprocessing (SSB). Flasks content were adjusted (pH 4.0 - 7.0), inoculated solely with two agar plugs (6 mm cork borer) of 5-day old cultures of *Aspergillus niger* and *Trichoderma harzianum*, and incubated (28°C - 40°C) for 6 - 14 days. All TSPs produced were statistically different ($p < 0.05$). The TSP produced by *A. niger* and *T. harzianum* ranged from 157-365 mg/L and 175-362 mg/L, respectively, with both optima at pH 5.0. However, TSP production by A. *niger* and *T. harzianum* ranged from 103-350 mg/L and 181-885 mg/L with optima at 35°C and 28°C, respectively. Both fungi produced the highest TSP on day 10 of incubation. The study established that *A. niger* and *T. harzianum* could be useful tools for efficient bio-processing of sawdust into TSP for industrial and biotechnological applications.

Keywords: *Anogeissus leiocarpus* sawdust, *Aspergillus niger*, *Trichoderma harzianum*, Total Soluble Protein, Solid State Bioprocessing.

INTRODUCTION

Lignocellulosic biomass is the most abundant renewable organic matter on earth with potentials for biosynthesis of many value-added products (Oshoma *et al*., 2020). Fungi possess biosynthetic machineries for the

upgrade of low-protein lignocellulosic substrates to macromolecules suitable for industrial and biotechnological applications (Haider, 2021). Sawdust, a low-protein substrate has been utilized by fungi species to produce crude soluble protein and single cell protein using appropriate nutrient (Haider, 2021). These fungi could be either mesophiles or thermophiles found in habitats where plant matter abound (Buraimoh *et al*., 2015).

Lignocellulosic wastes include cassava peels, banana peels, citrus peels, and sugarcane baggase, and wastes from forestry processing, agricultural practices and agro-based industries (Ezekiel and Aworh, 2013). Cellulose, hemicelluloses and lignin are the three main chemical components of lignocelluloses; of which cellulose is the most abundant biopolymer (Seddiqi *et al*., 2021). However, the lignin structure renders the other components inaccessible to hydrolytic activities of many chemicals and enzymes (Buraimoh *et al*., 2015); thus, limiting their value. Certain fungi play vital roles in the breakdown and use of lignocelluloses by producing accessory enzymes (Nguyen *et al*., 2018). The enzymes responsible for degradation of cellulose and hemicelluloses belong predominantly to the hydrolases which cleave glycosidic bonds, while the major groups of enzymes involved in lignin degradation are peroxidases and phenol oxidases (Chukwuma *et al*., 2020; Kumar and Chandra, 2020).

In spite of the enormous potentials, accumulation of lignocellulosic wastes poses a serious threat to the environment. Often, much of the wastes are disposed of by burning which leads to air pollution (Howard *et al*., 2003). In Nigeria, indiscriminate dumping of sawdust into water bodies is a common practice (Buraimoh *et al*., 2015). In the last two decades, Nigeria has put in concerted efforts in search of appropriate technologies to utilize agro-industrial wastes and alleviate their environmental hazards (Ezekiel and Aworh, 2013). Solid State Bioproccessing (SSB) stands out as the most appropriate technology to convert agro-industrial wastes to value-added products. This is due to the availability of low-cost substrates, high turnover, less downstream processing, increased quantity of protein, high market value of products, and as a valuable alternative to the problem of waste disposal (Chilakamarry *et al*., 2021).

Many studies have produced extracellular proteins including, cellulase, hemicellulase and xylanase, mannanase; organic acids and other platform chemicals for industrial applications using sawdust, bagasse, corncob and other lignocelluoloses by *Aspergillus niger (*Ojumu *et al*., 2003; Adesina *et al*., 2013; *Hamdy*, 2013; Buraimoh *et al*., 2015*). Trichoderma harzianum* is also reported as highly efficient in the production of many extracellular proteins. They are used commercially for production of cellulases and other enzymes that degrade complex polysaccharides in the food and textile industries (Kunamneni *et al*., 2014). The multi-sided activity of the genus *Trichoderma* provides a potential for extensive applications in different branches of bioeconomy (Blaszczyk *et al.,* 2014*).* It has been reported that total soluble proteins produced by *Trichoderma* could be a repository of a variety of bioactive secondary metabolites (*Khan et al., 2020).* Secondary metabolites *such as hydrophobins* are applied in medical, agricultural and industrial fields (Khan *et al.,* 2020). The presence of potential biocontrol agents, compounds like gluconic, citric, and coumaric acids which causes the release of phosphorus ions and microelements for enhanced growth of plants is also of environmental and consumer-friendly benefits (Blaszczyk *et al.,* 2014; *Khan et al.,* 2020). It is proposed that for efficacy, bioproducts from effective strains should be obtained from regions in which they are to be applied (Blaszczyk *et al.,* 2014). Thus, large-scale production of TSP could be a versatile tool

for the biorefining and assay of many value-added products.

However, the production of fungal soluble proteins is significantly affected by environmental factors such as pH, temperature, water activity, incubation period and available nutrients. Another work stated that the type of lignocellulosic substrate used has the greatest effect on the production of bioproteins (Cai *et al*., 2021; Ganash *et al*., 2021) which requires screening of agro-industrial wastes. Several research works have produced fungal proteins due to myriads and continued demand for biotechnological and industrial applications (Okunowo *et al*., 2010). Most of the extracellular proteins have been widely studied in Submerged Fermentation (SmF) (Howard *et al*., 2003). However, SSB holds tremendous potential for the production of extracellular proteins and can be of special interest in those processes where the crude fermented products may be used directly as enzyme sources or sources of other metabolites. Hence, there is a need to further investigate on the sets of environmental conditions for optimum production of fungal soluble proteins. Therefore, the objective of this work is to establish suitable conditions for the production of TSP, using *Anogeissus leiocarpus* sawdust as the sole source of carbon in SSB, by optimizing the effect of pH, temperature and incubation period on *Aspergillus niger* and *Trichoderma harzianum*.

MATERIALS AND METHODS

Sample collection and processing

Wood shavings of *Anogeissus leiocarpus* was collected from Bodija plank market, Ibadan North Local Government Area, Oyo State, Nigeria. Sample was sun-dried to reduce moisture content and crushed to sawdust using a motorised grinding machine (Immanuel *et al*., 2006). The sawdust was oven dried at 60˚C to constant weight and sieved through a 2.0 mm wire mesh to provide particles of even sizes for oxygen diffusion, nutrient absorption and assimilation by fungal mycelia (Goyal *et al*., 2008). Twenty five grams of sample was dispensed in duplicate Erlenmeyer flasks (250 mL), moistened with 75 mL distilled water (Adenipekun and Fasidi, 2005). Samples were thermally treated by autoclaving at 121° C (15 psi) for 1 hour to make the wood components more readily available for hydrolysis by the fungal enzyme (Adesina *et al*., 2013).

Isolation and identification of fungi

Sawdust sample was moistened with sterile distilled water and kept in the Green House, Department of Microbiology, University of Ibadan, for 7 days spontaneous inoculation. A 0.5 g of sample was directly inoculated on Potato Dextrose Agar (PDA Lab M) and incubated at room temperature for 7 days. Pure cultures were obtained by sub-culturing and store on PDA slants at 4°C. Fungi cultures at 5-day old were observed for both cultural and morphological characteristics and features compared with the Compendium of Soil Fungi (Domsch *et al*., 1980).

Production of growth medium and inoculation in SSB

The Mandel and Weber medium for cellulase production as modified by Chahal, (1985) was used. The mineral salt nutrient contained the following (g/L): KH_2PO_4 2.0; $(NH_4)_2SO_4$ 1.4; Urea 0.3; $MnSO_4.7H_2O$ 0.0016; $ZnSO_4.7H_2O$ 0.0014; $CaCl_2$ 0.3; $FeSO_4.7H_2O$ 0.005 and Yeast extract 0.1 (Singh *et al*., 2017). Thereafter, 25 g sawdust was moistened with the medium (15 mL) in a 250 mL Erlenmeyer flasks and autoclaved at 121°C (15 psi) for 15 minutes. Each flask was inoculated with two agar plugs of a 5-day old culture of *Aspergillus niger* and *Trichoderma harzianum* separately using a 6 mm cork borer. The fungal isolates were selected for fermentation on the basis of frequency of isolation from the wood substrate. A duplicate flask containing

sawdust and the mineral salt medium was incubated without inoculum as control. Prior to optimization of growth conditions the isolates were incubated at 25°C (pH 3.0) for 6 days.

The effect of pH and temperature on total soluble protein production

To determine the effect of pH, the medium was adjusted with 0.1M HCl to attain various pH values ranging from 4.0 - 7.0. The sample was set at 60 % moisture content (Adesina *et al*., 2013) and then incubated at 25°C. The effect of temperature was determined at optimum pH and incubated at 28°C, 35°C and 40°C for 6, 10, and 14 days.

Extraction and determination of total soluble proteins

A 50 mL cold 0.05M Sodium phosphate buffer (pH 7.0) was added into each fermented flasks (1:2), agitated vigorously for 10 minutes and filtered with 90 mm Whatman filter paper No. 1. The clear filtrate was collected and stored at 4°C (Singkaravanich and Vichitsoonthonkul, 2007). Total soluble proteins were determined by the method of Lowry *et al*. (1951). The reaction mixture consisted of 0.1 mL soluble protein, 0.5 mL sterile distilled water, 3 mL (2% Na_2Co_3 in 0.1 M NaOH and 1.0 mL of 0.5% $CuSO_4$. $5H_2O$ in 1% Sodium potassium tartarate) incubated for 10 minutes at room temperature. Folin-Ciocalteu reagent (BDH) (0.3 mL) was added and incubated for 30 minutes. The Optical Density (O.D.) was determined at 670 nm using a spectrophotometer (Uniscope 23D) and the protein content extrapolated from a standard curve using egg albumin (BDH) (Bradley and Markwell, 2007).

Statistical Analysis

Results obtained from the study were subjected to analysis of variance using one way ANOVA and differences between means of test samples were separated by Duncan Multiple Range Test (Ducan, 1955).

RESULTS

In the study, fungi belonging to three genera namely; *Aspergillus niger*, *Trichoderma harzianum* and *Fusarium oxysporum* where isolated on PDA from sawdust of *Anogeissus leiocarpus* after 7 days of spontaneous inoculation (Plate 1a-c). Prior to optimization of growth conditions *Aspergillus niger* and *Trichoderma harzianum* produced 121 mg/L and 144 mg/L of total soluble protein respectively (Figure 1). Figure 2 present the effect of pH on total soluble protein (TSP) produced by *Aspergillus niger* and *Trichoderma harzianum* on day 6 of fermentation. *Aspergillus niger* produced TSP in the range of 157 - 321 mg/L but was higher at pH 7.0 (321 mg/L). TSP produced by *Trichoderma* spp. ranged from 188 - 232 mg/L and was higher at pH 5.0 (232 mg/L). There was significant difference in TSP produced by the isolates at the different pH values ($p<0.05$) on day 6. The production of TSP on day 10 was best at pH 5.0 for both *Aspergillus niger* and *Trichoderma harzianum* and ranged from 172 - 365 mg/L and 175 - 362 mg/L, respectively with $p<0.05$ (Figure 3). However, production of TSP by *Aspergillus niger* and *Trichoderma harzianum* generally decreased but was high at pH 6.0 (217 mg/L) and pH 4.0 (268 mg/L) respectively on day 14 (Figure 4).

Figure 5-7 present the effects of temperature on TSP produced on days 6, 10 and 14 of fermentation. On day 6, TSP produced by *Aspergillus niger* ranged from 146 - 314 mg/L but was high at 35°C (314 mg/L) while TSP produced by *Trichoderma harzianum* ranged from 173 - 320 mg/L and was high at 40°C (320 mg/L). *Aspergiilus niger* again recorded high TSP at 35°C (350 mg/L) on day 10. The highest TSP obtained in the study was produced by *Trichoderma harzianum* at 28°C (855 mg/L) on day 10. The TSP production on day 14 declined generally with both *Aspergillus niger* and *Trichoderma harzianum* producing 194 mg/L and 230 mg/L, respectively at 28°C.

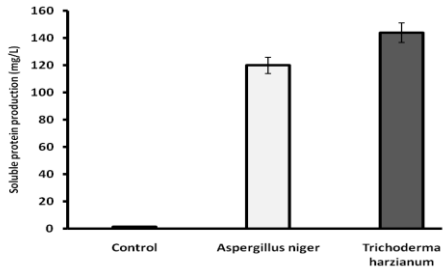

Fig. 1 Total soluble protein produced by *A. niger* and *T. harzianum* on day 6 before optimization of growth conditions. Bar represent standard error of duplicate determination.

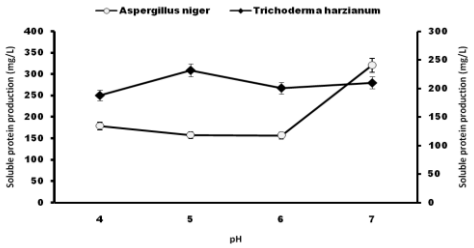

Fig. 2 Effect of pH on total soluble protein produced by *A. niger* and *T.* harzianum on day 6 of fermentation. Bar represent standard error of duplicate determination.

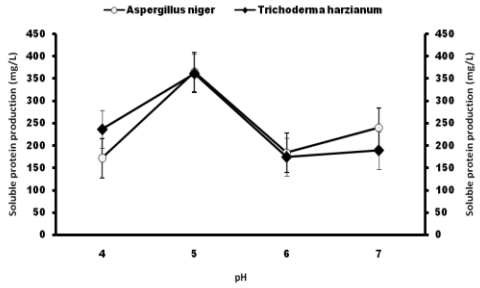

Fig. 3 Effect of pH on total soluble protein produced by *A. niger* and *T. harzianum* on day 10 of fermentation. Bar represent standard error of duplicate determination.

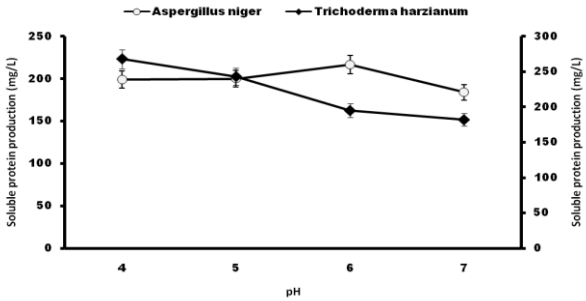

Fig. 4 Effect of pH on total soluble protein produced by *A. niger* and *T. harzianum* on day 14 of fermentation. Bar represent standard error of duplicate determination.

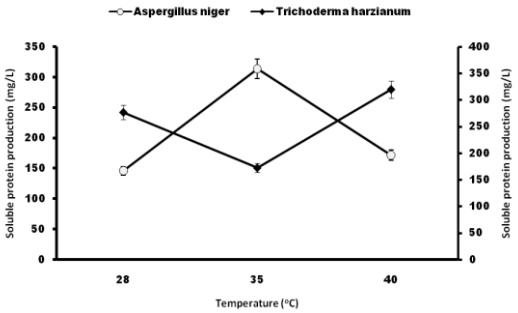

Fig. 5 Effect of temperature on total soluble protein produced by *A. niger* and *T. harzianum* on day 6 of fermentation. Bar represent standard error of duplicate determination.

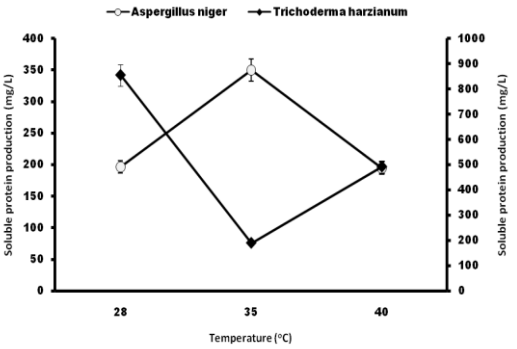

Fig. 6 Effect of temperature on total soluble protein production by *A. niger* and *T. harzianum* on day 10 of fermentation. Bar represent standard error of duplicate determination.

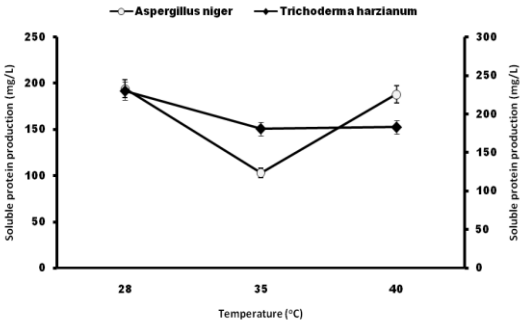

Fig. 7 Effect of temperature on total soluble protein produced by *A. niger* and *T. harzianum* on day 14 of fermentation. Bar represent standard error of duplicate determination.

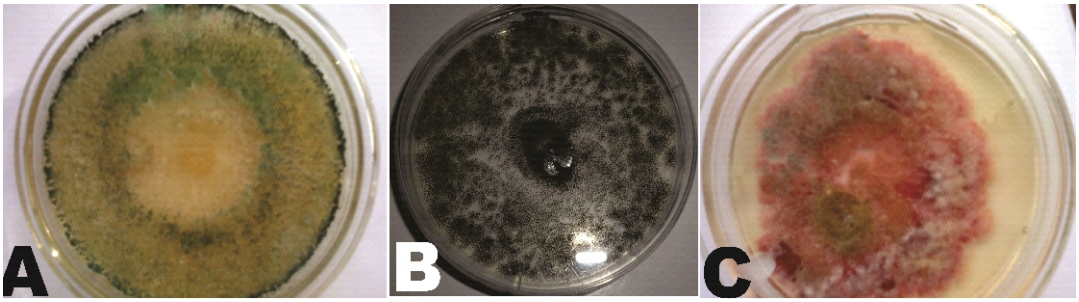

Plate 1 A – C: Fungi isolated from sawdust of *Anogeissus leiocarpus* on PDA.
(A) *Trichoderma harzianum*, (B) *Aspergillus niger*, (C) *Fusarium oxysporum*

DISCUSSION

Production of TSP by various species of fungi is reportedly affected by pH (Hakkinen *et al*., 2015; Barda *et al*., 2020). In this study, production of TSP by *A. niger* attained its peak at pH 5.0 in 10 days contrary to the findings of Yalemtesfa *et al*. (2010) who reported peak production of crude and soluble protein by *A. niger* using orange peels at pH 7.0 in 4 days. The differences in the peaks of production may be in part due to the different types of lignocellulosic substrates used in the fermentations. This is consistent with *Cai et al*. (2021) who stated that different carbon sources induce the production of different soluble proteins which affect the growth and development of fungi. Furthermore, the type of lignocellulosic substrate has the greatest effect on soluble protein production (Ganash *et al*., 2021). This is because, fungi vary in their abilities to utilize and convert different plant materials; a characteristic that plays a vital role in the recycling of nutrients, production of food, chemicals and enzymes for industries (*Cai et al*., 2021).

Reportedly, *A. niger* is an excellent producer of soluble proteins including cellulase and hemicellulase using sawdust as the sole source of carbon (Buraimoh *et al*., 2015). According to Immanuel *et al*. (2007) *A. niger* grown in sawdust-supplemented medium produced high amount of cellulase at pH 5.0 which coincided with the optimum pH for TSP production in our study. Since, the Mandel and Weber medium used for moistening of the sawdust was for cellulase production, it is plausible to suggest that the peak might represent the cellulase component of the TSP. Furthermore, *A. niger* is reported to quickly decrease the pH of fermenting substrates by secreting citric acid, which may account for the decline in pH from pH 7.0 - 5.0 in the study (Hamdy, 2013; *Odoni et al., 2017*). Hence, this optimized the condition for higher production of TSP. However, production of TSP declined at pH 6.0 on day 14. The corresponding dip in extracellular production of protein possibly signaled an end to the peak of metabolic activity and product synthesis by the fungus (Gefena *et al*., 2014). This may be due to many factors such as exhaustion of carbon and nitrogen sources, depletion in the concentration of oxygen, change in pH value of the fermenting substrates and production of certain toxic metabolite (Olorunnisola *et al*., 2018, Haider, 2021). In another study, Laba *et al*. (2017) also reported changes in the pH value of *Adansonia digitata* sawdust using *Neurospora intermedia* and *Pleurotus pulmonarius, and* attributed it to the increase in amino nitrogen content and metabolic waste products.

Studies have reported different pH optima for the production of proteins, including cellulases and xylanases. Bala and Singh (2017) reported concomitant production of cellulases and xylanases by *Sporotichum thermophile* at pH 5.0 on substrates containing hemicelluloses. In our study, *T. harzianum* produced high amount of

TSP at pH 5.0 (day 6) which is also close to the optimum pH 5.5 for cellulase and xylanase produced by *T. viride* and *T. reesei* on day 5 (Bilal *et al*., 2015). However, maximum TSP production was obtained at pH 5.0 on day 10. Also, Ezekiel and Aworh (2013), reported maximum production of crude protein by *T. viride* at pH 6.0 (day 8) using cassava peels which lies close to the peak of our study. The difference in the peaks of production may be due to differences in the fungal species and the substrates used in SSF. However, production of TSP generally declined on day 14; but, with the peak at pH 4.0. This corroborates another study that *Trichoderma sp.* maintains a more stable pH of the fermenting medium between pH 4.0 - pH 6.0 (Pandey *et al*., 2015).

Temperature has profound effects on the growth and production of protein by fungi in Solid State Fermentation (Singhaniaa *et al*., 2009). Optimum temperature for maximum production of bioprotein depends on the characteristics of the strain (Chimata *et al*., 2010). The production of TSP by *Aspergillus niger* on sawdust of *A. leiocarpus* differed significantly at all the temperatures (p<0.05). The production of TSP by *A. niger* was high at 35°C on day 6; however, the peak of production (350 mg/L) was at 35°C on day 10. This result lies close to the findings of Akula and Golla (2018) who reported maximum production of cellulases at 32°C by *A. niger* using various lignocellulosic substrates. However, another work stated that *A. niger* produced maximum amount of cellulase at 40°C using the lignocellulose *Arachis hypogaea* shells as substrate on day 5 (Sulyman *et al*., 2020). The difference in peak of production may be due to fungal morphogenesis and substrates. Also, it is possible to suggest that *A. niger* may have passed the primary stage of growth on day 6 to the secondary phase which was characterized by the sharp increase in TSP production on day 10 (Oskar, 2017).

In the study, the highest TSP of 855 mg/L was produced by *T. harzianum* at 28°C on day 10. Different workers have reported the effects of varying temperatures on the production of soluble proteins by *Trichoderma sp.* on various lignocellulosic substrates. Goyal *et al.* (2008) reported maximum production of xylanase on jowar straw by *T. viride* between 20 - 25°C. However, Pang *et al*. (2006) reported maximum production of xylanase using combination of sugar cane baggase and palm kernel cake by *Trichoderma sp*. in SSF at 25 - 30°C which is consistent with our study. The temperature range over which an enzyme maintains a stable, catalytically competent conformation depends upon (and typically moderately exceeds) the normal temperature of the cells in which it resides (Kennelly *et al*., 2016). Thus, the optimum temperature obtained for production of bioprotein by *Trichoderma sp*. in the study was naturally close to the optimum temperature for growth in its natural habitat (Signh *et al*., 2014). Similarly, Lee *et al*. (2017) reported a good correlation between growth and extracellular protein production. It is important to note that the best soluble protein was obtained on day 10 followed by a decline on day 14. The decline in production was probably because the fungus passed the active stage of extracellular protein production and possibly entered its death phase on day 14 (Oskar, 2017).

CONCLUSION

The study established *A. niger* and *T. harzianum* as efficient producers of TSP through optimization of the environmental factors using *Anogeissus leiocarpus* sawdust as the sole source of carbon. Optimum production of TSP for both isolates occurred at pH 5.0; while, temperature influenced the highest TSP production at 28°C by *T. harzianum* in the study. The best incubation period for TSP by both isolates was on day 10. Based on this work relevant industries may establish efficient strategies for maximum production of TSP by *A. niger* and *T. harzianum* as versatile tools for biorefining of many value-added products required for myriads of biotechnological and industrial applications.

Conflict of interest

The authors declared no potential conflicts of interest.

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