JJBS

Volume 15, Number 4,December 2022
ISSN 1995-6673
Pages 621 – 627
https://doi.org/10.54319/jjbs/150410

Jordan Journal of Biological Sciences

Consolidated Bioethanol Production using *Trichoderma asperellum* B1581

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Received: July 21, 2021; Revised: December 30, 2021; Accepted: January 31, 2022

Abstract

Consolidated bioprocessing (CBP) is an alternative commercial process that combines all essential processes in a single bioreactor for the conversion of lignocellulosic biomass into ethanol. The challenge in the development of CBP is to find microorganisms with crucial properties for the utilisation of lignocellulosic materials to produce bioethanol. Also, it can be difficult to determine the optimal culture conditions for all processes to occur simultaneously. Therefore, this study focused on the potential of *Trichoderma asperellum* B1581 to produce ethanol and optimised the physicochemical parameters required for paddy straw waste conversion via CBP. Six parameters (days of saccharification, saccharification temperature (°C), days of fermentation, fermentation temperature (°C), medium level (%, v/v), and substrates loading (%, w/v)) were optimised in one-factor-at-a-time (OFAT) analysis via Response Surface Methodology (RSM). The numerical optimisation was statistically validated by comparing the volume of ethanol produced to the volume predicted by the RSM. *T. asperellum* B1581 produced 0.94 g/L bioethanol in CBP and is a more convenient, manageable and cost-effective process as all the crucial steps were performed by only one organism.

Keywords: Bioethanol production; Consolidated bioprocessing; Fermentation; Optimization; Paddy straw; Response surface method; Saccharification.

1. Introduction

Non-renewable fossil fuels such as coal, oil and natural gas have been known to be the primary sources of energy for many decades (Lugani *et al.*, 2020). With the growing of human population as well as urbanization in the 21st century and to continue pursuing current development goals, energy availability has emerged as one of the key problems that needs to be resolved. (Awogbemi *et al.*, 2021). Several alternatives have been considered by researchers, particularly biofuels which primarily depend on the type of biomass (Afolalu *et al.*, 2021). Biofuels can be categorized into four generations: (1) food-based crops containing starch, (2) lignocellulosic-based biofuels, (3) algal biomass and (4) genetically modified algae with high lipid content (Robak and Balcerek, 2020). Since the first-generation biofuels faced major controversy due to the food versus fuel issue, the direction of research interest has been shifted towards the second-generation biofuels by utilizing lignocellulosic biomass as renewable feedstock for biofuel production (Dey *et al.*, 2020). Lignocellulosic biomass is one of the most abundantly available bioresource with an annual global yield of 1.3 billion tons, and hydrolysis of this material would result in the release of valuable reducing sugars which are crucial for the production of biofuels such as bioethanol and biogas (Baruah *et al.*, 2018). Bioethanol is a high-energy substitute fuel to gasoline with an excellent clean-burning system and commonly blended with conventional gasoline for the use in automobile as an effort to reduce greenhouse gases emissions (Chang *et al.*, 2018). In bioethanol production, several processes such as pretreatment, saccharification and fermentation are the predominant steps that need to be executed in an efficient way (Kumar *et al.*, 2020). Four significant process configurations are required for efficient lignocellulose-based biofuel production: separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF), and consolidated bioprocessing (CBP) (Parisutham *et al.*, 2014).

The consolidated process involves the integration of both saccharification and fermentation into one bioreactor, hence reducing the number of stages in the bio-refinery (Zoglowek *et al.*, 2016). The resulting process is like SSF, but without the addition of exogenous enzyme (Teter *et al.*, 2014). CBP also involves the use of a microorganism or a group of compatible microorganisms for substrate hydrolysis and fermentation within a single reactor (Olson *et al.*, 2012; Ho *et al.*, 2012). However, the most difficult step in CBP is the selection of an appropriate microorganism or microbial consortium that secretes the hydrolytic enzyme required for the lignocellulosic material to produce ethanol (Paulova *et al.*, 2015). A study by Bech *et al*. (2015) proved the ability of *T. asperellum* to hydrolyse pretreated duckweed, producing up to 60% glucose yield, and hence this fungus is a potential

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organism for on-site enzyme production. *Trichoderma asperellum* has also been used in several biochemical processes due to its impressive features to boost cellulose production and cause less catabolite suppression (Nava-Cruz *et al.*, 2016). *Trichoderma asperellum* B1581 was chosen as a subject for this study based on a previous study by Syazwanee *et al.* (2019) in which the strain was identified as the best producer of exoglucanase (at 2.37 ± 0.34 U/mL) and β-glucosidase (3.00 ± 0.15 U/mL). Exoglucanase, also known as cellobiohydrolase (CBH), is a primary exocellulase that converts cellulose into cellobiose where its high production is crucial because it makes up 60% of the enzyme cocktail (Brady et al., 2015). β-glucosidase also plays a significant role in bioethanol production to eliminate cellobiose inhibition (Wang *et al.*, 2012) and has become a conundrum in producing bioethanol.

Besides selecting a productive strain, optimising the culture conditions is crucial to systematically improve the efficiency of ethanol production by adding or eliminating components from the formulation, which also results in more stability and reproducible culture conditions (Dong *et al.*, 2012). Therefore, this study was designed to determine the effectiveness of *T. asperellum* B1581 as single culture in producing ethanol, as well as optimising the physicochemical parameters for paddy straw waste conversion based on one-factor-at-a-time (OFAT) analysis and Response Surface Methodology analysis (RSM).

2. Materials and Methods

2.1. Fungal isolates

Trichoderma asperellum B1581 was obtained from the Mycology Laboratory, Faculty of Science, Universiti Putra Malaysia and grown on Potato Dextrose Agar (PDA) at 28°C ± 2°C for 7 days.

2.2. Culture conditions

The culture medium was prepared by using 1% (w/v) pretreated paddy straw (Syazwanee *et al.*, 2018) mixed into 25 mL of 10% (v/v) basal medium ($(NH_4)2SO_4$ 1.4 g/L; KH_2PO_4 2.0 g/L; $CaCl_2$ 0.3 g/L; $MgSO_4.7H_2O$ 0.3 g/L; $CoCl_2$ 2.0 g/L) with 1 mL of trace element ($MnSO_4.H_2O$ 1.56 g/L; $FeSO_4.7H_2O$ 5.0 g/L; $ZnSO_4.7H_2O$ 1.4 g/L), then sterilised at 121 ± 0.5°C for 15 min. The medium was inoculated with fungal spore suspensions once cooled.

The spore suspensions of *T. asperellum* B1581 was prepared from well sporulated colonies and were suspended in sterilized water. The concentrations of the spore suspensions were calculated using haemocytometer, and the concentrations were adjusted to 1 x 10^6 spore/mL (Mauch *et al.*, 1988).

2.3. One-Factor-At-a-Time (OFAT) analysis

In order to determine dynamic variables, one-factor-at-a-time (OFAT) analysis was design to investigate one factor while other variables were kept constant (Abou-Taleb and Galal, 2018). The CBP culture conditions were based on a preliminary study, 150 rpm, 30°C ± 0.5°C for saccharification and fermentation processes, 3 days of saccharification and 3 days of fermentation. The effect of six parameters on ethanol production, days of saccharification, saccharification temperature (°C), days of fermentation, fermentation temperature (°C), medium level (%, v/v), and substrates loading (%, w/v) were assessed using the Megazyme® ethanol assay kit in triplicates (Cutzu and Bardi, 2017). The data obtained from OFAT was analysed by using mean ± standard deviation at 95% confidence limit ($p < 0.05$) (Wahid and Nadir, 2013).

2.4. Response Surface Methodology (RSM)

The optimisation of RSM was performed using a Central Composite Design (CCD) via Design-Expert software Version 6.0.8 (Stat-Ease Inc., Minneapolis, MN, USA) with full expression of the quadratic model. In Central Composite Design (CCD), all factors were studied in five levels (-α, -1, 0, +1, +α). The coded variables were used to explain the ranges used in CCD such as extreme predicted point (±α), central point (0) and axial point (±1). For each response, optimum points were predicted based on the variables input and followed the second-order polynomial in the quadratic model. The amount of ethanol was quantified for each set-up and was subjected to analysis of variance (ANOVA) by evaluating the goodness-of-fit and significance of each parameter in the regression model (Said and Amin, 2015).

The prediction of model on each species of fungi producing ethanol was based on the response of the independent variables and the interactions were developed from the following equation (Eq. 1):

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_5 E + \beta_6 F + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{55} E^2 + \beta_{66} F^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{15} AE + \beta_{16} AF + \beta_{23} BC + \beta_{24} BD + \beta_{25} BE + \beta_{26} BF + \beta_{34} CD + \beta_{35} CE + \beta_{36} CF + \beta_{45} DE + \beta_{46} DF + \beta_{56} EF \quad \text{(eq. 1)}$$

The equation of Y is the amount of ethanol produced, β_0 is the interception of coefficient, $\beta_1, \beta_2, \beta_3, \beta_4, \beta_5, \beta_6$ were linear coefficients, $\beta_{11}, \beta_{22}, \beta_{33}, \beta_{44}, \beta_{55}, \beta_{66}$ were quadratic coefficients, $\beta_{12}, \beta_{13}, \beta_{14}, \beta_{15}, \beta_{16}, \beta_{23}, \beta_{24}, \beta_{25}, \beta_{26}, \beta_{34}, \beta_{35}, \beta_{36}, \beta_{45}, \beta_{46}, \beta_{56}$ represent interactive coefficients and symbols A, B, C, D, E and F were hours of saccharification, temperature of saccharification, hours of fermentation, temperature of fermentation, medium level and substrate level respectively.

3. Results

3.1. One-Factor-At-A-Time (OFAT) analysis

The OFAT analysis was conducted in sequence with the temperature of fermentation was the first parameter tested from 25°C to 45°C with an interval of 5°C. The most ethanol (0.06 ± 0.02 g/L) was produced at 30°C for fermentation after 3 days of saccharification and 3 days of fermentation with the other parameters remaining constant. The fermentation at other points of temperature failed to produce any ethanol. Throughout the evaluation, the most optimum days for both saccharification and fermentation processes by *T. asperellum* B1581 were 2 days (48 h) respectively; making a total of 4 days (96 h) for both processes to complete. The amount of ethanol produced during this period was 0.05 ± 0.01 g/L. Pertain to medium and substrates loading, *Trichoderma asperellum* B1581 produced up to 1.35 ± 0.02 g/L and 1.41 ± 0.07 g/L with 3% (w/v) substrate loading and 10% (v/v) basal medium. Generally, the amount of ethanol produced by *T. asperellum* B1581 decreased as the substrate loading increased over 3 % (w/v) and the volume of medium increased more than 10% (v/v). The final parameter analysed was the temperature of saccharification and as for

T. asperellum B1581, the volume of ethanol produced was the highest during saccharification at 30°C. The OFAT analysis managed to identify a compromise temperature for both saccharification and fermentation processes in CBP, which was 30°C ± 0.5°C. The summary of OFAT analysis is shown in Table 1.

Table 1. The pre-determine ranges for each of the parameters in one-factor-at-a-time (OFAT) and the optimum point for the highest ethanol production by *Trichoderma asperellum* B1581

No.	Parameters	Control setting	Ranges	Optimum point
1.	Temperature of fermentation	30 ± 0.5°C	25°C - 45 ± 0.5°C	30°C
2.	Days of saccharification	3 days	1 day – 5 days	2 days
3.	Days of fermentation	3 days	1 day – 5 days	2 days
4.	Substrates loading (w/v)	1%	1% - 7%	3%
5.	Medium level (v/v)	10%	10% - 90%	10%
6.	Temperature of saccharification	30 ± 0.5°C	25°C - 45 ± 0.5°C	30°C

3.2. Response Surface Methodology (RSM) analyses for optimisation

In RSM, the period of saccharification and fermentation was converted into hours for a more precise value. Five-level and six factors were used in a fractional factorial design to evaluate the effects of synthesis parameters, including hours of saccharification (h), saccharification temperature (°C), hours of fermentation (h), fermentation temperature (°C), medium level (%, v/v) and substrates loading (%, w/v). The optimum point from the OFAT analysis was used as the centre point in the CCD. There were 86 settings, including a 10-centre point set-up generated by the software and experiments were carried out on *T. asperellum* B1581. The adequacy of the model was validated by checking the statistical properties listed in the fit summary table such as ANOVA, lack-of-fit, R-squared, adjusted R-squared, predicted R-squared and adequate precision. The F-value for *T. asperellum* B1581 at 7.92 (Table 2) implied that the significant value, with only a 0.01% chance, happened due to noise. The validity of the null hypothesis was significant with a Probable F-value of <0.05, indicating less than 5% possibility, in which regression parameters were zero.

Table 2. Statistical summary of ethanol production by *T. asperellum* B1581

Source	Value
Std. dev.	0.19
Mean	0.26
R-Squared (R^2)	0.79
Adjusted R-Squared	0.69
Predicted R-Squared	0.39
PRESS	5.98
Adequate Precision	11.82

A regression model displays lack-of-fit when it is unable to adequately describe the functional relation between the experimental factors and the response variable. The lack-of-fit for *T. asperellum* B1581 was 2.11 with 0 pure error. The R-square (R^2) value varied between 0 and 1, with the value closer to 1, accounting for a larger proportion of the variance by the model. The quadratic regression model showed that the value of the determination coefficient (R^2) was 0.79 with the fit explaining 79% of the total variation in the data.

The ratio for *T. asperellum* B1581 was 11.82, indicating an adequate signal. Despite the inadequacy in the predicted R^2 value for the *T. asperellum* B1581 model, the ratios in adequate precision proved these models could be used to navigate the design space, and thus effectively navigate three dimensional (3D) structures (Figure 1).

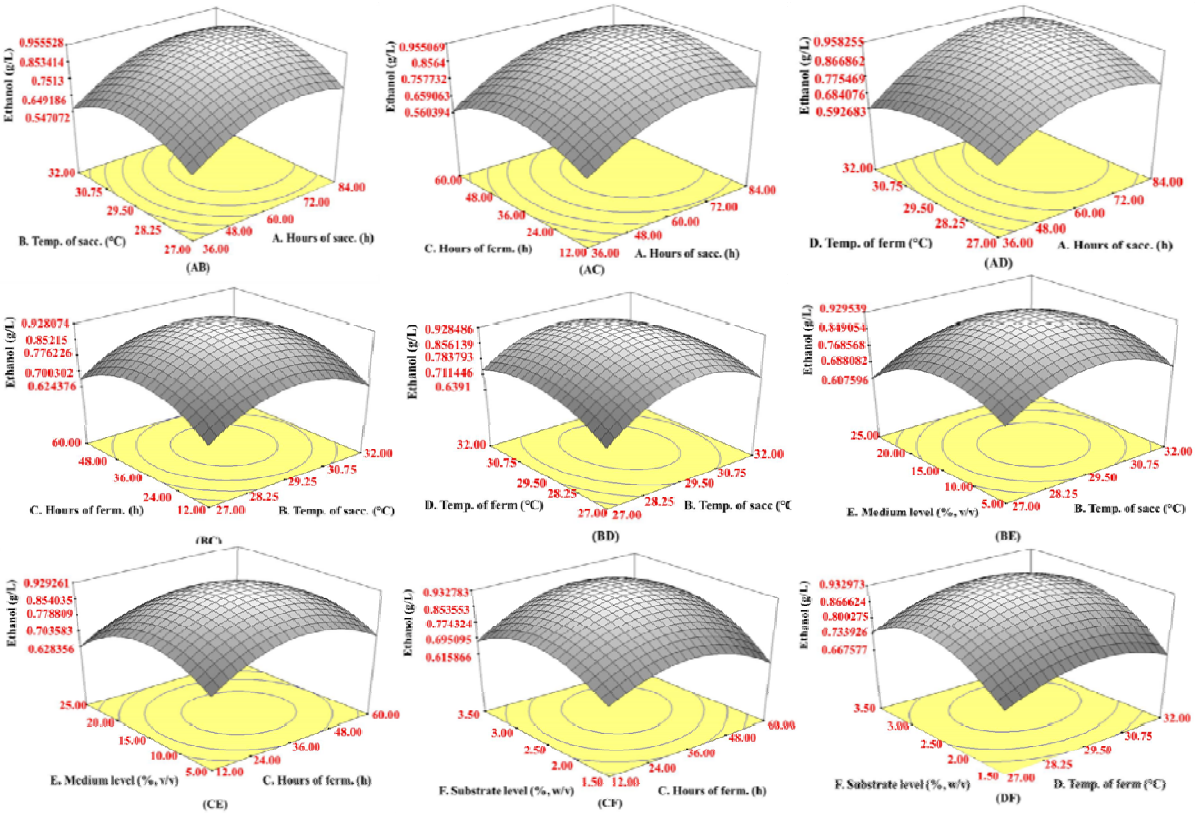

Figure 1. The development of 3D surface plot-based response of ethanol production produced by *T. asperellum* B1581 with interaction among parameters. (A) hours of saccharification, (B) temperature of saccharification, (C) hours of fermentation, (D) temperature of fermentation, (E) medium level and lastly (F) substrates loading.

The amount of ethanol produced by *T. asperellum* B1581 can be expressed by Equation (Y_1):

Y_1 = + 0.93 + 0.11A + 0.021B + 0.016C + 0.019 D - 0.031 E + 0.048 F - 0.12A2 - 0.14B2 - 0.13C2 - 0.082D2 - 0.13E2 - 0.12F2 + 0.013AB + 0.010AC + 0.036AD - 0.031AE + 0.042AF - 2.703E-003BC - 0.029BD - 6.719E-004BE - 0.020BF + 2.516E-003CD - 5.453E-003CE + 0.034CF - 0.010DE + 9.547E-003DF - 0.037EF (eq. 2)

Symbols A, B, C, D, E, F represent the coded variables used in CCD: (A) hours of saccharification, (B) saccharification temperature, (C) hours of fermentation, (D) fermentation temperature, (E) medium level and lastly (F) substrates loading. The positive and negative signs in these equations represent the synergy and antagonistic effects among the variables, respectively. The predicted ethanol production (0.96 g/L) was compared to the actual ethanol production (0.94 g/L) for validation purposes (Table 3), with no significant difference in production. The settings of OFAT analysis were also compared with the optimization set-up generated by RSM software to observe the differences between the set-up as well as the amount of ethanol produced. The response of ethanol production by *T. asperellum* B1581 using optimized set-up was rather low (0.94 g/L) compared to the value of ethanol produced in OFAT analysis (1.35 g/L).

Table 3: The optimization settings recommended by RSM with comparison to settings produced by OFAT analysis

	Saccharification		Fermentation		Basal medium		Ethanol (g/L)	
	Hours (h)	Temp. (°C)	Hours (h)	Temp. (°C)	Medium (%,v/v)	Substrate (%, w/v)	Predicted	Actual
RSM Set-up	67.72	29.58	32.9	29.79	12.42	2.84	0.96	0.94
OFAT analysis	48	30	48	30	10.00	3.00	1.35	

4. Discussion

One of the main challenges of consolidated bioprocessing (CBP) is the difficulty of establishing optimum culture conditions for microbial growth, saccharification and fermentation processes. Thus, the culture (medium and substrate loading), saccharification (temperature and days) and fermentation (temperature and days) were evaluated to determine the maximum ethanol production by lignocellulolytic fungi in aerobic conditions via CBP. The main problem in CBP is that the temperature needs to be a compromise between the optimal temperature for saccharification of biomass and fermentation to take place. Even though the saccharification temperature is best at 50°C (Amarasekara,

2013), the current findings with saccharification temperature at 30°C are essential for efficient ethanol production via CBP in tropical countries, helping to reduce the cooling and water costs during fermentation, subsequently reducing the total production costs (Murata *et al.*, 2015). Other parameters that influence the cost of production is the incubation period, with the optimum period for both saccharification and fermentation by *T. asperellum* B1581 being 2 days respectively, thus a total of 4 days for both processes to produce approximately 0.05 ± 0.01 g/L ethanol. Four days for CBP was reported previously (Nadeem *et al.*, 2015), and a longer incubation exceeding the optimum period will result in a low amount of ethanol due to nutrient depletion and diminished growth conditions (Fahrizal *et al.*, 2013).

Regarding medium and substrates loading, this study had observed the similar trend reported previously that high substrate concentrations severely limit the ethanol yield commercially, increasing the cost of processing, especially in downstream distillation (Zhao *et al.*, 2015). In order to create more economical process, substrate concentrations need to be optimized as the increasing of viscosity material may lead to end-product inhibition, reducing enzyme mobility and prevent hydrolysis process to take place (Mardawati *et al.*, 2019). In the context of medium volume, further increment will cause a high degree of aerobic metabolism, which utilizes sugar substrate but zero ethanol production (Arifa and Sarwar, 2012). Thus, optimisation of the fermentation or cultural conditions is a crucial step to achieve an optimum ratio of the metabolite production and cost before semi-pilot/pilot production plans (Shaymaa *et al.*, 2019).

The OFAT analysis developed a setting for optimisation of bioethanol production using *T. asperellum* B1581. However, the weakness of OFAT analysis was their incapability to establish interactions between factors that lead to inaccurate optimal conditions, particularly when interactions among different factors are significant (Humbird and Fei, 2016). Unlike the multivariate approach which offers global knowledge in its whole experimental area, OFAT only gives local knowledge where the experiment is performed (Ashgar *et al.*, 2014). Shaymaa *et al.* (2019) had shown the effectiveness of combination between Plackett–Burman design and Box-Behnken design as tools for the RSM. However, Central Composite Design (CCD) was chosen for this study as a statistical strategy rather than Plackett-Burman or Box-Behnken designs because CCD has axial points outside the region of interest to make up for OFAT's drawbacks.

Generally, different strains of fungi have their own optimal growth conditions, and this has led to the need to find the closest optimal condition for both strains to mutually co-exist for bioethanol production. In order to explore the relationships between several explanatory operating variables, Response Surface Methodology (RSM) has been extensively used for optimizing parameters for the production of ethanol from different substrates (Dasgupta *et al*., 2013). Besides, RSM is able to minimize the number of experiments required to develop a statistical relationship between factors and response, thus reducing the time consumption for optimization process. As seen in the RSM model produced in this study, the models provided an adequate fit for estimation with stability. A previous study reported that a combination of 5:1 *Aspergillus niger* B2484 and *Trichoderma asperellum* B1581 produced the most ethanol (1.03 g/L). The single culture organism is more economically efficient, reducing the risk of contamination or any possible complication during consortium development (Syazwanee *et al.*, 2021). A single microbial community can produce all the necessary enzymes to convert sugars into ethanol in a single reactor, thus reducing the overall costs (Sarabana *et al.*, 2015).

Industrial yeasts like *Saccharomyces cereviseae* have been used to produce alcohol for hundreds of years and have been extensively researched as the main strain for sugar-based bioethanol industries. The fermentation process for ethanol typically requires multiple setups for saccharification and fermentation and typically takes several hours to complete. On the overhand, CBP offers a combination of enzyme secretion, saccharification, and fermentation process in the same bioreactor has been known for economical manufacturing of bioethanol (Hasunuma *et al*., 2013). The key to cost reduction in CBP comes from either fermentative organisms that secrete vital cellulolytic enzymes for the breakdown of biomass, or from fermentative cellulolytic organisms that do not require a separate step for enzyme production (Linger and Darzins, 2013).

The effort to use *T. asperellum* B1581 crude enzyme in CBP for direct fermentation of ethanol has not been reported in any previous reports, and this study has become the first to investigate their potentials. Therefore, RSM is a valuable tool to plan the strategy in developing and optimizes the setup for CBP. Even though the RSM set-up produces a lower ethanol yield compared to OFAT set-up, the setting generated by RSM was still chosen over OFAT because the RSM analysis tends to overlook the overall interactions between physical and other factors affecting fermentation (Zambare and Christopher, 2012). Lower ethanol output may be caused by the fact that the filamentous fungi in CBP require several days to complete the fermentation process and usually consume the ethanol it produces (Anasontzis and Christakopoulos, 2014). Lower ethanol output in optimization setup by RSM was believed due to several drawbacks: (i) the productivity and ethanol yields are low and the fermentation process is time–consuming, (ii) optimum rate for hydrolysis of cellulases is usually greater than the ethanol producing microorganisms, (iii) unclear number of cellulase genes to be introduced into a single strain of host organism to become a viable CBP organism, and (iv) some of the secretory cellulolytic proteins may not fold properly (Jouzani and Taherzadeh, 2015). Although ethanol concentrations produced by filamentous fungi such as *T. asperellum* B1581 are unexpectedly high for organisms normally considered non-fermentative, the amount of ethanol produced is still too low for industrial bioethanol production and further analysis on the CBP setup is expected.

5. Conclusion

The OFAT analysis revealed that the optimum culture conditions of *T. asperellum* B1581 were 2 days of both saccharification and fermentation at 30°C with 3% (w/v) substrates loading and 10% (v/v) medium level. The optimised physicochemical conditions (67.7 h

saccharification, 32.9 h fermentation, 2.8% (w/v) substrate, 12.4% (v/v) medium level, 30°C for both processes) generated through RSM achieved ethanol production of 0.94 g/L, indicating the potential of *T. asperellum* B1581 as a single culture for bioethanol production in consolidated bioprocessing (CBP).

Acknowledgements

The authors would like to thank Universiti Putra Malaysia for the financial support through Grant No. GP-IPS/2016/9485600 and all staff of the Plant Systematic and Microbe Laboratory, Biology Department, Universiti Putra Malaysia for their efforts.

Conflict of Interest Statement

There are no conflicts of interest in this publication.

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