Pertanika J. Sci. & Technol. 29 (1): 301 - 316 (2021)

PERTANIKA JOURNALS

SCIENCE & TECHNOLOGY

Journal homepage: http://www.pertanika.upm.edu.my/

# Improvement of Bioethanol Production in Consolidated Bioprocessing (CBP) via Consortium of *Aspergillus niger* B2484 and *Trichoderma asperellum* B1581

**Mona Fatin Syazwanee Mohamed Ghazali, Muskhazli Mustafa*, Nur Ain Izzati Mohd Zainudin and Nor Azwady Abd Aziz**

*Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia*

## ABSTRACT

Consolidated bioprocessing (CBP) in bioethanol production involves the combination of four essential biological procedures in a single bioreactor, using a mixture of organisms with favourable cellulolytic ability without the addition of exogenous enzymes. However, the main disadvantage of this process is the complexity to optimise all factors considering both enzymes and microbial activity at the same time. Hence, this study aimed to optimise suitable culture conditions for both organisms to work efficiently. Six single factors that are considered crucial for bioethanol production were tested in one-factor-at-a-time (OFAT) analysis and analysed using Response Surface Methodology (RSM) software for *Aspergillus niger* B2484 and *Trichoderma asperellum* B1581 strains. The formulation of a new consortia setting was developed based on the average of two settings generated from RSM testing several combinations of consortia concentrations (5:1, 2:4, 3:3, 4:2, and 1:5). The combination of 5:1 *Aspergillus niger* B2484 and *Trichoderma asperellum* B1581 produced the most ethanol with 1.03 g/L, more than *A. niger* B2484, alone with 0.34 g/L of ethanol, indicating the potential of the combination of *A. niger* B2484 and *T. asperellum* B1581 co-culture for bioethanol production in CBP.

*Keywords*: Bioethanol, consolidated bioprocessing, consortium; one-factor-at-a-time analysis, response surface methodology

ARTICLE INFO

*Article history:*
Received: 9 September 2020
Accepted: 30 November 2020
Published: 22 January 2021

DOI: https://doi.org/10.47836/pjst.29.1.17

*E-mail addresses*:
mfsyazwanee_ghazali@yahoo.com (Mona Fatin Syazwanee Mohamed Ghazali)
muskhazli@upm.edu.my (Muskhazli Mustafa)
ainizzati@upm.edu.my (Nur Ain Izzati Mohd Zainudin)
azwady@upm.edu.my (Nor Azwady Abd Aziz)
*Corresponding author

ISSN: 0128-7680
e-ISSN: 2231-8526

## INTRODUCTION

Bioethanol, commonly known as ethyl alcohol ($C_2H_5OH$), is generated from the fermentation of fermentable sugars, such as glucose and sucrose, from plant sources using microorganisms (Chin & H'ng, 2013). The production of bioethanol represents as an alternative source of energy which also helps to minimise greenhouse gases effects (Artifon et al., 2018). The first-generation bioethanol production was based on food crops but due to competition between the food supply and bioethanol development, there was a sudden increase in food prices (Naik et al., 2010). This led to the development of second-generation bioethanol production using non-food based and readily available resources, such as lignocellulosic materials (Singh & Trivedi, 2013). These materials primarily originate from biomass sources, such as wheat straw, corn stover, and paddy straw, which comprise two structural polysaccharides, namely cellulose and xylan, that can be transformed into simple sugars (Park et al., 2010). Biodegradation of cellulose into glucose has become more popular as it offers low investment costs and is a non-polluting bioprocess (Liu et al., 2011).

Consolidated bioprocessing (CBP) in bioethanol production involves the combination of four biological procedures, secretion of cellulolytic enzymes, degradation of polysaccharides present in biomass, and the fermentation of hexose (C6) and pentose sugars (C5), in a single bioreactor (Kaneko et al., 2012). The challenge in the development of CBP is to identify an appropriate microorganism, which has all crucial properties for the utilisation of lignocellulosic materials, such as cellulolytic enzymes for degradation and capacity to ferment all mono-saccharides available, to produce ethanol via fermentation (Huang et al., 2014; Suhag & Singh, 2014).

Due to unavailability of a single strain to produce all essential enzymes for efficient lignocellulose degradation, a recent study focussed on the development of fungal consortia with benefits of evading feedback regulations and metabolite suppression (Wongwilaiwalin et al., 2010; Cui et al., 2015). The 'on-site' production of cellulolytic enzyme results from the co-cultivation of fungi in a single system (Ray & Behera, 2017), which can be achieved by co-cultivation of compatible fungal strains in a single bioreactor, cultivation of genetically modified strain with some good cellulolytic genes, or cultivation of several monocultures by blending enzymes (Kolasa et al., 2014). In comparison to single cultures, co-cultivation cultures of fungi may result in better utilisation of substrate, enhanced adaptability to changing conditions, improved resistance to contamination by undesirable microbes and most importantly, increased production yield (Tesfaw & Assefa, 2014). Before fungi application as consortia, a compatibility test is mandatory to avoid further complication during CBP. According to Syazwanee et al. (2019) there was mutual interaction between *Aspergillus niger* B2484 and *Trichoderma asperellum* B1581, indicating that these species can mutually live together in the same medium or environment without suppressing each

other's growth. However, the major obstacle in using microbial consortia for CBP is the difficulty in controlling both single microbes and the whole system simultaneously (Shong et al., 2012). The initiation of a stable co-culture system involves a complex process, in which all culture conditions, such as the pH of the medium, temperature of saccharification, the concentration of the substrates and enzyme, substrates size, carbon sources and pressure must be adjusted to be optimal for each strain (Cheng & Zhu, 2013; Shah et al., 2016). Hence, determination of the appropriate and stable conditions for fungi consortium to produce the maximal amount of bioethanol is required. This study aimed to develop a fungi consortium of *A. niger* B2484 and *T. asperellum* B1581 to produce bioethanol.

## MATERIALS AND METHODS

### Fungi Stock Culture

*Aspergillus niger* B2484 and *Trichoderma asperellum* B1581 were obtained from the Mycology Laboratory, Faculty of Science, Universiti Putra Malaysia. All strains were grown on Potato Dextrose Agar (PDA) at 28°C ± 2°C for 7 days.

### Preparation of Culture

The culture was prepared using 1% (w/v) paddy straw with size 5 mm and pretreated with 2% (w/v) NaOH (Syazwanee et al., 2018). The compositions of paddy straw after pretreatment were; 72.47% cellulose, 19.42% hemicellulose, 1.02% lignin and 5.44% ash content. The paddy straw was mixed in 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 elements ($MnSO_4.H_2O$ 1.56 g/L; $FeSO_4.7H_2O$ 5.0 g/L; $ZnSO_4.7H_2O$ 1.4 g/L) and sterilised at 121 ± 0.5°C for 15 min (Ja'afaru, 2013). The culture medium was inoculated with fungal spore suspensions of *T. asperellum* B1581 and *A. niger* B2484 once it had cooled. In order to ensure the growth of both fungi were constant throughout the entire experiment, 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).

### One-factor-at-a-time (OFAT) Analysis

The culture conditions were based on a preliminary study and are as follows: 150 rpm, 30°C ± 0.5°C for saccharification and fermentation processes, 3 days of saccharification and 3 days of fermentation. Six parameters, duration of saccharification, saccharification temperature (°C), duration of fermentation, fermentation temperature (°C), media level (%, v/v), and substrate level (%, w/v), were tested using a Megazyme® Ethanol Assay Kit (Table 1). In this study, both saccharification and fermentation process were carried out in

Infors HT- Multitron incubator shaker; the only difference during fermentation process was the samples were allowed to rest at controlled temperature without any agitation occurs. This approach was performed sequentially to identify the level of the factors influencing the yield (Shaw et al., 2002). The data obtained from OFAT was analysed using mean ± standard deviation at the 95% confidence limit ($p < 0.05$).

Table 1

*The pre-determine ranges for each of the parameters in one-factor-at-a-time (OFAT)*

| Parameters tested | Control setting | Ranges |
|---|---|---|
| Temperature of fermentation | 30 ± 0.5°C | 25°C - 45 ± 0.5°C |
| Days of saccharification | 3 days | 1 day – 5 days |
| Days of fermentation | 3 days | 1 day – 5 days |
| Substrate level | 1% | 1% - 7% |
| Media level | 10% | 10% - 90% |
| Temperature of saccharification | 30 ± 0.5°C | 25°C - 45 ± 0.5°C |

### 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 the full expression of the quadratic model. For each response, optimum points were predicted based on the variable input, followed by 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) to determine the optimum set-up for bioethanol production.

### Consortium Development

The compatibility of *A. niger* B2484 and *T. asperellum* B1581 was tested before the development of fungal consortia. The consortia of *A. niger* B2484 and *T. asperellum* B1581 was designed based on 6% v/v ($10^6$ spores/mL) in the combination of 1:5, 2:4, 3:3, 4:2 and 5:1. The amount of ethanol produced was quantified by the Megazyme® ethanol assay kit according to the manufacturer's instructions at 340 nm.

## RESULTS AND DISCUSSION

### Determination of Parameters via OFAT Analysis

The optimisation of all parameters is essential to ensure the maximum production of bioethanol. The classical method of optimisation involves varying one-factor-at-a time

(OFAT) while keeping the others constant (Czitrom, 1999). In the OFAT analysis, the parameter range was tested from large scale and narrowed down to a smaller scale, which was later used in the RSM software. The analysis also allows fast identification of the influence of the factors involved and the experimental results can be easily understood (Pambi & Musonge, 2016). One of the main disadvantages in the SSF process and CBP is the identification of the optimal temperature required for the saccharification and fermentation stages (Hasunuma & Kondo, 2012). Hence, the first parameter tested was the fermentation temperature. All samples were incubated at different temperatures from 25°C to 45 ± 0.5°C, with the most ethanol produced by both *A. niger* B2484 (0.04 ± 0.01 g/L) and *T. asperellum* B1581 (0.06 ± 0.02 g/L) at 30°C (Figure 1a), thus, narrowing the range of fermentation temperature for RSM to 27–32°C ± 0.5°C. The fermentation process in this study was carried out by filamentous fungi under aerobic condition. As this process is an exergonic, controlling the fermentation temperature with proper handling has become a compulsory (Cutzu & Bardi, 2017). According to Satyakala et al. (2017) maximum growth for *A. niger* and *Trichoderma harzianum* are recorded at 30°C and it is significantly highest over all other temperature tested between 20°C to 35°C. The unsuitable temperature for the microbial growth causes an inhibitory effect on the production of bioethanol (Selim et al., 2018). The increment of temperature improves the rate of biological reactions up to a certain temperature but further increment in temperature may cause in lesser product formation (Kanagasabai et al., 2019). The duration of saccharification was manipulated from 1 day to 5 days using the optimal fermentation temperature, with *A. niger* B2484 producing 0.04 ± 0.01 g/L ethanol after 3 days, while *T. asperellum* B1581 produced most ethanol (0.05 ± 0.01 g/L) after 2 days of saccharification (Figure 1b). Regarding the amount of ethanol produced, *T. asperellum* B1581 produced more ethanol than *A. niger* B2484, which is in line with Jena and Satpathy (2017) who showed that *Trichoderma* strains produced more ethanol from the fermentation of cellulose into ethanol than *Aspergillus.*

The duration of fermentation was shorter than saccharification, especially for *A. niger* B2484, with most ethanol produced after 1 day of fermentation (0.03 ± 0.00 g/L), decreasing thereafter (Figure 1c), whereas *T. asperellum* B1581 produced most ethanol after 2 days of fermentation (0.03 ± 0.00 g/L), with no ethanol detected from day 3 onwards. The fermentation time influences fungal growth, hence, a shorter fermentation time will cause inefficient fermentation due to insufficient fungal growth, while a longer period of fermentation results has toxic effects on growth due to the high concentration of ethanol in the fermented broth (Azhar et al., 2017). Even though the OFAT analysis was performed sequentially, it fails to consider the interactions between variables (Kanmani et al., 2013), explaining why the amount of ethanol produced suddenly drops. Therefore, to explore the relationships between several explanatory operating variables, RSM has been extensively used to optimise parameters for the production of ethanol from different substrates (Dasgupta et al., 2013).

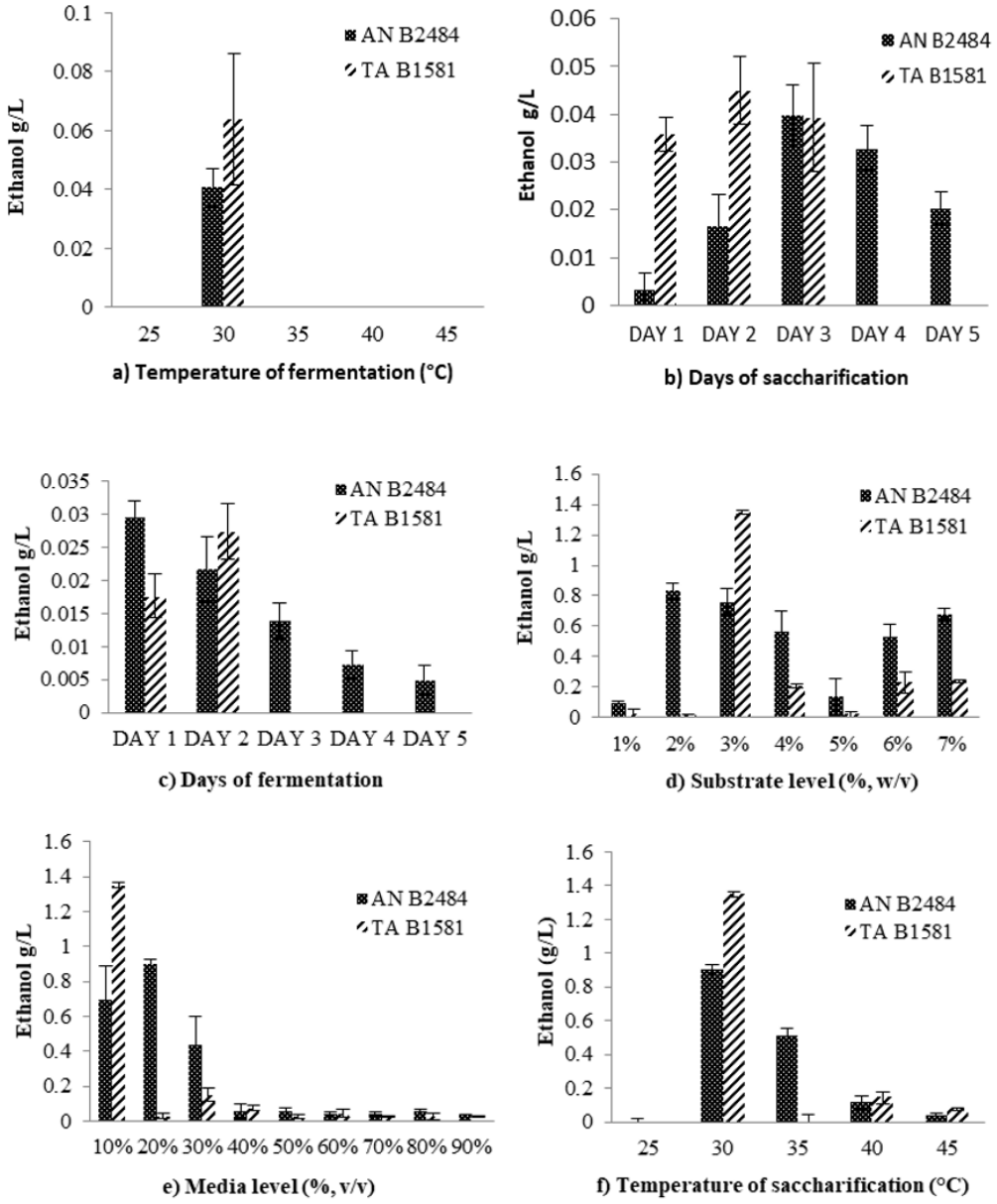


*Figure 1.* Optimization of all significant parameters using OFAT analysis, which were carried out in sequential pattern from parameter (a) to parameter (f) for both *A. niger* B2484 and *T. asperellum* B1581

The substrate was paddy straw pretreated with 2% NaOH and the range was set below 10% (w/v). In this study, *A. niger* B2484 produced 0.83 ± 0.05 g/L ethanol using 2% substrate loading and *T. asperellum* B1581 produced 1.35 ± 0.02 g/L ethanol using 3%

substrate loading mixed 10% (v/v) basal media (Figure 1d). Next, the media level (%, v/v), also referred to as the amount of basal media, was manipulated from 10% to 90% (v/v), with the amount of ethanol produced by *A. niger* B2484 increasing from 0.83 ± 0.05 g/L to 0.90 ± 0.03 g/L using 20% (v/v) media (Figure 1e). However, there was no improvement in the volume of ethanol produced by *T. asperellum* B1581. The optimal temperature in normal CBP for saccharification was 50°C, 30°C for fermentation, thus, a compromise was required to achieve both processes (Mutreja et al., 2011). In this study, the last parameter tested was the saccharification temperature, which ranged from 25°C to 45 ± 0.5°C, showing that the optimal temperature for saccharification for *A. niger* B2484 and *T. asperellum* B1581 was 30 ± 0.5°C (Figure 1f). This analysis suggested a compromised optimal temperature for both the saccharification and fermentation process of 30 ± 0.5°C in CBP. However, the OFAT approach proved to be time consuming and unreliable, leading to inaccurate optimal conditions without considering the interactions between factors (Wahid & Nazir, 2013). Such complications can be reduced by varying several variables at the same time, by designing experiments using statistical methods such as RSM (Bhaumik et al., 2013; Biswas et al., 2017). Despite the drawbacks, the OFAT analysis played an important role in determining the selection range for RSM evaluation for bioethanol production.

**Response Surface Methodology (RSM)**

The OFAT analysis results were used to optimise bioethanol production using *A. niger* B2484 and *T. asperellum* B1581 by RSM. To measure how adequate the suggested model suits the experimental data, the parameters such as $R^2$, p-value, standard deviation and adequate precision are used to describe the quadratic model. The p-value (the values of "Prob > F") is the probability of a given statistical model, whether it is similar to or larger than the actual experimental results when the null hypothesis is true. If the p-value is small, the probability of the null hypothesis is small, hence, a smaller p-value corresponds to more significant results (Liu et al., 2018). In this study, the p-value for both organisms (*A. niger* B2484 and *T. asperellum* B1581) was <0.0001, indicating significant bioethanol production. For *A. niger* B2484, the quadratic regression model yielded a determination coefficient ($R^2$) of 0.60, with the fit explaining 60% of the total variation in the data, while the $R^2$ value for *T. asperellum* B1581 was 0.79, explaining 79% of the results (Table 2). A value of $R^2$ which is close to 1 indicates an almost flawless relationship with all data points falls perfectly on the regression line, while a value of $R^2$ close to 0 indicates that the mean is corresponding to the model fitted (Saunders et al., 2012). Nevertheless, a high coefficient of determination is not a definite guarantee in indicating a 'goodness of fit' and similarly there is also no guarantee that a small $R^2$ value specifies a weak relationship as the statistic is mostly influenced by variation in the independent variable (Hamilton, 2015). In this study, despite having a low $R^2$ value, the independent variables were significant

and important relationship between variables can be clearly seen in the Equations 1 ($Y_1$) and 2 ($Y_2$).

Table 2

*The summary of quadratic model statistics of* A. niger *B2482 and* T. asperellum *B1581*

| Source | Std.dev | Mean | $R^2$ | Adjusted $R^2$ | Predicted $R^2$ | Adequate Precision |
|---|---|---|---|---|---|---|
| 1. AN B2484 | 0.19 | 0.22 | 0.60 | 0.42 | -0.01 | 8.23 |
| 2. TA B1581 | 0.19 | 0.26 | 0.79 | 0.69 | 0.39 | 11.82 |

1. *Aspergillus niger* B2484
2. *Trichoderma asperellum* B1581

The signal to noise ratio was evaluated by adequacy precision, which involved the predicted value at the design points and the average prediction error (Behera et al., 2018). In the present study, the adequacy precision ratio for *A. niger* B2484 was 8.23, 11.82 for *T. asperellum* B1581, which was desirable as the required ratio should be greater than 4. Hence, the developed model can be used to guide the design space.

To simultaneously optimise the responses, the RSM uses a set of mathematical and statistical procedures to explain a polynomial equation that relates to the experimental data (Bezerra et al., 2008; Akintunde et al., 2015). $Y_1$ and $Y_2$ represent the ethanol production by *A. niger* B2484 and *T. asperellum* B1581 respectively in the CBP process. The symbols A, B, C, D, E, F represent coded variables used in CCD: (A) duration of saccharification, (B) saccharification temperature, (C) duration of fermentation, (D) fermentation temperature, (E) media level and lastly, (F) substrate level.

$$Y_1 = + 0.58 + 0.11A + 0.02B + 0.02C + 0.03D + 0.02E + 0.03F -0.07A2 - 0.07B2 -0.06C2 - 0.05D2 - 0.07E2 - 0.06F2 - 0.01AB + 0.02AC + 9.25E\text{-}003AD + 7.25E\text{-}003AE + 0.01AF - 5.50E\text{-}003BC + 0.01BD - 4.97E\text{-}003BE - 0.02BF + 0.02CD - 0.01CE + 0.02CF + 8.75E\text{-}004DE + 9.41E\text{-}003DF - 7.03E\text{-}003EF \quad [1]$$

$$Y_2 = + 0.93 + 0.11A + 0.02B + 0.02C + 0.02\ D - 0.03E + 0.05F - 0.12A2 - 0.14B2 - 0.13C2 - 0.08D2 - 0.13E2 - 0.12F2 + 0.01AB + 0.01AC + 0.04AD - 0.03AE + 0.04AF - 2.70E\text{-}003BC - 0.03BD - 6.72E\text{-}004BE - 0.02BF + 2.52E\text{-}003CD - 5.45E\text{-}003CE + 0.03CF - 0.01DE + 9.55E\text{-}003DF - 0.037EF \quad [2]$$

The positive and negative signs in Equations 1 and 2 represent synergy and antagonistic effects among the variables. Hence, the model terms showing a positive synergistic effect in ethanol production by *A. niger* B2484 ($Y_1$ or Equation 1) were A, B, C, D, E, F, AC, AD, AE, AF, BD, CD, CF, DE, DF, with the interaction between A, B, C, D, F, AB, AC, AD, AF, CD, CF, DF showing positive synergy in ethanol production by *T. asperellum* B1581, with the other terms showing antagonistic effects ($Y_2$ or Equation 2).

The standard deviation for both models was 0.19, indicating that the predicted and actual values were close. To test the adequacy of the model developed, the numerical optimisation of ethanol production by *A. niger* B2484 and *T. asperellum* B1581 were tested, in which the model predictions were compared with the actual outcome for validation purposes (Table 3). To check the optimal points predicted by the software, a series of five replicate experiments were performed and the outcome analysed using a one-sample t-test (Safa et al., 2017). The values of the predicted amount of ethanol showed no significant difference to the actual amount of ethanol produced, confirming that the experimental values were in agreement with the predicted values, thus the model was validated.

Table 3

*The optimization settings recommended by RSM for* A. niger *B2484 and* T. asperellum *B1581 along with formulation of new consortia setting based from the average settings*

| RSM Settings | | | | | | | Outcome | |
|---|---|---|---|---|---|---|---|---|
| Saccharification | | | Fermentation | | Basal Media | | Ethanol (g/L) | |
| No. | Hour (h) | Temp (°C) | Hour (h) | Temp (°C) | Media (%,v/v) | Subs. (%,w/v) | Predicted | Actual |
| 1. | 66.75 | 29.76 | 32.3 | 30.18 | 14.74 | 2.59 | 0.61 ± 0.11 | 0.63 ± 0.19 |
| 2. | 67.72 | 29.58 | 32.9 | 29.79 | 12.42 | 2.84 | 0.96 ± 0.14 | 0.94 ± 0.27 |
| **Avg** | **67.24** | **29.67** | **32.6** | **29.99** | **13.58** | **2.72** | | |

1. *Aspergillus niger* B2484

2. *Trichoderma asperellum* B1581

The new RSM setting for both strains suggests the same saccharification and fermentation temperature, 30°C ± 0.5 with an average total time of 99.84 h or approximately 4 days. The optimum level of media (%, v/v) used for *A. niger* B2484 and *T. asperellum* B1581 were 14.74% and 12.42% respectively. Theoretically, the usage of high substrate concentration should achieve a great ethanol yield during fermentation but the concentrated

substrate creates an inhibitory effect to the fermentation process owing to the osmotic stress (Hamouda et al., 2015). With optimum amount of substrate level used, 2.59% managed to produce approximately 0.63 g/L ethanol using *A. niger* B2484 in CBP. As for *T. asperellum* B1581, 0.94 g/L ethanol was produced using 2.84% substrate. In this study, the optimized RSM set-up helped to provide better result with good reproducibility and reliable estimation as it evaluated the effects and learn the interactions between all the important parameters involved for an efficient bioethanol production using paddy straw.

## Formation of the Fungal Consortium

Generally, different strains of fungi have different optimal growth conditions, so the most appropriate optimal conditions for both strains to mutually co-exist for bioethanol production must be determined (Table 3). The combination of different species influences the productivity of biomass degradation through species interactions, such as mutual intermingling, inhibition and mutual intermingling with inhibition (Correa et al., 2018). The compatibility of the fungi from different genera, *Trichoderma* and *Aspergillus* have been studied, indicating that both species show mutual intermingling interactions and can co-exist in the same environment for the production of ethanol (Syazwanee et al., 2019). This led to the development of a consortium of 6% v/v ($10^6$ spores/mL) *A. niger* B2484 and *T. asperellum* B1581 in the ratio of 1:5, 2:4, 3:3, 4:2 and 5:1 (Table 4).

Table 4

*The amount of ethanol produced from different consortium composition between* A. niger *B2484 and* T. asperellum *B1581*

| Species | Consortium ratio ($10^6$ spore/mL) | Species | Ethanol (g/L) ± S.D |
|---|---|---|---|
| *A. niger* B2484 | 1:5 | *T. asperellum* B1581 | $0.93 \pm 0.16^{a}$ |
| *A. niger* B2484 | 2:4 | *T. asperellum* B1581 | $0.17 \pm 0.04^{bc}$ |
| *A. niger* B2484 | 3:3 | *T. asperellum* B1581 | $0.04 \pm 0.02^{c}$ |
| *A. niger* B2484 | 4:2 | *T. asperellum* B1581 | $0.16 \pm 0.03^{bc}$ |
| ***A. niger* B2484** | **5:1** | ***T. asperellum* B1581** | **$1.03 \pm 0.10^{a}$** |

Values are means of three replicates with ±SD.

Conventionally, ethanol is manufactured from processing starch, followed by fermentation of glucose using *Saccharomyces cerevisiae*, but this particular yeast failed to fully utilise the main C5 sugar (xylose) of the hydrolysate produced from agricultural waste hydrolysis (Sarkar et al., 2012; Ire et al., 2016). In comparison with pure cultures, co-cultivation systems could widen up the substrate utilisation scales (Jiang et al., 2019). Based on the advantages of these two fungi, their combination performs better in reducing sugar (Kartini & Dhokhikah, 2018). Indeed, the co-cultivation of fungi has been suggested to be more efficient for CBP compared to mono-cultivation (Grewal et al., 2020). However, fine-tuning and balancing the inoculation ratio significantly affects overall production and can be very challenging (Jawed et al., 2019). Hence, several combination ratios of *A. niger* B2484 and *T. asperellum* B1581 were tested, demonstrating that the combination of *A. niger* B2484 and *T. asperellum* B1581 in the ratio 5:1 produced the most ethanol, 1.03 g/L, compared to the single culture of *A. niger* B2484, 0.63 g/L. These results indicate that CBP for bioethanol production from cellulosic material can be accomplished by the combination of *A. niger* B2484 and *T. asperellum* B1581 in the ratio 5:1 without using microbes with gene recombinant (Horisawa et al., 2019). The enhancement of the ethanol titre occurred only within a proper range and appropriate proportion (Du et al., 2015). Under these circumstances, the beneficial effects of microbial consortium could become limited due to environmental stress factors and competition for the same resources (Bradáčová et al., 2019). In a microbial consortium, interactions such as mutualism and competition between two different species in the same ecological environment will affect the metabolism and influence the production of the target product in the fermentation process (Jiang et al., 2017).

Instead of using gas chromatography (GC) analysis, the overall process of ethanol quantification was done using Megazyme® Ethanol Assay Kit as the process was more cost efficient and time saving considering the large number of samples that needed to be quantified. The Single Lab Validation from Ivory et al. (2020) demonstrates that the Ethanol Assay Kit is suitable and relevant for the quantification of ethanol in low alcohol samples, fruit juices as well as fermented drinks with quick, easy and robust method. Hence, suggesting the fitness of this kit for the measurement of ethanol in this study.

## CONCLUSION

The assessment of consolidated process for ethanol production using fungal consortium between *A. niger* B2484 and *T. asperellum* B1581 has been carried out using newly formulated setting derived from an average of initial settings suggested by RSM for both strains. Out of five possible combination ratios, the 5:1 combination of *A. niger* B2484 and *T. asperellum* B1581 produced the most ethanol, 1.03 g/L; verifying the potential of *A. niger* B2484 and *T. asperellum* B1581 as co-culture for bioethanol production in CBP.

## ACKNOWLEDGEMENT

The authors would like to acknowledge Universiti Putra Malaysia for 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.

## REFERENCES

Akintunde, A. M., Ajala, S. O., & Betiku, E. (2015). Optimization of *Bauhinia monandra* seed oil extraction via artificial neural network and response surface methodology: A potential biofuel candidate. *Industrial Crops and Products, 67*, 387-394. doi: https://doi.org/10.1016/j.indcrop.2015.01.056

Artifon, W., Bonatto, C., Bordin, E. R., Bazoti, S. F., Dervanoski, A., Alves, S. L., & Treichel, H. (2018). Bioethanol production from hydrolyzed lignocellulosic after detoxification via adsorption with activated carbon and dried air stripping. *Frontiers in Bioengineering and Biotechnology, 6*, 1-6. doi: https://doi.org/10.3389/fbioe.2018.00107

Azhar, S. H. M., Abdulla, R., Jambo, S. A., Marbawi, H., Gansau, J. A., Faik, A. A. M., & Rodrigues, K. F. (2017). Yeasts in sustainable bioethanol production: A review. *Biochemistry and Biophysics Reports, 10*, 52-61. doi: https://doi.org/10.1016/j.bbrep.2017.03.003

Behera, S. K., Meena, H., Chakraborty, S., & Meikap, B. C. (2018). Application of response surface methodology (RSM) for optimization of leaching parameters for ash reduction from low-grade coal. *International Journal of Mining Science and Technology, 28*(4), 621-629. doi: https://doi.org/10.1016/j.ijmst.2018.04.014

Bezerra, M. A., Santelli, R. E., Oliveira, E. P., Villar, L. S., & Escaleira, L. A. (2008). Response surface methodology (RSM) as a tool for optimization in analytical chemistry. *Talanta, 76*(5), 965-977. doi: https://doi.org/10.1016/j.talanta.2008.05.019

Bhaumik, R., Mondal, N. K., Chattoraj, S., & Datta, J. K. (2013). Application of response surface methodology for optimization of fluoride removal mechanism by newly developed biomaterial. *American Journal of Analytical Chemistry, 4*(8), 404-419. doi: 10.4236/ajac.2013.48051

Biswas, G., Kumari, M., Adhikari, K., & Dutta, S. (2017). Application of response surface methodology for optimization of biosorption of fluoride from groundwater using *Shorea robusta* flower petal. *Applied Water Science, 7*, 4673-4690. doi: https://doi.org/10.1007/s13201-017-0630-5

Bradáčová, K., Florea, A. S., Bar-Tal, A., Minz, D., Yermiyahu, U., Shawahna, R., … & Poşta, G. (2019). Microbial consortia versus single-strain inoculants: An advantage in PGPM-assisted tomato production? *Agronomy, 9*(2), 1-23. doi: https://doi.org/10.3390/agronomy9020105

Cheng, J. R., & Zhu, M. J. (2013). A novel co-culture strategy for lignocellulosic bioenergy production: A systematic review. *International Journal of Modern Biology and Medicine, 1*(3), 166-193.

Chin, K. L., & H'ng, P. S. (2013). A real story of bioethanol from biomass: Malaysia perspective. In M. D. Matovic (Ed.), *Biomass now: Sustainable growth and use* (pp. 329-346). Rijeka, Croatia: InTech Open Access Publisher. doi: http://dx.doi.org/10.5772/51198

Correa, S. J., Jaramillo, A. C., Merino, R. A., & Hormaza, A. (2018). Evaluation of individual fungal species and their co-culture for degrading a binary mixture of dyes under solid-state fermentation. *Biotechnology, Agronomy and Society and Environment, 22*(4), 242-251. doi: 10.25518/1780-4507.16675

Cui, Y., Dong, X., Tong, J., & Liu, S. (2015). Degradation of lignocellulosic components in un-pretreated vinegar residue using an artificially constructed fungal consortium. *BioResources, 10*(2), 3434-3450.

Cutzu, R., & Bardi, L. (2017). Production of bioethanol from agricultural wastes using residual thermal energy of a cogeneration plant in the distillation phase. *Fermentation, 3(*2), 1-8. doi: https://doi.org/10.3390/fermentation3020024

Czitrom, V. (1999). One-factor-at-a-time versus designed experiments. *American Statistician, 53*(2), 126-131.

Dasgupta, D., Suman, S. K., Pandey, D., Ghosh, D., Khan, R., Agrawal, D., … & Adhikari, D. K. (2013). Design and optimization of ethanol production from bagasse pith hydrolysate by a thermotolerant yeast *Kluyveromyces* sp. IIPE453 using response surface methodology. *SpringerPlus, 2*, 1-10. doi: https://doi.org/10.1186/2193-1801-2-159

Du, R., Yan, J., Li, S., Zhang, L., Zhang, S., Li, J., … & Qi, P. (2015). Cellulosic ethanol production by natural bacterial consortia is enhanced by *Pseudoxanthomonas taiwanensis*. *Biotechnology for Biofuels, 8*, 1-10. doi: https://doi.org/10.1186/s13068-014-0186-7

Grewal, J., Tiwari, R., & Khare, S. K. (2020). Secretome analysis and bioprospecting of lignocellulolytic fungal consortium for valorization of waste cottonseed cake by hydrolase production and simultaneous gossypol degradation. *Waste and Biomass Valorization*, *11*, 2533-2548. doi: https://doi.org/10.1007/s12649-019-00620-1

Hamilton, D. F. (2015). Interpreting regression models in clinical outcome studies. *Bone and Joint Research, 4*(9), 152-153. doi: https://doi.org/10.1302/2046-3758.49.2000571

Hamouda, H. I., Nassar, H. N., Madian, H. R., Abu-Amr, S. S., & El-Gendy, N. S. (2015). Response surface optimization of bioethanol production from sugarcane molasses by *Pichia veronae* strain hsc-22. *Biotechnology Research International, 2015*, 1-10. doi: https://doi.org/10.1155/2015/905792

Hasunuma, T., & Kondo, A. (2012). Development of yeast cell factories for consolidated bioprocessing of lignocellulose to bioethanol through cell surface engineering. *Biotechnology Advances, 30*(6), 1207-1218. doi: https://doi.org/10.1016/j.biotechadv.2011.10.011

Horisawa, S., Inoue, A., & Yamanaka, Y. (2019). Direct ethanol production from lignocellulosic materials by mixed culture of wood rot fungi *Schizophyllum commune*, *Bjerkandera adusta* and *Fomitopsis palustris*. *Fermentation, 5*(1), 1-8. doi: https://doi.org/10.3390/fermentation5010021

Huang, J., Chen, D., Wei, Y., Wang, Q., Li, Z., Chen, Y., & Huang, R. (2014). Direct ethanol production from lignocellulosic sugars and sugarcane bagasse by a recombinant *Trichoderma reesei* strain HJ48. *The Scientific World Journal, 2014*, 1-9. doi: https://doi.org/10.1155/2014/798683

Ire, F. S., Ezebuiro, V., & Ogugbue, C. J. (2016). Production of bioethanol by bacterial co-culture from agro-waste-impacted soil through simultaneous saccharification and co-fermentation of steam-exploded bagasse. *Bioresources and Bioprocessing, 3*, 1-12. doi: https://doi.org/10.1186/s40643-016-0104-x

Ivory, R., Delaney, E., Mangan, D., & McCleary, B. V. (2020). Determination of ethanol concentration in kombucha beverages: Single-laboratory validation of an enzymatic method, first action method 2019.08. *Journal of AOAC International, qsaa122*, 1-25. doi: doi.org/10.1093/jaoacint/qsaa122 doi: https://doi.org/10.1093/jaoacint/qsaa122

Ja'afaru, M. I. (2013). Screening of fungi isolated from environmental samples for xylanase and cellulase production. *International Scholarly Research Notices, 2013*, 1-7.

Jawed, K., Yazdani, S. S., & Koffas, M. A. G. (2019). Advances in the development and application of microbial consortia for metabolic engineering. *Metabolic Engineering Communications, 9*, 1-10. doi: https://doi.org/10.1016/j.mec.2019.e00095

Jena, N., & Satpathy, S. (2017). Production of ethanol by *Trichoderma* spp. in solidstate fermentation of sugarcane molacess. *International Journal of Engineering Mathematics, 6*(6), 281-288.

Jiang, L. L., Zhou, J. J., Quan, C. S., & Xiu, Z. L. (2017). Advances in industrial microbiome based on microbial consortium for biorefinery. *Bioresources and Bioprocessing, 4*(1), 1-10. doi: https://doi.org/10.1186/s40643-017-0141-0

Jiang, Y., Wu, R., Zhou, J., He, A., Xu, J., Xin, F., … & Dong, W. (2019). Recent advances of biofuels and biochemicals production from sustainable resources using co-cultivation systems. *Biotechnology for Biofuels, 12*, 1-12. doi: https://doi.org/10.1186/s13068-019-1495-7

Kanagasabai, M., Maruthai, K., & Thangavelu, V. (2019). Simultaneous saccharification and fermentation and factors influencing ethanol production in SSF process. In Y. Yun (Ed.), *Alcohol fuels - Current technologies and future prospect* (pp. 1-15). London, UK: InTech Open Access Publisher. doi: 10.5772/intechopen.86480

Kaneko, S., Mizuno, R., Maehara, T., & Ichinose, H. (2012). Consolidated bioprocessing ethanol production by using a mushroom. In M. A. P. Lima (Ed.), *Bioethanol* (pp. 191-208). Rijeka, Croatia: InTech Open Access Publisher.

Kanmani, P., Karthik, S., Aravind, J., & Kumaresan, K. (2013). The use of response surface methodology as a statistical tool for media optimization in lipase production from the dairy effluent isolate *Fusarium solani*. *International Scholarly Research Notices, 2013*, 1-8. doi: https://doi.org/10.5402/2013/528708

Kartini, A. M., & Dhokhikah, Y. (2018). Bioethanol production from sugarcane molasses with simultaneous saccharification and fermentation (SSF) method using *Saccaromyces cerevisiae-Pichia stipites* consortium. *IOP Conference Series: Earth and Environmental Science, 207*(1), 1-9.

Kolasa, M., Ahring, B. K., Lübeck, P. S., & Lübeck, M. (2014). Co-cultivation of *Trichoderma reesei* RutC30 with three black *Aspergillus* strains facilitates efficient hydrolysis of pretreated wheat straw and shows promises for on-site enzyme production. *Bioresource Technology, 169*, 143-148. doi: https://doi.org/10.1016/j.biortech.2014.06.082

Liu, D., Zhang, R., Yang, X., Wu, H., Xu, D., Tang, Z., & Shen, Q. (2011). Thermostable cellulase production of *Aspergillus fumigatus* Z5 under solid-state fermentation and its application in degradation of agricultural wastes. *International Biodeterioration and Biodegradation, 65*(5), 717-725. doi: https://doi.org/10.1016/j.ibiod.2011.04.005

Liu, J., Wang, J., Leung, C., & Gao, F. (2018). A multi-parameter optimization model for the evaluation of shale gas recovery enhancement. *Energies, 11*(3), 1-29. doi: https://doi.org/10.3390/en11030654

Mauch, F., Mauch-Mani, B., & Boller, T. (1988). Antifungal hydrolases in pea tissue and inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. *Plant Physiology*, *88*, 936-942. doi: https://doi.org/10.1104/pp.88.3.936

Mutreja, R., Das, D., Goyal, D., & Goyal, A. (2011). Bioconversion of agricultural waste to ethanol by SSF using recombinant cellulase from *Clostridium thermocellum*. *Enzyme Research, 2011*, 1-6.

Naik, S. N., Goud, V. V., Rout, P. K., & Dalai, A. K. (2010). Production of first and second generation biofuels: A comprehensive review. *Renewable and Sustainable Energy Reviews, 14*(2), 578-597. doi: https://doi.org/10.1016/j.rser.2009.10.003

Pambi, R. L. L., & Musonge, P. (2016). Application of response surface methodology (RSM) in the treatment of final effluent from the sugar industry using Chitosan. In C. A. Brebbia (Ed.), *WIT Transactions on Ecology and the Environment* (Vol. 209 pp. 209-219). Southampton, UK: WIT Press.

Park, J. Y., Shiroma, R., Al-Haq, M. I., Zhang, Y., Ike, M., Arai-Sanoh, Y., … & Tokuyasu, K. (2010). A novel lime pretreatment for subsequent bioethanol production from rice straw - Calcium capturing by carbonation (CaCCO) process. *Bioresource Technology, 101*(17), 6805-6811. doi: https://doi.org/10.1016/j.biortech.2010.03.098

Ray, R. C., & Behera, S. S. (2017). Solid state fermentation for production of microbial cellulases. In G. Brahmachari, A. L. Demain & J. L. Adrio (Eds.), *Biotechnology of microbial enzymes: Production, biocatalysis, and industrial applications* (pp. 43-79). Amsterdam, Netherland: Academic Press. doi: https://doi.org/10.1016/B978-0-12-803725-6.00003-0

Safa, Z. J., Aminzadeh, S., Zamani, M., & Motallebi, M. (2017). Significant increase in cyanide degradation by *Bacillus* sp. M01 PTCC 1908 with response surface methodology optimization. *AMB Express, 7*, 1-9. doi: https://doi.org/10.1186/s13568-017-0502-2

Sarkar, N., Ghosh, S. K., Bannerjee, S., & Aikat, K. (2012). Bioethanol production from agricultural wastes: An overview. *Renewable Energy, 37*(1), 19-27. doi: https://doi.org/10.1016/j.renene.2011.06.045

Satyakala, K., Alladi, A., & Thakur, K. D. (2017). Effect of physiological parameters on growth of *Aspergillus niger* and *Trichoderma harzianum*. *Indian Journal of Pure and Applied Biosciences, 5*(4), 1808-1812.

Saunders, L. J., Russell, R. A., & Crabb, D. P. (2012). The coefficient of determination: What determines a useful $R^2$ statistic? *Investigative Ophthalmology and Visual Science, 53*, 6830-6832. doi: https://doi.org/10.1167/iovs.12-10598

Selim, K. A., El-Ghwas, D. E., Easa, S. M., & Hassan, M. I. A. (2018). Bioethanol a microbial biofuel metabolite: New insights of yeasts metabolic engineering. *Fermentation, 4*(1), 1-27. doi: https://doi.org/10.3390/fermentation4010016

Shah, S. R., Ishmael, U. C., Palliah, J. V., Asras, M. F. F., & Ahmad, S. S. N. W. (2016). Optimization of the enzymatic saccharification process of empty fruit bunch pretreated with laccase enzyme. *Bioresources, 11*(2), 5138-5154.

Shaw, R., Festing, M. F. W., Peers, I., & Furlong, L. (2002). Use of factorial designs to optimize animal experiments and reduce animal use. *ILAR Journal, 43*(4), 223-232. doi: https://doi.org/10.1093/ilar.43.4.223

Shong, J., Diaz, M. R. J., & Collins, C. H. (2012). Towards synthetic microbial consortia for bioprocessing. *Current Opinion in Biotechnology, 23*(5), 798-802. doi: https://doi.org/10.1016/j.copbio.2012.02.001

Singh, D. P., & Trivedi, R. K. (2013). Acid and alkaline pretreatment of lignocellulosic biomass to produce ethanol as biofuel. *International Journal of ChemTech Research, 5*(2), 727-734.

Suhag, M., & Singh, J. (2014). Recent Advances in fermentation of lignocellulosic biomass hydrolysate to ethanol. *Journal of Advances in Science and Technology, 7*(13), 1-8.

Syazwanee, M. M. F., Izzati, M. N. A., Azwady, A. N., & Muskhazli, M. (2019). Screening of lignocellulolytic fungi for hydrolyzation of lignocellulosic materials in paddy straw for bioethanol production. *Malaysian Journal of Microbiology, 15*(4), 379-386. doi: http://dx.doi.org/10.21161/mjm.180250

Syazwanee, M. M. F., Shaziera, A. N., Izzati, M. N. A., Azwady, A. N., & Muskhazli, M. (2018). Improvement of delignification, desilication and cellulosic content availability in paddy straw via physico-chemical pretreatments. *Annual Research and Review in Biology, 26*(6), 1-11. doi: https://doi.org/10.9734/ARRB/2018/40947

Tesfaw, A., & Assefa, F. (2014). Co-culture: A great promising method in single cell protein production. *Biotechnology and Molecular Biology Reviews, 9*(2), 12-20. doi: https://doi.org/10.5897/BMBR2014.0223

Wahid, Z., & Nadir, N. (2013). Improvement of one factor at a time through design of experiments. *World Applied Sciences Journal, 21*, 56-61. doi: 10.5829/idosi.wasj.2013.21.mae.99919

Wongwilaiwalin, S., Rattanachomsri, U., Laothanachareon, T., Eurwilaichitr, L., Igarashi, Y., & Champreda, V. (2010). Analysis of a thermophilic lignocellulose degrading microbial consortium and multi-species lignocellulolytic enzyme system. *Enzyme and Microbial Technology, 47*(6), 283-290. doi: https://doi.org/10.1016/j.enzmictec.2010.07.013