Optimization of Medium Composition and Culture Conditions for Cell Multiplication of a High Quality Milk Beer Fermentation Yeast (*Kluyveromyces marxianus*)

Liang Wang1*, Yuting He1, Clifford S. Swanson2, Qiang He2, Benjamin Kumah Mintah3, Enyan Gao1, Xiaoyan Zheng1 and Mingying He1

1School of Food and Biological Engineering, Jiangsu University, Xuefu Road 301, Zhenjiang 212013, Jiangsu, China
2Department of Civil and Environmental Engineering, University of Tennessee, Knoxville, Tennessee, USA
3Department of Nutrition & Food Science, University of Ghana, Legon, Accra, Ghana

Received October 27, 2019 ; Accepted January 20, 2020

Milk beer is a distinctive alcoholic milk drink created in China, and increasingly popular with consumers. Yeast is key factor to its fermentation production, and determines the flavor and quality. In this investigation, a high-quality yeast *Kluyveromyces marxianus* MJ1 suitable for milk beer fermentation was utilized in an optimization study of proliferation culture. Results showed that using the optimal medium composition: wort-based medium supplemented with molasses 2.94 %, wheat bran 1.56 %, CaCO3 0.21 %; and applying the optimal culture conditions: 28 °C, rotation speed 220 rpm, inoculation concentration 1.5×10⁶ CFU·mL⁻¹, the viable count of MJ1 reached a maximum of 7.8×10⁸ CFU·mL⁻¹. Compared to YPD broth, the viable count of MJ1 increased to 4.11 times, while the raw material cost decreased to about 1/6 of the cost of YPD broth. The study achieved high-density proliferation culture of MJ1 through usage of low cost crop by-products; and laid the foundation for preparation of active freeze-dried powder for applications in the milk beer industry.

Keywords: milk beer, *Kluyveromyces marxianus*, proliferation culture, response surface methodology, orthogonal test

Introduction

Milk beer (MB) is a new type of beverage made from whole milk, skim milk, or whey that is achieved through fermentation by utilizing lactic acid bacteria and yeast in a two-step process. Besides having the characteristics of beer - low alcohol content, slight carbonation, and white foam - it also possesses the characteristics of a milk-beverage - sweet and sour, refreshing, and milky aroma. MB is nutritious (i.e. rich in protein/amino acids and vitamins) and is easy to absorb. MB also has the function of improving blood circulation, quenching thirst, and eliminating fatigue. Existing data shows that it becomes a consumer favorite following its introduction into a market, and that it has a very high market value and development space (Qi and Qu, 2007; Yang et al., 2007).

Yeast is a key microorganism for MB fermentation, which can contribute to the main flavor substances such as alcohols, acids, and esters. Its fermentation characteristics determine the quality and flavor of the MB (Wang et al., 2018). At present, there are not many enterprises producing MB in China, and the existing enterprises often use commercially available *Saccharomyces cerevisiae*, which leads to a similar taste, a single variety, and high sucrose content in MB (Li et al., 2008).

Despite China’s MB companies constantly evolving - as well as the innovation in beverage production technology - the strict, new regulations being applied to the food and beverage manufacturing industry are creating challenges with MB production. First, the fermentation process requires wort, hops, and other auxiliary materials, which complicates the process, prolongs the production cycle, and significantly increases the cost. Secondly, the product has a single flavor, high alcohol...
content, and heavy beer taste, making it difficult to meet the needs of consumers. These have greatly affected the competitiveness of the MB products on the beverage market.

Therefore, there is an urgent need for MB enterprises to find high-quality, milk-derived yeasts with rich metabolites, low alcohol yield, and that are suitable for direct fermentation of MB (Hu et al., 2017). Studies have shown that Kluyveromyces marxianus as a milk-derived yeast can efficiently ferment lactose, producing low alcohol and good aroma when applied in food fermentation (Li et al., 2013). Li et al. (Li et al., 2013) and Wang et al. (Wang et al., 2017) applied K. marxianus in MB fermentation, with the findings indicating that the strain performed superbly and thus can replace S. cerevisiae for the industrial production of MB.

In this study, a high-quality K. marxianus strain MJ1 isolated from Xinjiang wild kefir grains was utilized. In an earlier investigation it was tested in a MB fermentation and was compared to a commercial strain MJ (provided by cooperative companies). The test encompassed the quality index of the fermented product, including protein content, total solids, titratable acidity, ethanol content, total viable counts of coliforms, yeast and molds, as well as sensory evaluation of products, and analysis of volatile components. And the results showed that the product made by MJ1 meets the national and industry standards for MB, and the quality of its product was superior to MJ (Hu et al., 2017).

In order to further meet the requirements of large-scale production of MB, the preparation of yeast active dry powder has become an inevitable trend, which requires the strain to have a higher concentration of live organism. Therefore, the present investigation focused on the optimization of the medium composition and culture conditions to realize the high-concentration proliferation culture of K. marxianus strain MJ1. The study finding could provide reliable research basis for subsequent active dry powder production.

Materials and Methods

Materials YPD broth was obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Potato, soybean sprout, white granulated sugar, wort concentrate, molasses, corn flour, tapioca starch, wheat bran, rice bran, fish meal, soybean cake powder, soy flour, and peanut cake powder were bought from local supermarkets. Skim milk powder was obtained from Anchor, Fonterra Co-operative Group Ltd. (New Zealand). All chemical reagents were analysis grade and purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Yeast extract was obtained from Oxoid Ltd. (England).

Activation of strain and preparation of seed solution K. marxianus strain MJ1 was placed in a 250 mL Erlenmeyer flask containing 100 mL of sterilized YPD broth and cultured with a shaker (Fu-ma QYC 200, Shanghai Foma Experimental Equipment Co., Ltd., China) at 28°C, 160 rpm for 24 h. The bacterial solution was then transferred to a new YPD broth for an additional 24 hours to complete activation. The activated strain was inoculated onto YPD broth at a ratio of 2% (v/v) and cultured at 28°C and 160 rpm for logarithmic phase to obtain the seed solution of MJ1, and the viable count was noted.

Screening of basic medium The seed solution of MJ1 was inoculated onto 4 different media: potato - dextrose broth (PDB (20% potato; 2% dextrose)), soybean sprout medium (20% soybean sprout; 2% dextrose), skim milk medium (12% skim milk powder; 7% white granulated sugar), and wort basic medium (16.67% wort concentrate (2 Brix)) at a concentration of 1×10^6 CFU·mL⁻¹, and cultured at 28°C and 160 rpm for 24 hours. The optical density value (OD600) was measured every 4 hours to plot the growth curve of MJ1. The uninoculated medium was used as a blank control group, respectively, and each test was repeated thrice.

Screening and optimization of supplemental components of proliferation medium Studies have shown that the composition of a medium directly affects the growth of yeast, especially the type and content of carbon, nitrogen and inorganic salts (Angulo-Montoya et al., 2019; Shariati et al., 2019). Therefore, on the basis of the optimal basal medium obtained, it is necessary to study the optimal carbon source, nitrogen source and inorganic salt of strain MJ1. Furthermore, cost of proliferation is also one of the necessary factors to consider for industrial fermentation. That said, under the premise of meeting the growth and proliferation requirements, low-cost materials such as agricultural product processing by-products may be preferred choice (Aini et al., 2018).

In order to screen for the optimal carbon source, different concentrations of glucose, sucrose, molasses, white granulated sugar, potato, corn flour, and tapioca starch were evaluated. The concentration of glucose and sucrose were set to 5, 10, 15, 20, 25 g·L⁻¹, and the remaining carbon sources were 15, 30, 45, 60, 75 g·L⁻¹.

Regarding screening for optimal nitrogen source, different concentrations and different types of nitrogen source materials were evaluated - wheat bran, rice bran, fish meal, soybean cake powder, soy flour, peanut cake powder, yeast extract and fish meal peptone as organic nitrogen source; and ammonium chloride, ammonium sulfate and ammonium nitrate as inorganic nitrogen sources. Among them, the concentration of wheat bran and rice bran was set to 7.5, 15, 22.5, 30, 37.5 g·L⁻¹, whereas the remaining organic nitrogen sources was 1.5, 3, 4.5, 6, 7.5 g·L⁻¹, and the inorganic nitrogen source was 0.4, 0.8, 1.2, 1.6, 2.0 g·L⁻¹.

To screen for optimal inorganic salts, different concentrations of KH₂PO₄, K₂HPO₄, MgSO₄, CaCO₃, and KCl were evaluated. The concentrations were set to 0.4, 0.8, 1.2, 1.6, 2.0 g·L⁻¹ for KH₂PO₄, K₂HPO₄, MgSO₄, and 1, 2, 3, 4, 5 g·L⁻¹ for CaCO₃ and KCl.

The seed solution of MJ1 was centrifuged at 4°C, 8,000 rpm for 5 min, and the cells were collected, washed with
Optimization Culture of *K. marxianus* MJ1 Suitable for MB Fermentation

sterile water, and re-suspended to obtain an inoculum suspension. Then, it was inoculated onto the aforementioned media at a concentration of $1\times10^6$ CFU·mL$^{-1}$, and the viable cell counts were measured after culturing at 28°C and 160 rpm for 18 hours. A blank control test was also set, and each test was repeated three times.

Based on the screening results, the optimal carbon and nitrogen source as well as inorganic salt were selected as the investigation factors. Using central combination design (CCD) of response surface methodology (RSM) (Arumugam and Kumar, 2017), a total of 20 experiments were conducted with three factors and three levels.

**Optimization of culture conditions**  The growth of yeast is also affected by the culture conditions, which mainly include liquid volume, initial pH, culture temperature, rotation speed and inoculum concentration (Hu, 2017).

First, the optimization experiment of liquid volume was carried out. Different volumes (30, 40, 50, 60, 70 and 80 mL in six 250 mL Erlenmeyer flasks) of proliferation medium of natural pH (pH 5.36) was measured, and the seed solution of MJ1 ($1\times10^6$ CFU·mL$^{-1}$) was inoculated respectively. The optimum liquid volume was obtained by measuring the viable count after incubation at 28°C and 160 rpm for 18 h. Then, the optimization test of initial pH, culture temperature, rotation speed, and inoculum concentration were sequentially performed. Likewise, the test was carried out at six levels of initial pH (pH 4.5, 5, 5.36, 5.5, 6, and 6.5, adjusted with 1 M HCl or 1 M NaOH); culture temperature (24, 26, 28, 30, 32 and 34°C), rotation speed (140, 160, 180, 200, 220 and 240 rpm), and inoculum concentration (0.1×10$^6$, 0.5×10$^6$, 1×10$^6$, 1.5×10$^6$, 2×10$^6$ and 2.5×10$^6$ CFU·mL$^{-1}$).

Based on the test data, the factors that had major influence on the growth of MJ1 were selected as the influencing factors for orthogonal test - L$_{0}$ (3$^3$), and the experiment was carried out to obtain the optimum culture conditions.

**Determination of the viable count**  The viable count of the yeast was achieved by the plate-count method (Wang *et al.*, 2016), and the result was expressed as the CFU·mL$^{-1}$. Each test was done three times at the same time, and the results were averaged.

**Calculation of raw material cost of the proliferation medium**  For cost comparison of optimized proliferation medium and conventional YPD broth, the raw material cost per liter of medium (excluding water) was calculated based on average commercial price.

**Statistical analysis**  The design and data analysis of RSM experiment were performed in the Design Expert Software (Version 8, State-Ease, USA). The plotting of figures was performed in the Origin Software (Version 8.0; Microcal Software Inc., Northampton, MA, USA), and the error bars of figures showed standard deviation (SD). The analysis of variance was performed using the SPSS Software (Version 16.0, SPSS Inc., Chicago, USA). The statistics significance was evaluated using Duncan’s multiple’s range test and $P < 0.05$ was taken as significant.

**Results and Discussion**

**Counting of seed solution of strain MJ1**  By determining, the average viable count of the MJ1 seed solution obtained was $1.9\times10^6$ CFU·mL$^{-1}$.

**Selection of basic medium**  As shown in the growth curve (Fig. 1), OD600 reached a maximum of 0.899 in wort medium, followed by PDB of 0.745, and only reached 0.548 and 0.410 in skim milk medium and soybean sprout medium, respectively. The optical density (OD600) can indirectly monitor the growth of cells, and it is directly proportional to the concentration of microorganisms (Lobete *et al.*, 2015). Therefore, it can be concluded that the wort medium is most beneficial to the growth of strain MJ1. Wort is processed from barley as the main raw material. It is cheap, easy to obtain, and rich in oligosaccharides, maltose, peptides, and amino acids, which can provide nutritional conditions for the growth of microorganisms (Chong *et al.*, 2007). Finally, wort medium was considered the best basic medium (compared to the others).

In these four media, MJ1 reached the logarithmic and stable phases of the growth curve almost simultaneously, where the logarithmic growth phase began around the 4th hour and turned into the stable phase around the 16th hour. The harvest time of the cells is preferably selected in the late logarithmic phase (the early stable phase) (Corcoran *et al.*, 2010). Therefore, 18 hours of culture was taken as the optimal time for collection of strain MJ1 cells.

**Selection of optimal carbon source for proliferation medium**  The carbon source is the main source of nutrients and energy required by yeast. Sufficient carbon source must be provided during fermentation to ensure growth and proliferation of yeast. However, excessive concentrations of

![Fig. 1. Growth curve of MJ1 in four different basic medium](image-url)
carbon sources can also inhibit yeast growth (Pereira et al., 2010). Different yeast strains have different optimal carbon source preference due to their different physiological metabolic pathways. Therefore, screening for the optimal carbon source and concentration has a positive effect on increases in growth rate as well as cell concentration.

For the test of glucose and sucrose, compared with the blank control, the viable count of MJ1 showed a decreasing trend as the glucose concentration increased, which may be affected by the Crabtree effect (JoRgensen, 2009). Unlike glucose, the viable count increased initially and then decreased with increasing sucrose concentration, and reached the highest value of $2.27\times10^8$ CFU·mL$^{-1}$ at the concentration of 15 g·L$^{-1}$ ($P < 0.05$; Fig. 2A). Deducting from this is that, sucrose provided a more suitable osmotic pressure environment than glucose at a certain concentration, and thus could be more favorable for yeast growth. When molasses, white granulated sugar, potato and corn flour were used as carbon sources, the viable count increased initially and then decreased with increasing concentration. However, the addition of tapioca starch did not have positive effect on the proliferation of MJ1 within the range of concentrations tested (Fig. 2B). And the proliferation effect of molasses was the most significant, and the viable count reached a maximum of $3.42\times10^8$ CFU·mL$^{-1}$ at 30 g·L$^{-1}$ ($P < 0.05$). At the respective optimum concentrations, molasses obtained a higher viable count than sucrose. As a by-product of the sugar industry, molasses contains a significant amount of sucrose and a small amount of other saccharides such as raffinose, glucose, lactose, etc. It provides a good carbon source for yeast cell growth and is considered an ideal low-cost material (Santos et al., 2010; Leelavatcharamas et al., 2018). Therefore, molasses was labelled as the optimal carbon source for the proliferation medium of strain MJ1.

Selection of optimal nitrogen source for proliferation medium  
Nitrogen is one of the essential components of cells and plays a vital role in the growth and development of yeast. Nitrogen sources are important sources of nitrogen-containing metabolites, mainly including organic nitrogen sources and inorganic nitrogen sources (Zhang et al., 2018). The organic nitrogen sources commonly used in the fermentation industry include wheat bran, rice bran, fish meal, soybean cake powder, peanut cake powder, peptone, yeast extract, etc., while the inorganic nitrogen source includes ammonium salts, nitrates, ammonia water, etc (Kang, 2015).

As a by-product of crop processing, wheat bran and rice bran contain ample amount of crude protein, fat, crude fiber and multivitamins, which can be used as the main nitrogen source in the fermentation process (Hammami et al., 2018). In the test of these two nitrogen sources, by comparison, the viable count of MJ1 reached the highest value of $2.83\times10^8$ CFU·mL$^{-1}$ when the wheat bran concentration was 15 g·L$^{-1}$ ($P < 0.05$; Fig. 3A). In Fig. 3B, the soybean cake powder significantly increased the viable count of MJ1 within the tested concentration range, and the peanut cake powder had little effect, while the soy flour and fish meal caused the decrease in the viable count. The results of the comparison showed that the viable count reached a maximum of $2.75\times10^8$ CFU·mL$^{-1}$ with adding 4.5 g·L$^{-1}$ soybean cake powder ($P < 0.05$). When the yeast extract and the fish meal peptone were used as the nitrogen source, with the concentration increasing, the viable count increases initially and then decreases, and reached the highest value of $2.37\times10^8$ CFU·mL$^{-1}$ at 4.5 g·L$^{-1}$ yeast extract concentration ($P < 0.05$; Fig. 3C). At the respective optimal concentrations, compared with soybean cake powder and yeast extract, wheat bran had the best effect on increasing the viable count of MJ1. For the three inorganic nitrogen sources, their gradual addition resulted in a decrease in the viable count of MJ1 – suggesting a non-conducive condition for the proliferation of the strain (Fig. 3D). Thus,
Optimization Culture of *K. marxianus* MJ1 Suitable for MB Fermentation

Through the test of organic nitrogen source and inorganic nitrogen source, wheat bran was selected as the optimal nitrogen source for the proliferation medium of strain MJ1.

**Selection of optimal inorganic salt for proliferation medium** During the growth and development of yeast, some inorganic salt ions are needed to regulate their physiological metabolic activities. For example, K\(^+\) and Ca\(^{2+}\) can regulate cell osmotic pressure and permeability, and Mg\(^{2+}\) can regulate intracellular enzyme activity (Xue *et al.*, 2008).

In the test of KH\(_2\)PO\(_4\), K\(_2\)HPO\(_4\), MgSO\(_4\), CaCO\(_3\), and KCl as inorganic salts, the addition of the other four inorganic salts (except CaCO\(_3\)) resulted in a decrease in the viable count of the strain MJ1 as the concentration increased. The reason for this phenomenon may be that the strain has a low demand for inorganic salts, and the rich minerals in the wort are reasonably sufficient; and thus excess may not be suitable for the growth of the strain. On the contrary, the viable count was significantly increased after the addition of CaCO\(_3\), and reached a maximum of \(2.66 \times 10^8 \, \text{CFU·mL}^{-1}\) at \(2 \, \text{g·L}^{-1}\) (\(P < 0.05\); Fig. 4). This may be due to the fact that MJ1 produced some acidic substances that are not conducive to growth during fermentation, while CaCO\(_3\) can play a role of neutralization regulation. Therefore, CaCO\(_3\) was selected as the optimal inorganic salt for the proliferation medium of strain MJ1.

**RSM modeling of optimum proliferation medium components** RSM is a statistical method used to find the optimal response factors and has been widely used to optimize the composition of culture medium (Coninck *et al.*, 2000; Haddar *et al.*, 2010; Yoo *et al.*, 2018). Table 1 shows the experimental findings using the viable count as response variables. By applying the statistical technique - multiple regression analysis on the experimental data, the following
second-order polynomial equation giving the viable count (Y) as a function of molasses (A), wheat bran (B) and CaCO$_3$ (C) concentrations was obtained:

\[ Y = -1.3751 + 2.20436A + 3.76778B + 20.53899C - 0.03333AB + 0.58333AC + 0.83333BC - 0.38668A^2 - 1.23244B^2 - 56.95033C^2 \]  

Eq. 1

The ANOVA analysis (Table 2) revealed that the model is extremely significant ($P < 0.0001$); the parameters of A, B, C, AC, BC, $A^2$, $B^2$, and $C^2$ had significant effects on the proliferation of strain MJ1 ($P < 0.05$); the model mismatch term is not significant ($P > 0.05$); the correction coefficient $R^2$ and correlation coefficient $R$ are 0.9954 and 0.9976, respectively. This model can thus be used to analyze changes in response values.

The model predicted that when the concentration of molasses, wheat bran and CaCO$_3$ were 2.94%, 1.56% and 0.21%, respectively, the viable count would reach the highest value of $6.9 \times 10^8$ CFU·mL$^{-1}$. Three independent experiments were conducted at this concentration, and the actual viable count was $6.8 \times 10^8$ CFU·mL$^{-1}$, which was very close. It can be inferred that the parameters of the proliferation medium components obtained by the RSM modeling are accurate and reliable. Moreover, the viable count was about 3.58 times that of YPD broth ($1.9 \times 10^8$ CFU·mL$^{-1}$), which indicated that the optimized medium had a remarkable proliferative effect. The above results laid the experimental basis for optimizing the culture conditions to further increase the viable count of the strain MJ1.

**Effects of different culture conditions on the growth of strain MJ1**

The amount of liquid in the microbial shake flask culture process can affect the oxygen transfer, and many studies have tested the optimal liquid volume (Liu and Yang, 2006; Xu et al., 2010; Qi, 2014; Jiang et al., 2018). If the liquid volume is too low, the water will easily evaporate and the test error will be large. Conversely, if the volume of liquid is too high, the amount of dissolved oxygen will be insufficient, which is not conducive to the proliferation and culture of microorganisms. Most yeast grow optimally in the pH range 4.5 ~ 6.5, and thus too high or too low will affect their growth and proliferation (Yin et al., 2010; Guo et al., 2019). Temperature has a significant effect on the growth, proliferation, and metabolic activity of microorganisms. A certain increase in temperature can increase the activity of intracellular enzymes and membrane fluidity, as well as the sensitivity of cells to alcohol toxicity (Reddy and Reddy, 2011). The rotation speed directly determines the level of oxygen supply in the medium. If the rotation speed is too low, the dissolved oxygen content of the medium will be lowered, which is not conducive to the absorption and metabolism of nutrients by the strain. On the contrary, excessively high rotation speed can cause high shear forces on cells and inhibit cell growth (Garcia-Ochoa et al., 2010). A suitable inoculum concentration is vital for the proliferation of the strain. Insufficient inoculum will prolong the time to reach the stable phase, and excessive inoculum will result in insufficient nutrient in the medium to affect the metabolism and proliferation of the cells (Singh et al., 2007).

The effects of different culture conditions (liquid volume, initial pH, temperature, rotation speed, and inoculum concentration) on the proliferation of MJ1 were shown in Fig. 5. The viable count showed a trend of increasing initially and then decreasing with the change of certain condition, which was consistent with the above description. And at temperature 28°C, rotation speed 220 rpm, and inoculation concentration $1.5 \times 10^8$ CFU·mL$^{-1}$, the viable count of MJ1 reached the highest value ($P < 0.05$), respectively. For the liquid volume, although the viable count achieved the maximum at 50 mL, the difference was not significant in the range of 40 mL to 60 mL.
Similarly, the viable count reached the highest value at pH 5.36, but the difference was not significant within pH 5.0 ~ 5.36 ($P > 0.05$). Therefore, the liquid volume and pH were not suitable as the influencing factors for the orthogonal.

**Optimization of culture conditions by orthogonal test** The culture temperature, rotation speed and inoculum concentration that had significant influence on the MJ1 growth were selected as the influencing factors for the orthogonal [$L_9(3^4)$] test. From Table 3 and Table 4, the effects of factors A (temperature), B (rotation speed) and C (inoculation concentration) on the viable

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of square</th>
<th>df</th>
<th>Mean square</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>19.36</td>
<td>9</td>
<td>2.15</td>
<td>461.07</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>A-Molasses</td>
<td>0.074</td>
<td>1</td>
<td>0.074</td>
<td>15.84</td>
<td>0.0026</td>
</tr>
<tr>
<td>B-Wheat bran</td>
<td>0.14</td>
<td>1</td>
<td>0.14</td>
<td>30.96</td>
<td>0.0002</td>
</tr>
<tr>
<td>C-CaCO$_3$</td>
<td>0.079</td>
<td>1</td>
<td>0.079</td>
<td>16.86</td>
<td>0.0021</td>
</tr>
<tr>
<td>AB</td>
<td>0.011</td>
<td>1</td>
<td>0.011</td>
<td>2.41</td>
<td>0.1515</td>
</tr>
<tr>
<td>AC</td>
<td>0.061</td>
<td>1</td>
<td>0.061</td>
<td>13.13</td>
<td>0.0047</td>
</tr>
<tr>
<td>BC</td>
<td>0.031</td>
<td>1</td>
<td>0.031</td>
<td>6.70</td>
<td>0.0270</td>
</tr>
<tr>
<td>A$^2$</td>
<td>10.91</td>
<td>1</td>
<td>10.91</td>
<td>2337.95</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>B$^2$</td>
<td>6.93</td>
<td>1</td>
<td>6.93</td>
<td>1484.39</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>C$^2$</td>
<td>4.67</td>
<td>1</td>
<td>4.67</td>
<td>1001.76</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Residual</td>
<td>0.047</td>
<td>10</td>
<td>4.67E-03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack of Fit</td>
<td>0.018</td>
<td>5</td>
<td>3.67E-03</td>
<td>0.65</td>
<td>0.6779</td>
</tr>
<tr>
<td>Pure Error</td>
<td>0.028</td>
<td>5</td>
<td>5.67E-03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cor Total</td>
<td>19.41</td>
<td>19</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$R^2$=0.9976
$R^2(adj)$=0.9954

*The P-values less than 0.05 are significant, and the P-values less than 0.01 are extremely significant.*
count of MJ1 were $R_A > R_B > R_C$. Except for the inoculation concentration, the other factors had significant effects on the viable count ($P < 0.05$). The optimal growth condition for strain MJ1 was $A_2B_2C_2$ (temperature 28 °C, rotation speed 220 rpm, and inoculation concentration $1.5 \times 10^6$ CFU·mL$^{-1}$). Three independent experiments were done under the optimal conditions, and the average viable count was $7.8 \times 10^8$ CFU·mL$^{-1}$, which was higher than the optimal combination $A_2B_2C_3$ of $7.6 \times 10^8$ CFU·mL$^{-1}$ in the orthogonal experiment. And the test had good repeatability, which indicated $A_2B_2C_2$ can be used as the optimal culture condition for strain MJ1. Finally, the viable count of strain MJ1 obtained after optimization of proliferation culture was as high as $7.8 \times 10^8$.

Fig. 5. Effect of liquid volume (A), pH (B), temperature (C), rotation speed (D) and inoculum concentration (E) on the growth of MJ1. Different lowercase letters indicate significant difference ($P < 0.05$).
Optimization Culture of *K. marxianus* MJ1 Suitable for MB Fermentation

Optimization Culture of *K. marxianus* MJ1 Suitable for MB Fermentation

Comparison of the cost of the optimal proliferation medium and YPD broth

The optimal proliferation medium consisted of wort base medium (16.67% wort concentrate), molasses 2.94%, bran 1.56%, and CaCO₃ 0.21%. Reference to the average commercial price, the unit price (per kilogram) of wort concentrate, molasses, wheat bran, and CaCO₃ are 23, 10, 4, and 8 Yuan, respectively, therefore it costs 4.21 Yuan per liter of proliferation medium. The unit (per kilogram) price of YPD broth is 548 yuan, and it costs 27.4 Yuan per liter of medium (50 g L⁻¹), meaning the cost of the proliferation medium optimized was less than 1/6 of YPD broth. This study not only achieved high-density fermentation of strain MJ1, but also significantly reduced the cost of proliferation and improved the utility value of agricultural product processing by-products.

Moreover, this laid the foundation for further mass cultivation of MJ1 live cells using fermenters.

Conclusion

*K. marxianus* strain MJ1 suitable for milk beer fermentation production was used in an optimization study of proliferation culture. Using the optimized proliferation medium (molasses 2.94%, wheat bran 1.56%, CaCO₃ 0.21%) and applying the optimal culture conditions (28°C, rotation speed 220 rpm, inoculation concentration 1.5×10⁸ CFU mL⁻¹), the viable count of strain MJ1 increased from 1.9×10⁹ CFU mL⁻¹ (YPD broth) to 7.8×10⁹ CFU mL⁻¹, which was increased to 4.11 times. In addition, this study utilized crop by-products as a raw material for proliferation, with a total cost of 4.21 Yuan per liter. And the price of YPD broth was 27.4 Yuan per liter, which represented nearly 5 times reduction in cost.

### Table 3. Results of orthogonal test for culture conditions

<table>
<thead>
<tr>
<th>Run</th>
<th>Temperature (A, °C)</th>
<th>Rotation speed (B, rpm)</th>
<th>Inoculum concentration (C, ×10⁶ CFU mL⁻¹)</th>
<th>Blank (D)</th>
<th>The viable count (Y, ×10⁷ CFU mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1(26)</td>
<td>1(200)</td>
<td>1(1.0)</td>
<td>1</td>
<td>62</td>
</tr>
<tr>
<td>2</td>
<td>1(26)</td>
<td>2(220)</td>
<td>2(1.5)</td>
<td>2</td>
<td>71</td>
</tr>
<tr>
<td>3</td>
<td>1(26)</td>
<td>3(240)</td>
<td>3(2.0)</td>
<td>3</td>
<td>65</td>
</tr>
<tr>
<td>4</td>
<td>2(28)</td>
<td>1(200)</td>
<td>2(1.5)</td>
<td>3</td>
<td>72</td>
</tr>
<tr>
<td>5</td>
<td>2(28)</td>
<td>2(220)</td>
<td>3(2.0)</td>
<td>1</td>
<td>76</td>
</tr>
<tr>
<td>6</td>
<td>2(28)</td>
<td>3(240)</td>
<td>1(1.0)</td>
<td>2</td>
<td>74</td>
</tr>
<tr>
<td>7</td>
<td>3(30)</td>
<td>1(200)</td>
<td>3(2.0)</td>
<td>2</td>
<td>64</td>
</tr>
<tr>
<td>8</td>
<td>3(30)</td>
<td>2(220)</td>
<td>1(1.0)</td>
<td>3</td>
<td>71</td>
</tr>
<tr>
<td>9</td>
<td>3(30)</td>
<td>3(240)</td>
<td>2(1.5)</td>
<td>1</td>
<td>67</td>
</tr>
</tbody>
</table>

K1 198 198 207 205
K2 222 218 210 209
K3 202 206 205 208
k1 66 66 69 68.333
k2 74 72.667 70 69.667
k3 67.333 68.667 68.333 69.333
R 8.000 6.667 1.667 1.334

Factor order A > B > C > D
Optimal level 2 2 2
Optimum combination A₂B₂C₂

### Table 4. ANOVA analysis of orthogonal test

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of squares</th>
<th>df</th>
<th>Mean square</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>110.222</td>
<td>2</td>
<td>55.111</td>
<td>38.154</td>
<td>0.026</td>
</tr>
<tr>
<td>Rotation speed</td>
<td>67.556</td>
<td>2</td>
<td>33.778</td>
<td>23.385</td>
<td>0.041</td>
</tr>
<tr>
<td>Inoculum concentration</td>
<td>4.222</td>
<td>2</td>
<td>2.111</td>
<td>1.462</td>
<td>0.406</td>
</tr>
<tr>
<td>Error</td>
<td>2.889</td>
<td>2</td>
<td>1.444</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The P-values less than 0.05 are significant, and the P-values less than 0.01 are extremely significant.*
The results set a good foundation for further mass production of the strain MJ1 active cells, as well as the preparation of the active freeze-dried powder and the industrial application of the strain MJ1 in MB production.

Acknowledgements This work was supported by Jiangsu University senior professional talent research start-up funds (12JDG069), Xinjiang Production and Construction Corps Industry and High-tech Science and Technology Research and Achievement Transfer Project (2015AB032) and Xinjiang Production and Construction Corps 12th Division Industrial Research Project (SR2016001, SR2017003).

References


