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Research Article

Pretreatment Strategies to Improve Crude Glycerol Utilisation and Metabolite Production by *Aspergillus terreus*

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Crude glycerol (CG) can be used as a substrate for microbial bioconversion. However, due to presence of many impurities, many microorganisms are unable to utilise this substrate efficiently. The present study is trying to improve CG using as the feedstock of *Aspergillus terreus* for the production of lovastatin, (+)-geodin, and sulochrin. The CG was pretreated chemically (solvents) and physically (activated carbon (AC) and water softener (WS)) to separate most of the impurities from the CG. For solvent pretreatments, petroleum ether (PE) produced the largest increase of lovastatin (92.8%) when compared to positive control and pure glycerol (PG) and up to 820% when compared to negative control (CG). In contrast, diethyl ether (DE) produced the largest increase in (+)-geodin at 80.81% (versus CG) and 176.23% (versus PG). The largest increase in toluene (Tol) was observed in sulochrin production, at 67.22% (versus CG) and 183.85% (versus PG). For physical pretreatments, the pattern of metabolite production in AC (lovastatin: 20.65 mg/L, (+)-geodin: 7.42 mg/L, sulochrin: 11.74 mg/L) resembled PG (lovastatin: 21.8 mg/L, (+)-geodin: 8.60 mg/L, sulochrin: 8.18 mg/L), while WS (lovastatin: 11.25 mg/L, (+)-geodin: 15.38 mg/L, sulochrin: 16.85 mg/L) resembled CG (lovastatin: 7.1 mg/L, (+)-geodin: 17.10 mg/L, sulochrin: 14.78 mg/L) at day 6 of fermentation. These results indicate that solvent pretreatments on CG are excellent for metabolites production in *A. terreus*, depending on the solvents used. In contrast, physical pretreatments are only feasible for (+)-geodin and sulochrin production. Therefore, different strategies can be employed to manipulate the *A. terreus* bioconversion using improved CG by using a few simple pretreatment strategies.

1. Introduction

CG waste was previously considered a valuable by-product derived from the biodiesel industry. However, the rapid growth of the biodiesel industry resulted in an overproduction of CG, leading to its significant devaluation. CG waste has become a burden, especially for small biodiesel producers, as improper disposal is harmful to the environment, and purification processes are not cost-effective [1]. The bioconversion of CG is particularly challenging as biodiesel-derived CG waste contains a significant amount of impurities. CG contents vary widely with the biodiesel manufacturer, ranging from 38% to 96% glycerol; with the remaining normally comprised of water, methanol (MeOH), free fatty acids, and salts [2]. MeOH and fatty acids are formed during the transesterification process, while salt originating from the catalyst is used in the biodiesel production process. High contents of impurities in CG may inhibit the production of metabolites in most microorganisms [3–6] and hence can reduce the economic potential of chemical processes using CG as feedstock.

The most common method of crude glycerol purification includes laborious process of distillation, which involves specific temperature, pressure, and pH control. Furthermore, such a process is employed to produce industrially pure glycerol, which is not required in microbial bioconversion as microorganisms may be able to tolerate certain amount of impurities. Simple techniques, such as the addition of solvent, water softener, and activated charcoal [7], may reduce the amount of impurities and enhance the

microbial conversion significantly. Solvent extraction is capable of forming two heterogonous layers of impurities and glycerol, while water softener removes hard chemicals and activated carbon is efficient in capturing ash and MONG of crude glycerol. Some microorganisms displayed excellent use of improved CG, including *Schizochytrium limacinum* [5], yeast *Yarrowia lipolytica* [8], and bacteria *Clostridium butyricum* [9, 10].

Aspergillus terreus ATCC 20542 is a filamentous fungus that can be found in soil. It is a well-known strain that can produce lovastatin, a cholesterol-lowering drug. The mode of action of lovastatin involved the inhibition of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, a rate-limiting enzyme involved in cholesterol biosynthesis in the liver. Its lesser known metabolites, namely, (+)-geodin and sulochrin, can also be pharmaceutically important. (+)-Geodin has been shown to inhibit the plasminogen activator inhibitor (PAI-1), a molecule that is important in fibrinolysis mechanism and for the stimulation of glucose uptake in rat cells [11]. Likewise, sulochrin can inhibit the activation of human immune cell (eosinophil), endothelial cells, and certain cytokine release (IL-8 and LTC4) [12, 13].

A. terreus is of particular interest to be used with CG, mainly due to their fungus characteristic which is known for their ability to withstand different contaminants present in CG. The cultivation of *A. terreus* in CG significantly reduces its abilities to produce certain metabolites, although the growth of the fungus was uninterrupted [14]. It was found that the contaminants in CG, namely, methanol, sodium chloride, and fatty acids can lead to different responses of *A. terreus*. For lovastatin, certain fatty acids are inhibitory to *A. terreus*, while methanol and sodium chloride are stimulatory up to a certain concentration [14]. In contrast, (+)-geodin was inhibited in the presence of methanol and palmitic acid [14].

This current study reports the efficiency of pretreated CG on the growth and metabolite production of *A. terreus* ATCC 20542. Three strategies of pretreatments are applied, by using nonpolar solvents, activated carbon, and water softener pillow. The growth, in the form of dry cell weight, and the production of metabolites were compared against the CG (negative control) and PG (positive control). This study is a continuation from the previous investigation by Abd Rahim et al. [14]. This is the first study involving the use of improved CG for the production of lovastatin, (+)-geodin, and sulochrin from *A. terreus*.

2. Materials and Methods

2.1. Description of Crude Glycerol Used in the Study. CG samples were donated by Biodiesel Producers Limited (Melbourne, VIC). The CG is a waste by-product derived from a biodiesel process that uses tallow and uses cooking oil as feed. The CG exhibits dark yellow colour and has a pH of 7. It was stored in a sealed aluminium container at room temperature. Depending on the type of experiment conducted, the CG was either filtered to remove the undissolved contents, autoclaved to remove MeOH, or vigorously shaken to evenly distribute all the contents.

The pretreatment using solvent washing was performed to dissolve hydrocarbons present in CG. Different nonpolar solvents (hexane, heptane, octane, and petroleum ether) were used to wash the CG at room temperature using 1 : 1 volume ratio, followed by shaking at 200 rpm. After three hours of mixing, it was centrifuged at 3000g for 2 minutes to separate the mixture into two distinct phases, namely, the upper phase of solvent and the lower aqueous phase of glycerol containing residual impurities. The lower phase was subjected to washing again with fresh solvent, and the process was repeated to collect the final treated CG.

The physical pretreatments involved the use of activated carbon and water softener pillow purchased from MARS Fishcare North America (PA, USA). The CG used in this pretreatment was diluted with the same volume of deionised water to reduce its viscosity. Around 20% (wt/v) of activated carbon or water softener pillow was added directly to the diluted samples, following the incubation at 200 rpm at room temperature for 3 hours. The samples were later centrifuged at 3000 g for 15 minutes, and the deposit (activated carbon or resins of water softener pillow) was removed to obtain the CG.

2.2. Culture Conditions. The fungal strain used in this study is *Aspergillus terreus* ATCC 20542. The cultivation of *A. terreus* into the shake flask was done as previously described [15]. In short, the freeze-dried fungus was reactivated using sterile deionised water and maintained on potato dextrose agar at 30°C for 7 days. The number of spores used was 10^7 spores/mL (counted using the haemocytometer) and inoculated into the 125 mL Erlenmeyer flasks and shaken at 185 ± 5 rpm and a temperature of 30 ± 1°C. The basic basal salt medium was used for all experiments (0.4 g/L KH_2PO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.4 g/L NaCl, and 0.001 g/L $ZnSO_4{\cdot}7H_2O$). Carbon sources (pretreated CG, CG, and PG) were adjusted to 30 g/L, and yeast extract was used as the sole nitrogen source at 4.0 g/L. The preculture was prepared in a similar basal salt medium, but with glycerol carbon source (10 g/L) and yeast extract nitrogen source at 8 g/L between 24 and 30 hours. The fungus was used for cultivation when the diameter of the fungal pellet reached 1.5 ± 0.5 mm, measured using a digital calliper. The day of the preculture is considered as Day 1.

2.3. Analytical Methods. The quantification of lovastatin, (+)-geodin, and sulochrin was carried out by high-performance liquid chromatography (HPLC), Agilent 1200, using a C-18 column and UV detector at a wavelength of 238 nm, with reference wavelength of 360 nm, as previously described [15]. The preparation of lovastatin standard involved a treatment of methanol and sodium hydroxide solution [14]. The (+)-geodin and sulochrin standards were purchased from Sapphire Bioscience (Sydney, Australia). Sulochrin, (+)-geodin, and lovastatin appeared at a retention time of around 4, 7, and 10 minutes, respectively, in the HPLC chromatogram. The quantification of glycerol in the samples was performed using the glycerol calorimetric detection reagent purchased from Sigma-

Aldrich (Sydney, Australia). This reagent measures glycerol by using enzymatic reaction. As the detection is very sensitive, the acceptable range of glycerol concentration is around 20 mg/L. The plate was read using a microplate reader (Biorad model 680) at 450 nm. The biomass yield was determined gravimetrically. Fungus biomass was recovered by filtration using No. 2 Whatman filter paper and washed twice with distilled water, followed by drying at 80°C for 24 hours or until a constant weight is achieved.

2.4. Statistical Analysis. All experiments were conducted at least using triplicates. Data obtained for metabolite production involving lovastatin, (+)-geodin, and sulochrin were analysed using one-way ANOVA with the Tukey post hoc test. The results are considered significant when $p < 0.05$. All statistical analyses were performed using Graphpad Prism, version 6.01. For the plotting of the graph, 95% confidence interval was used for the error bar.

3. Results and Discussion

3.1. Comparison between the Growth, Substrate Consumption, and Metabolite Production Using Crude, Pure, and Nonpolar Solvent Pretreated Glycerol. Solvent washing is a simple method of separating a compound based on the different solubilities of two immiscible liquids. This method is efficient in reducing certain fatty acids in the solution [16]. In our investigation, we opted to use petroleum ether (PE), diethyl ether (DE), and toluene (Tol) as our solvent of interest. Rehman et al. were among the first team to use this technique on CG, achieving a good microbial bioconversion [17].

The growth of *A. terreus* in PG exhibited better growth than in CG. The use of pretreated CG produced good biomass, which was comparable or exceeded that observed with PG (Table 1). DE showed the highest biomass production (11.28 g/L), followed by PE (10.60 g/L), PG (10.07 g/L), Tol (9.80 g/L), and CG (9.75 g/L). The high production of biomass was likely from the residual fatty acids that are still present in pretreated CG, given that the solvent washing cannot entirely remove them from the solution [16]. The increase in biomass in the presence of fatty acids in CG has also been shown previously [14]. Although in theory, CG should contain the highest amount of fatty acids, the biomass produced was the lowest due to the presence of certain type growth inhibitory fatty acids, as shown with the soap [9]. Nevertheless, the biomass production is not as important in this type of cultivation, as the main goal is not to produce biomass, but rather, to produce metabolites. In many previous studies, the increase or decrease in biomass is not a direct indication of the metabolite production [18].

There was little change in the glycerol content between the CG before and after the chemical solvent pretreatments. The pretreatments of CG with these solvents improved the glycerol consumption significantly (up to 50% improvement) when compared to nontreated CG media (Figure 1). This may be possible as a result of the solvent reducing most of the free and methyl ester fatty acids in the CG. However, the rate of consumption of pretreated CG still did not reach the level of consumption when PG was used, as the treatments were still unable to remove other type of contaminants such as MeOH and salts [16]. The lower glycerol consumption in CG is most likely due to the presence of fatty acids, which may lead to the substrate competition (as fatty acids may probably be the preferable carbon source) or other inhibitory effect of fatty acids on carbon uptake [14].

The production of lovastatin, (+)-geodin, and sulochrin using pretreated CG is depicted in Figure 2. PE, in particular, showed a significant increase of 92.8% in lovastatin production at day 6 compared to the PG (positive control). In contrast, the final titre of lovastatin at day 6 for both DE and Tol was insignificant to the positive control. Interestingly, a set of different responses were observed in (+)-geodin and sulochrin production. Instead of PE (5.58 mg/L), DE (8.95 mg/L)-induced (+)-geodin is the strongest, while sulohrin is more responsive to Tol pretreatment (24.78 mg/L). These results are still considered excellent nonetheless, because substantial improvement in metabolite production, as well as glycerol consumption, was achieved in all pretreated samples when compared to the CG (negative control) (Figure 2).

The improvement of lovastatin production can be attributed to the ability of the solvent to remove some of the contaminants, while leaving a number of stimulatory substances. Previous investigation showed that certain solvents can remove several key fatty acids in CG, such as linoleic acid methyl ester, palmitic acids, and oleic acids [16]. Although some the beneficial fatty acids, such as oleic acid, are also being removed, the removal of inhibiting saturated fatty acids such palmitic acids was shown to have a larger effect on the lovastatin production by this fungus [14, 16]. It is possible that the induction of lovastatin production in PE is caused by the incomplete removal of unsaturated fatty acids in the treatment. The incomplete removal may also be the reason why we observed a mixed response of (+)-geodin and sulochrin as well (Figure 2). It might be that each of these metabolites is induced more strongly by different impurities, which resulted in a unique response in different solvent pretreatments [14].

3.2. The Efficiency of Nonliquid Pretreatment Technique of Crude Glycerol on Substrate Consumption, Growth, and Lovastatin Production. In this section, activated carbon (AC) and water softener pillow (WS) were used to improve the CG. AC is a form of carbon material that has been treated with oxygen by thermal decomposition to create millions of pores that increase the surface area available for adsorption. Solvent washing reduces the impurities by transferring the impurities from one liquid to another. However, AC traps and filters the impurities inside the CG. In contrast, WS reduces the "hardness" of the solution by reducing the presence of certain minerals such as calcium and magnesium. While there is no evidence that associate the effect of hard water on the growth or production of metabolites by fungus, recent evidence showed that divalent metal cations can influence lovastatin biosynthesis [19]. Moreover, the

Table 1: The biomass production of *A. terreus* under different pretreatments of nonpolar solvents. The standard errors represent 95% confidence.

Treatments	DE	PE	Tol	CG control	PG control
Biomass (g/L)	11.28 ± 0.30	10.60 ± 0.27	9.80 ± 0.22	9.75 ± 0.12	10.07 ± 0.10

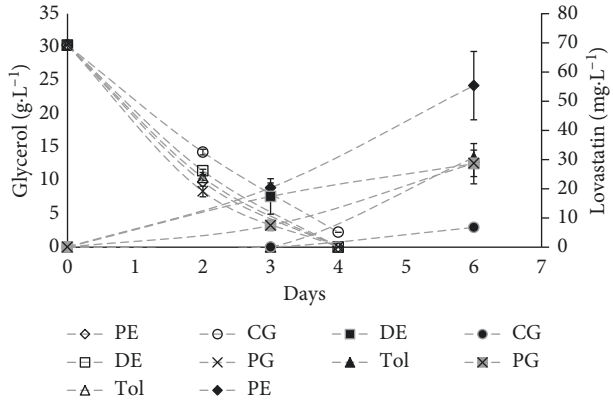

Figure 1: The glycerol consumption and lovastatin production of *A. terreus* under different substrates. The glycerol consumption is the slowest in CG and the fastest in PG. Lovastatin is consistently the highest under PE pretreatment, while CG produced the lowest lovastatin.

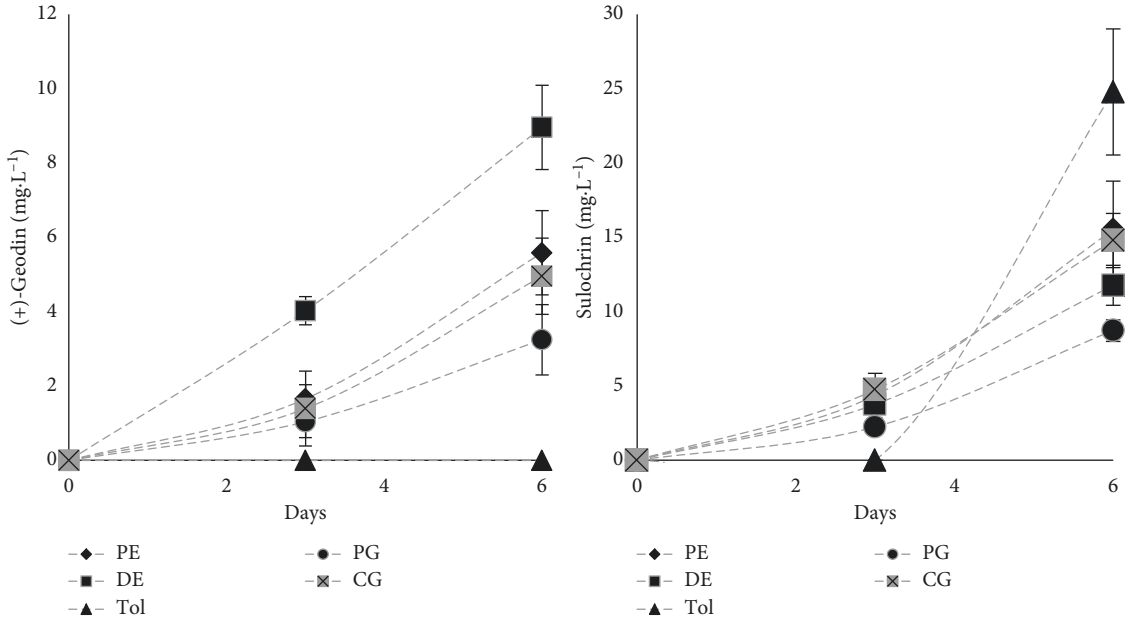

Figure 2: The (+)-geodin and sulochrin production under different substrates. (+)-geodin is the highest under the DE pretreatment, while sulochrin is the highest when Tol is used as pretreatment.

presence of certain metal ions together in certain concentrations may be inhibitory to the lovastatin production.

As in solvent pretreatment, these physical treatments produced very little change in terms of glycerol content. Our subsequent analysis showed an improvement in terms of substrate consumption when compared to CG when AC and WS (42% and 22% improvement, respectively, at day 2) was used (Figure 3). However, no improvement in biomass growth was detected under both treatments. The higher consumption improvement when AC was used instead of WS indicated that the presence of nondissolved solids inside the CG played a major role in the uptake of substrate (glycerol). Nevertheless, the removal of those cations still improves the substrate consumption by a significant margin.

Figure 4 shows the production of lovastatin, (+)-geodin, and sulochrin using pretreated CGs (AC and WS), nontreated CG, and PG during the 6-days cultivation. The production of lovastatin (21.80 mg/L), (+)-geodin (7.43 mg/L), and sulochrin (11.74 mg/L) following AC treatment was very similar to PG (20.65 mg/L, 8.60 mg/L, and 8.18 mg/L, respectively). In contrast, WS metabolite productions (lovastatin = 11.25 mg/L, (+)-geodin = 12.34 mg/L, and sulochrin = 12.85 mg/L)

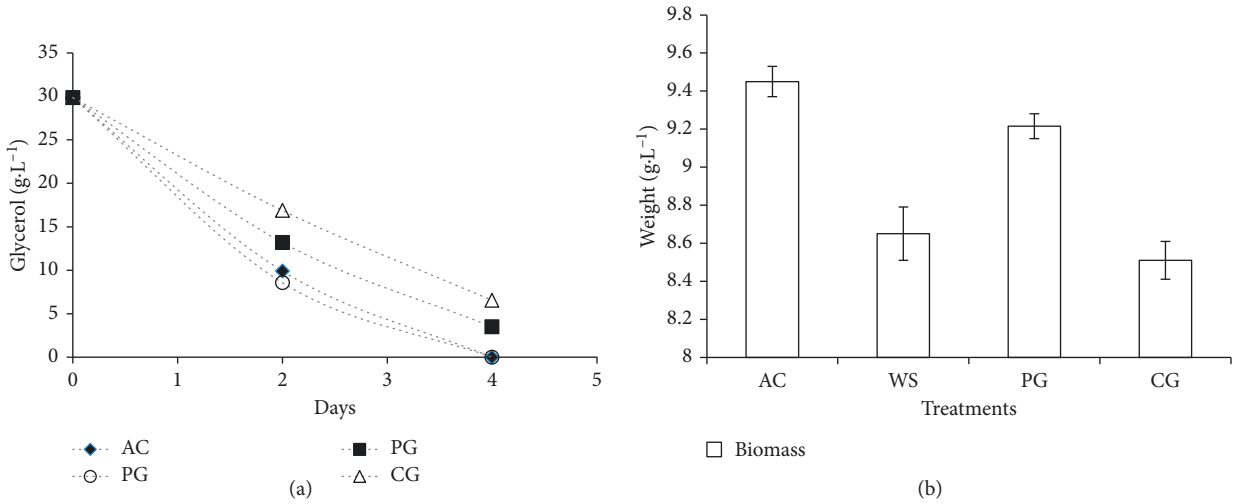

Figure 3: The glycerol consumption and biomass growth by *A. terreus* under the treatment of AC and WS. (a) Glycerol consumption. (b) Biomass production taken on day 6. In (a), the glycerol consumption in AC improved considerably, similar to positive control. In (b), the biomass production was not affected by the pretreatments.

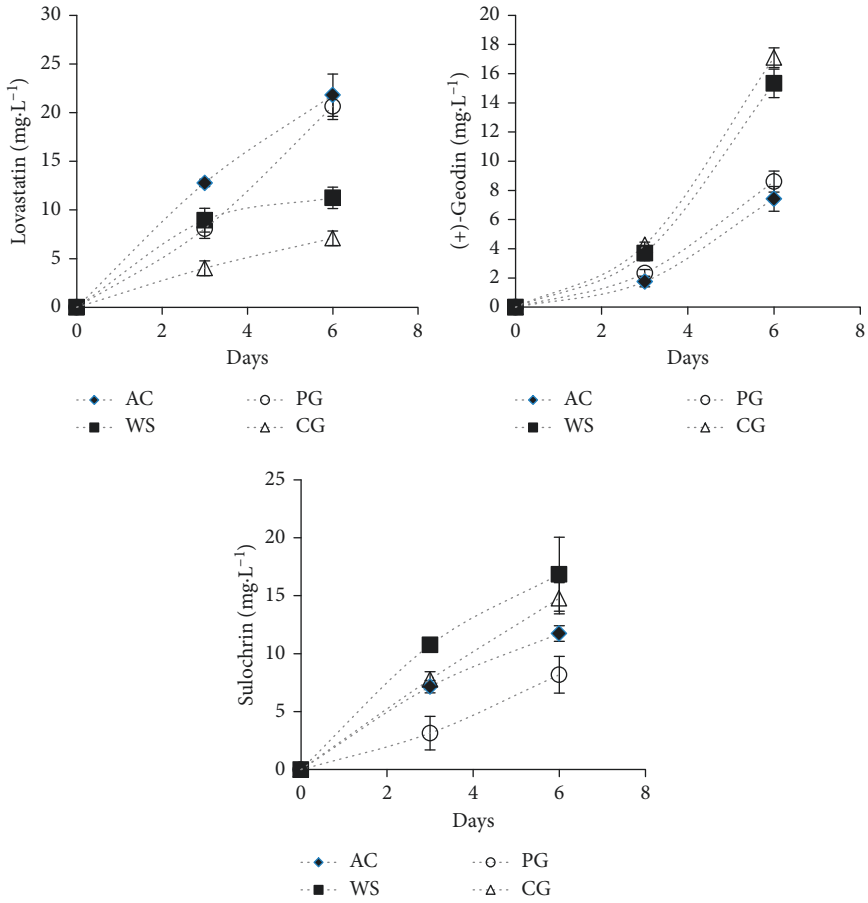

Figure 4: The production of lovastatin, (+)-geodin, and sulochrin by *A. terreus* under the treatment of AC and WS. Lovastatin, (+)-geodin, and sulochrin production in AC mirrored positive control (PG), while the metabolite production in WS mirrored negative control (CG).

mirrored CG (lovastatin = 7.10 mg/L, (+)-geodin = 17.10 mg/L, and sulochrin = 14.78 mg/L). This suggests that the AC treatment produced almost "pure" substrate, comparable to PG, while WS treatment is less efficient in improving the quality of the cultivation given that its production pattern resembles that of CG. Nevertheless, WS still produced significantly higher lovastatin than that of CG, although it is not as good as the production in PG. As

expected, WS treatment and CG control produced higher (+)-geodin and sulochrin due to the higher presence of impurities. This observation supports that (+)-geodin and sulochrin are more responsive towards impurities and may be indicative of their role in stress-related mechanism in *A. terreus* [14].

4. Conclusion

The use of pretreated CG can increase the production of metabolites, even higher than PG (positive control). Although CG may not be a better carbon source than PG for lovastatin production, it is indeed a promising economic and environmental alternative to make full use of easily accessible bio-wastes.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Disclosure

This publication contains an excerpt from a previously published work (PhD thesis) of the first author [20].

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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