Application of *Agaricus bisporus* industrial wastewater to produce the biomass of *Pichia burtonii*

Jiafu Huang

**ABSTRACT**

By using Plackett–Burman combined with Box–Behnken design, the fermentation conditions of *Pichia burtonii* using *Agaricus bisporus* industrial wastewater as culture medium were optimized. The biomass of *P. burtonii* in the fermentation broth was analyzed by multispectral imaging flow cytometry. Plackett–Burman design was used to screen out three factors from six factors affecting the biomass of *P. burtonii* as major factors. The Box–Behnken response surface method was used to optimize the interaction of the three main factors to predict the optimal fermentation conditions. The significant factors affecting the biomass of *P. burtonii*, such as shaking speed, solubility and culture temperature, were screened. The optimum conditions for *P. burtonii* were as follows: a shaking speed of 265 rpm, a solubility of 8%, a culture temperature of 25 °C, an initial pH of 6.0, an inoculation amount of 8%, and an amount of 30 mL liquid in 250 mL, and the total living yeast can reach $1.27 \pm 0.02 \times 10^8$ Obj/mL, which was within the 95% confidence interval of the predicted model ($1.08 - 1.32 \times 10^8$ Obj/mL).

**Key words** | *Agaricus bisporus* industrial wastewater, multispectral imaging flow cytometry, *Pichia burtonii*, Plackett–Burman design, response surface

**INTRODUCTION**

Edible fungi are mainly produced in China, the United States, the Netherlands and Japan, and according to the data of the FAO (Food and Agriculture Organization), the yield of edible mushroom in China ranks first in the world. Among the edible mushrooms, *Agaricus bisporus*, *Lentinus edodes* and parts of wild fungus, viz., *Tricholoma matsutake*, are the main trade products in the world, and more, due to the short storage period of fresh edible mushroom, the main form of mushroom in international trade is canned products. Therefore a large amount of industrial wastewater is produced during canned processing each year in China. The discharge of the wastewater into the surrounding areas is a cause of environmental pollution. However, the wastewater contains a large amount of soluble components from the mushroom. According to the reports there are 0.65% protein, 0.17% total sugar, 0.08% reducing sugar, and 0.04% amino acid nitrogen in the industrial wastewater (Duan *et al.* 2015; Chen *et al.* 2018), which could provide sufficient carbon and nitrogen sources for microorganisms (Huang *et al.* 2018a, 2018b) or plants (Zhan *et al.* 2017).

*P. burtonii* belongs to the genus *Pichia*, which is widely distributed (Zhou *et al.* 1991; Middelhoven 2002; Rai *et al.* 2010; Ghosh *et al.* 2015), has a simple nutrient type and is easy to culture. It is often used in the fields of fermentation, animal feed additives and bacteriostatics (Chen *et al.* 2015b; Wang *et al.* 2016). Therefore, the industrial wastewater can be used as a natural medium for *P. burtonii*, which would provide theoretical support for microbial fertilizer fermentation and the development of the downstream industry of *A. bisporus*.

**MATERIALS AND METHODS**

**Microorganisms and culture conditions**

*P. burtonii* (GIM 2.179) was purchased from the Guangdong Culture Collection Center. Seed medium: glucose 10 g, peptone 5 g, malt extract 3 g, yeast powder 3 g, distilled water 1 L, pH 6.2, sterilized at 121 °C for 15 min. Preparation of seed suspension: activated *P. burtonii* was put into sterilized
seed liquid medium and shaken in a shaker (28 °C, 150 rpm) for 48 h. *A. bisporus* processing wastewater was collected from processing enterprises (Fujian KEREN Biological Co., Ltd), filtered, concentrated, and sterilized according to different experimental requirements.

### Plackett–Burman design

Plackett–Burman design was applied to evaluate the relative importance of several fermentation parameters that influence the desired response, and enabled one to screen *N* variables in at least *N* + 1 experiments (Ekpenyong et al. 2017). To increase the response, each factor selected was tested at two levels, high levels (+1) and low levels (−1). A Plackett–Burman design of the experiments was formulated for six factors that affect the total number of viable bacteria using the Design-Expert version 8.0.6 software (Stat-Ease, Inc., Minneapolis, MN, USA). For the present study, the selected factors included the levels of industrial wastewater solubility, initial pH, inoculum dose, loaded liquid, culture temperature, and shaking speed. The levels of each factor shown in Table 1 were determined based on prior experience. Each trial run was performed in triplicate, and the biomass of *P. burtonii* in the fermentation broth was taken as the response. Pareto chart analysis was used to evaluate the variables, which presents the impact of each variable on the total number of living yeast, and the most significant variables were further investigated by steepest ascent design and Box–Behnken design of response surface methodology.

### Steepest ascent design

After the three most significant variables were identified through the Box–Behnken design, the steepest ascent experiment was performed to move the experimental region of the response in the direction of the optimum, by appropriately changing the range of the selected variables. The path was initiated from the design center of the factorial design (the screening design) and receded when no further improvement in the response could be achieved. When the maximum value was gained, the point could be considered as the center point for the optimization experimental design (Chen et al. 2015a). Table 2 summarizes the steepest ascent experimental design, the variables, and their values.

### Box–Behnken design

The response surface methodology is a collection of statistical tools and techniques for constructing and exploring a putative functional relationship between a response variable (i.e., the total number of living yeast) and a set of design variables (i.e., shaking speed, solubility, culture temperature). It is possible to derive an expression for performance measurement on the basis of the response values obtained from experiments using a particular combination of input

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**Table 1** Experimental design and results of Plackett–Burman

<table>
<thead>
<tr>
<th>Run</th>
<th>A (Solubility (%))</th>
<th>B (pH)</th>
<th>C (Inoculation dose (%))</th>
<th>D (Load liquid (mL/250 mL))</th>
<th>E (Temperature (°C))</th>
<th>F (Speed (rmp))</th>
<th>Total number of live <em>P. burtonii</em>(10^7 Obj/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
<td>7.0</td>
<td>–1</td>
<td>2</td>
<td>90</td>
<td>1</td>
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<tr>
<td>2</td>
<td>1</td>
<td>4</td>
<td>7.0</td>
<td>1</td>
<td>8</td>
<td>90</td>
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<td>2</td>
<td>–1</td>
<td>90</td>
</tr>
<tr>
<td>4</td>
<td>–1</td>
<td>0.25</td>
<td>7.0</td>
<td>1</td>
<td>8</td>
<td>150</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>4</td>
<td>–1</td>
<td>6.0</td>
<td>2</td>
<td>150</td>
<td>–1</td>
</tr>
<tr>
<td>6</td>
<td>–1</td>
<td>0.25</td>
<td>7.0</td>
<td>1</td>
<td>8</td>
<td>–1</td>
<td>90</td>
</tr>
<tr>
<td>7</td>
<td>–1</td>
<td>0.25</td>
<td>–1</td>
<td>6.0</td>
<td>2</td>
<td>–1</td>
<td>90</td>
</tr>
<tr>
<td>8</td>
<td>–1</td>
<td>0.25</td>
<td>–1</td>
<td>6.0</td>
<td>–1</td>
<td>150</td>
<td>–1</td>
</tr>
<tr>
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<td>4</td>
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<td>–1</td>
<td>2</td>
<td>150</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
<td>4</td>
<td>–1</td>
<td>6.0</td>
<td>1</td>
<td>150</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>–1</td>
<td>0.25</td>
<td>–1</td>
<td>6.0</td>
<td>1</td>
<td>–1</td>
<td>90</td>
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<tr>
<td>12</td>
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<td>7.0</td>
<td>–1</td>
<td>2</td>
<td>150</td>
<td>1</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± standard deviation (SD) of three batches of independent experiments.
variables (Wu et al. 2018). In the present study, by employing Box–Behnken design and response surface methodology, the effects of the three independent variables (shaking speed, 200–300 rmp; solubility, 4–8%; culture temperature, 20–28 °C) and three levels (high, middle, and low) on the response (the total number of living yeast) were investigated to determine the optimal conditions, which maximized the total number of living P. burtonii from shake cultivation. Each independent variable was coded at three levels: –1, 0, and +1. The Box–Behnken design comprised 17 experiments with five center points (to allow for estimation of pure error) and facilitated calculations of response function at intermediate levels, fitting a second-order response surface. Table 3 shows the variables and their values and the experimental design.

### Determination of the total number of living P. burtonii

The fermentation broth was diluted ten times with PBS, and 1 mL diluted solution was mixed with 3 μL LIVE/DEAD Baclight™ (L7012, Thermo) staining reagent (containing SYTO 9 and propidium iodide (PI)) and incubated for 50 min in the dark, followed by analysis with multispectral imaging flow cytometry (Calvert et al. 2008). PBS was used as a flow sheath, the SYTO 9 signal was the X axis, the PI signal was the Y axis, and the scatter plot was made to distinguish the numbers of dead and living bacteria.

### RESULTS AND DISCUSSION

#### Total number of living P. burtonii in the fermentation liquid analyzed by multispectral imaging flow cytometry

A scatter plot comparing fluorescence intensities of SYTO 9 in the green channel and PI in the red channel was generated for the in-focus population. Two segregated regions were identified and gated (Figure 1(a)). The SYTO 9 stain generally labels all yeast, including both those with intact and with damaged membranes. By contrast, PI penetrates only yeast with damaged membranes, causing a reduction in SYTO 9 stain fluorescence when both dyes are present. Therefore, when using a combination of SYTO 9 and PI stains, yeast with intact cell membrane stains fluorescent green, whereas yeast with damaged membrane stains fluorescent red. Dead yeast had PI fluorescent signals or both SYTO 9 and PI signals (Figure 1(b)). Yeast with only the SYTO 9 fluorescent signal was considered living (Figure 1(c)).

### Plackett–Burman experiment

The Plackett–Burman experimental design matrix and results are shown in Table 1. Plackett–Burman design methods can be used in the screening of the key influential factors compared with other statistical designs on the experimental response. Six variables were selected in the screening approach with the Plackett–Burman experimental design, suggesting 12 trial runs. The total number of viable
yeast was selected as the observed response to determine the effects of the variables studied. Pareto analysis was used to determine the contribution of the screened variables (Figure 2), which showed that the shaking speed had the highest positive effect on the total number of living yeast, followed by solubility, while culture temperature was a negative factor. These three factors were determined to be of the highest relative importance in influencing the total number of living yeast compared with the other fermentation parameters tested and, hence, each was selected for further optimization.

**Steepest ascent design**

According to the positive and negative effects of the three factors in Figure 2, the rotational speed and solubility exhibited positive effects and temperature exhibited negative effects. Five sets of experiments of the steepest ascent and the corresponding experimental results are shown in Table 2. The total number of living *P. burtonii* peaked at the fourth step and no further improvement could be achieved in the response; when the speed was 250 rpm, the solubility of the pre-boiling liquid was 6% and the culture temperature was 24 °C, which suggested that it was proximal to the region of maximum response. Accordingly, these levels of the three factors in the fourth set were considered the center point of the Box–Behnken design.

**Optimization of fermentation parameters by response surface methodology**

In the present analysis, experiments were designed to obtain a second-order polynomial equation consisting of 12 trials plus five central points. The design matrix of the variables is shown in Table 3 along with the experimental values of the response. Through multiple regression analysis of the experimental data, shown in Table 4, the following second-order polynomial equation was derived for the total number of living *P. burtonii* by considering only the significant factors.

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean squares</th>
<th>F value</th>
<th>p-value prob &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>82.22</td>
<td>9</td>
<td>9.14</td>
<td>48.61</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>$X_1$</td>
<td>4.20</td>
<td>1</td>
<td>4.20</td>
<td>22.37</td>
<td>0.0021</td>
</tr>
<tr>
<td>$X_2$</td>
<td>1.32</td>
<td>1</td>
<td>1.32</td>
<td>7.02</td>
<td>0.0329</td>
</tr>
<tr>
<td>$X_3$</td>
<td>5.93</td>
<td>1</td>
<td>5.93</td>
<td>31.57</td>
<td>0.0008</td>
</tr>
<tr>
<td>$X_1X_2$</td>
<td>5.02</td>
<td>1</td>
<td>5.02</td>
<td>26.70</td>
<td>0.0013</td>
</tr>
<tr>
<td>$X_1X_3$</td>
<td>7.95</td>
<td>1</td>
<td>7.95</td>
<td>42.31</td>
<td>0.0003</td>
</tr>
<tr>
<td>$X_2X_3$</td>
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<td>1</td>
<td>2.24</td>
<td>11.89</td>
<td>0.0107</td>
</tr>
<tr>
<td>$X_1^2$</td>
<td>9.21</td>
<td>1</td>
<td>9.21</td>
<td>48.99</td>
<td>0.0002</td>
</tr>
<tr>
<td>$X_2^2$</td>
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<td>1</td>
<td>2.06</td>
<td>10.94</td>
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<td>$X_3^2$</td>
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<td>1</td>
<td>43.28</td>
<td>230.30</td>
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<tr>
<td>Residual</td>
<td>1.32</td>
<td>7</td>
<td>0.19</td>
<td></td>
<td></td>
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<tr>
<td>Lack of fit</td>
<td>0.77</td>
<td>3</td>
<td>0.26</td>
<td>1.91</td>
<td>0.2699</td>
</tr>
<tr>
<td>Pure error</td>
<td>0.54</td>
<td>4</td>
<td>0.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor total</td>
<td>83.54</td>
<td>16</td>
<td></td>
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</tbody>
</table>

$R^2 = 0.9843$, $R_{adj}^2 = 0.9640$, adequate precision = 25.083, CV = 4.98%.
terms: \[ Y = 10.59 - 0.72X_1 + 0.41X_2 + 0.86X_3 + 1.12X_1X_2 + 1.41X_1X_3 + 0.75X_2X_3 - 1.48X_1^2 + 0.70X_2^2 - 3.21X_3^2 \]

where \( Y \) is the predicted response of the total number of living \( P. \) \( \textit{burtonii} \), and \( X_1, X_2, \) and \( X_3 \) are the coded values of shaking speed, wastewater solubility, and culture temperature, respectively. Statistical significance of the second-order model and all the coefficient estimates were assessed using ANOVA, and the data are shown in Table 4. The \( p < 0.0001 \) of the regression model shows that when the regression equation is used to describe the relationship between every factor and response value, the linear relationships between dependent variable and all independent variables are very significant, that is, the experimental method is reliable. The value of adj-\( R^2 \) (0.9640) suggests that the total variation of 96.40% for the biomass of \( P. \) \( \textit{burtonii} \) was attributable to the independent variables. The determination coefficient (\( R^2 = 0.9843 \)), which is commonly used to assess the goodness of the model, exhibited an excellent correlation between the experimental and predicted response values. A low CV (CV = 4.98%) value clearly revealed that the deviations between experimental and predicted values were low and it displayed not only a high degree of precision but also a high reliability in the conducted experiments. Adequate precision measures the signal-to-noise ratio, and a ratio greater than 4 is desirable. In this study, a ratio of 25.083 indicated an adequate signal. Therefore, the quadratic model was selected in this optimization study.

This multiple nonlinear model resulted in three response surface graphs through canonical analysis of the response surface. Interpretation of the response surface 3D model and contour plot were as the graphical representations of the regression equation. They provided visual interpretations of the relationship between responses and experimental levels of each variable, and the type of interactions between two test variables (Long et al. 2018). Figure 3 shows the fitted response surface 3D models and their corresponding contour plots for the total number of living \( P. \) \( \textit{burtonii} \) produced by the predicted model. As can be seen from Figure 3, the response surface map of the speed and temperature, and the response surface between the solubility and temperature are all convex downward curved surfaces, indicating that the total number of viable bacteria of \( P. \) \( \textit{burtonii} \) has a high value. The contour centers of the three response surfaces are located within the set range, which indicates that the optimal design conditions exist within the designed level of factors.

From Figure 3(a) and 3(b), it is known that the response surface is steep, indicating that the influence of speed and solubility on the total number of living bacteria is obvious; at the same time, the analysis of variance shows that the interaction between speed and pre-cooking liquid solubility is significant (\( p < 0.05 \)). When the culture temperature was 24°C (the temperature test level is 0), the change of the total number of living bacteria gradually decreased with the increase of shaking speed and the solubility of the pre-cooking liquid; when the shaking speed was 250 rmp, the solubility of the pre-cooking liquid was 6%, and the culture temperature was 24°C, the total number of living bacteria gradually increased and reached the actual maximum close to the design points.

From Figure 3(c) and 3(d), it is known that the response surface is steep, indicating that the influence of speed and solubility on the total number of living bacteria is obvious; the contour line is oval, and the interaction between speed and temperature is significant. Under the condition of constant solubility, the biomass of \( P. \) \( \textit{burtonii} \) increased first and then decreased with the increase of rotational speed and temperature, and the vertex of the surface is the maximum point of the biomass.

From Figure 3(e) and 3(f), the effect of solubility and temperature on the total number of living bacteria was obvious. At the same time, the interaction between solubility and temperature was significant by the analysis of variance (\( p < 0.05 \)). When the shaking speed was 250 rmp, that is, the test level is 0, the total number of living yeast increased first and then decreased with the increase of temperature; the change trend of the total number of living yeast tended to be gentle with increase of solubility. Under a constant shaking speed (the level is 0), the total number of living bacteria first increased and then decreased as the temperature, and the vertex of the surface indicates the maximum amount of total living yeast.

These observations were also verified through canonical analysis of the response surface. By solving the inverse matrix from the second-order polynomial equation, the optimum values of the test variables were shaking speed 264.16 rmp, wastewater solubility 8%, culture temperature 25.25°C, and the predictive value of the total number of viable \( P. \) \( \textit{burtonii} \) was \( 1.20 \times 10^8 \) Obj/mL. To confirm the validity of the model for predicting the maximum total number of viable \( P. \) \( \textit{burtonii} \), an additional experiment using these optimum operation conditions was performed under shake-flask culture. The total number of viable \( P. \) \( \textit{burtonii} \) was \( 1.27 \pm 0.02 \times 10^8 \) Obj/mL \( (N = 3) \), which was within the 95% confidence interval of the predicted model \( (1.08-1.32 \times 10^8 \) Obj/mL\), suggesting that the model was adequate for reflecting the expected optimization, and the response surface methodology model was satisfactory and accurate.
Figure 3 | The effect of cross-interaction among shaking speed, different concentration and culture temperature on total number of living *P. burtonii*: (a) response surface plot of effects of interaction between shaking speed and different concentrations on total number of live *P. burtonii*; (b) contour line of effects of interaction between shaking speed and different concentrations on total number of live *P. burtonii*; (c) response surface plot of effects of interaction between shaking speed and culture temperature on total number of live *P. burtonii*; (d) contour line of effects of interaction between shaking speed and culture temperature on total number of live *P. burtonii*; (e) response surface plot of effects of interaction between different concentrations and culture temperature on total number of live *P. burtonii*; (f) contour line of effects of interaction between different concentrations and culture temperature on total number of live *P. burtonii*. 
Response surface methodology is one more effective method that reduces development costs, optimizes experimental conditions, improves production efficiency and solves practical production problems. Compared with one-factor-at-a-time experiments, statistically designed experiments are able to describe the effect of the interactions between the factors in linear and quadratic terms (Rane et al. 2017). In the present study, the optimization of fermentation conditions for *P. burtonii* was divided into two phases: a screening of the main effects of the selected variables and a response optimization. Reduction in the initial number of variables was carried out through Plackett–Burman design that was used to screen out three factors from six factors (industrial wastewater solubility, initial pH, inoculum dose, loaded liquid, culture temperature and shaking speed) affecting the total number of viable bacteria as major factors, which were the shaking speed, wastewater solubility, and culture temperature. They were taken for the Box–Behnken design of response surface methodology to assess their effects on the biomass of *P. burtonii*. We used response surface methodology to further optimize the biomass of *P. burtonii* by Box–Behnken design. Response surface methodology not only helped locate the optimum levels of the most significant factors but also proved to be useful and satisfactory in this process-optimizing practice. Through these optimization experiments, the maximum biomass of *P. burtonii* at 1.27 ± 0.02 × 10^8 Obj/mL was obtained under the optimum conditions of speed 264 rmp, pre-cooking liquid solubility 8%, temperature 25 °C, initial pH 6, inoculum 8%, and liquid volume 30 mL/250 mL, after 48 hours.

SYTO 9 and PI were used for double staining of viable and dead cells. With an appropriate mixture of SYTO 9 and PI stains, bacteria with intact cell membranes stain fluorescent green, scored as viable; and bacteria with injured membranes stain fluorescent red, scored as non-viable (Leuko et al. 2004; Hu et al. 2017). The fluorescent staining of bacterial cells was quantified with multispectral imaging flow cytometry by fluorescent signals and microscopic images (Berney et al. 2007; Jenner et al. 2016). Therefore, the *P. burtonii* viable and dead cells and particles in the fermented liquid were quickly and accurately identified and quantified by flow cytometry in a statistically sound manner.

There is a positive correlation between the shaker speed and the amount of dissolved oxygen, and the amount of the dissolved oxygen can also reflect the growth of the bacteria from the side. When the speed is too low, the dissolved oxygen is not enough, resulting in uneven mixing of the various substances in the fermentation system, which can not normally utilize the large amount of organic acids produced during the growth of the aerobic decomposition consuming strain and greatly reduces the yield of the bacteria; when the speed is too high, the amount of dissolved oxygen increases rapidly, and it produces a large number of metabolites, which also affect the growth of the bacteria. Therefore, *P. burtonii* is one kind of facultative aerobe that forms large aggregates when the shaking speed is at a high level.

In the course of production and processing, *A. bisporus* is treated with hot water many times, so that some of its water-soluble nutrients are lost into the processing wastewater. Although the amount is small, after the wastewater was further concentrated, it is sufficient to meet the growth requirement of bacteria, yeast and other microorganisms. Temperature influences microbial life mainly by affecting the mobility of the microbial cell membrane and the activity of biological macromolecules (Baweja et al. 2016; Taniguchi et al. 2017). As temperature increases, the rates of intracellular enzymatic reactions increase, resulting in an increase in cell metabolism and growth. However, once the temperature becomes too high, bioactive substances become denatured, resulting in decreased cell functions and even death (Kunze et al. 2014). In accordance with this, the total number of viable *P. burtonii* first increased and then decreased as the temperature increased. The most suitable temperature range was 24–32 °C with an optimal incubation temperature of 26 °C.

### CONCLUSIONS

In this paper, three major influencing factors including shaking speed, wastewater solubility and culture temperature were screened by a Plackett–Burman design test, and the optimal fermentation conditions of *P. burtonii* were designed by Box–Behnken: speed 264 rmp, pre-cooking liquid solubility 8%, temperature 25 °C, initial pH 6, inoculum 8%, liquid volume 30 mL/250 mL; after 48 hours, the total number of viable bacteria reached 1.27 ± 0.02 × 10^8 Obj/mL, reaching the requirements of agricultural microbial inoculants, which could be used as the best fermentation conditions to ferment *P. burtonii* by using wastewater from *A. bisporus* processing.

### ACKNOWLEDGEMENTS

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REFERENCES


Wu, K., Ding, L., Zhu, P., Li, S. & He, S. 2018 Application of the response surface methodology to optimize the fermentation parameters for enhanced docosahexaenoic acid (DHA) production by Thraustochytrium sp. ATCC 26185. Molecules 23 (4), 974.


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