

Study on the Novel Strain *Rhizopus Oryzae* ATCC2809's Growth and Fermentation

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Abstract: *The high quality saccharifying power strain is very important for fermentation in China. R.oryzae ATCC2809 was screened from Chinese traditional alcohol starters "JiuQu", which is an excellent strain possessing strong saccharifying power in musk fermentation and generating aroma taste flavor. For further exploring the characteristics of strain R.oryzae ATCC2809 matched the standard species description of growth characteristics and productivity of Rhizopus oryzae strain ATCC2809, and saccharifying power of the strain Rhizopus oryzae ATCC2809 as the main indexes, the main growing factors including trace element, temperature, saccharification, culture medium, amino acid concentrations after the fermentation by R.oryzae ATCC2809 in the mash, etc., were analyzed by comparison experiment. When the fermentation was over, the content of the amino acid of the mash of R.oryzae ATCC2809 was contrasted by the other two traditional strains of R.oryzae F14 and R.oryzae Q303, because the later two strains were widely used in industrial production and achieved good economic profit for the enterprises. At last, we got the conclusion: the essential trace elements of strain R.oryzae ATCC2809 were: P(phosphorus), Mg(magnesium). The strain R.oryzae ATCC2809 grew very well in PDA. Its saccharification power in Czapek's bran medium was better than the others culture mediums. The optimal production yields were achieved at 32 °C*

Keywords: R. oryzae ATCC2809, saccharification power, sweet wine, rice wine

1. Introduction

1.1 The fungi of *Rhizopus oryzae* in "JiuQu" produce amylolytic and proteolytic enzymes, which have important roles in starch saccharification and protein or peptide digestion, respectively [Park et al., 2009; Kim et al., 1997; 1998; 2006; Yu et al., 1998]. Most of the wild-type yeast strains cannot directly utilize starch because of their inability to produce starch decomposing enzymes (Jamai et al., 2007). Numerous studies have demonstrated that, both α -amylase and glucoamylase are required for efficient starch hydrolysis in industrial ethanol production using *S. cerevisiae* strains (Knox et al., 2003; Kosugi et al., 2009). In previous study, *R. oryzae* α -amylase was secreted through an *S. cerevisiae* strain. However, the recombinant strain in the starch-containing culture medium showed extremely low α -amylase activity (approximately 0.06 U/ml), a level insufficient for efficient starch degradation without exogenous glucoamylase, while α -amylase is main trait of *R.oryzae* (Song Li et al., 2011). In this study, the *R. Oryzae* ATCC2809 strain was able to hydrolyse soluble starch efficiently because of its ability to express α -amylase at high concentrations in the starch-containing culture. Amylolytic starters used for alcoholic fermentation, consisting of rice flour and a range of starch. *R. oryzae* have a strong power to produce amylase (Robert et al., 2010).

1.2 *R.oryzae* α -amylase can be used to produce high-maltose syrup (Doyle et al., 1989), in which the maltose concentration is up to 50% (w/w) and the glucose concentration is usually around 10% (w/w). In addition, considerable amounts of maltotriose, which also yielded higher final biomass than in the presence of glucose (Zastrow et al., 2000), can be released during starch hydrolysis (Doyle et al., 1989). In previous study, soluble starch hydrolysed with *R. oryzae* α -amylase yielded a final concentration of glucose and maltose at 12 and 67% (w/w),

respectively and a large amount of maltotriose was still released at the initial hydrolysis stage of the gelatinised starch. Overall, it can be concluded that the ability of *R. oryzae* α -amylase to produce high levels of maltose and maltotriose accounts for the higher cell density obtained by the *R.oryzae* in the presence of soluble starch.

1.3 *R.oryzae* ATCC2809 is screened from Guangdong zhaoqing traditional koji which has a strong ability in saccharification power which derive from the plenty secretory α -amylase and producing a special flavor. This strain has been widely used in many area of Asia as starter of alcohol fermentation, which has been got predominant economic benefits.

1.4 Among physical parameters, cultivation temperature and pH of growth media are important for organism growth and enzyme secretion (Gupta et al., 2003). Similar to the temperatures used in many studies (Rocha et al., 1996; Mincheva et al., 2002), 28 to 30°C yielded the highest α -amylase activity and biomass. In order to improve the fermentation capability and reduce the industrial production cost, we must examine the most optimum growing condition of *R. oryzae* ATCC2809, which include the premium composition of the culture medium and growing temperature even the fermentation condition.

1.5 This paper tested the hyphae premium growing situation and saccharification power. According to comparison with *R.oryzae* F14 and *R.oryzae* Q303 which are two strains possessing high-saccharification power and was widely used in industrial production, strain *R. oryzae* ATCC2809 showed dominantly advantage productivity, So it is worthy of further study and promote the application.

2. Materials and Methods

2.1 Microorganisms, Media and Reagents

R.oryzae ATCC2809, *R. oryzae* F14, *R.oryzae* Q303 were collected from microbiological laboratory of College of Food Science of Jiangxi Agriculture University. All strain samples collected were made from wheat Qu. All culture medium have Czapek's bran medium, Potato Dextrose Agar (PDA), Malt Extraction medium (ME), Bean Sprouts medium (BS), Czapek's medium, 0.85% saline solution (ss), Neutralizer used: 0.85%, NaCl containing 0.5% Na₂S₂O₃, sodium hydroxyl (0.1M), phenolphthalein solution, distilled water. One gram of strain sample was suspended in 9 mL of sterilized, distilled water containing 0.85% sodium chloride, and 10-fold serial dilutions were made. Each diluent was plated in triplicate on Difco Cooke Rose Bengal Agar (BD Biosciences, Sparks, MD) containing chloramphenicol (100 µg/mL; Sigma-Aldrich, St. Louis, MO). After 5–7 days incubation at 25°C, fungal colonies were counted and characterized by observing the morphological characteristics, including the colony type and spore morphology. The fungal colonies were sub-cultured on potato dextrose agar (PDA; BD Biosciences) for single conidium isolation. All strains were stored in 15% glycerol at –70°C.

2.2 Morphological and Physiological Identification

For each strain, one inoculating loop of spores was suspended in 500 µL of 0.2% agar with 0.05% Tween 80. The suspension was used for one- or three-point inoculations on 9 cm diameter Petri dishes containing approximately 25 mL of media. All strains were cultivated on PDA, malt extract agar (MEA; 2% malt extract, 2% glucose, 0.1% peptone, and 2% agar), Bean Sprouts medium (BS) and Czapek's medium at 25°C for 7–10 days. All strains were grown for 7 days on Czapek yeast agar (CYA) at either 25 or 37°C, CYA with 20% sucrose (CY20S) at 25°C or MEA at 25°C. For micromorphological observations, the fruiting bodies of fungi were observed with a SMZ1500 stereoscopic microscope (Nikon, Tokyo, Japan), and the vegetative and asexual stages were observed with a DE/Axio Imager A1 microscope (Carl Zeiss, Oberkochen, Germany) after staining MEA colonies with lactophenol cotton blue (BD Biosciences). The morphological features of the isolates were characterized, and the species were identified according to the methods of previous studies on Mucorales and *Rhizopus* [Ciesla et al., 2000; Ribes et al., 2000; Garcia-Hermoso et al., 2009].

2.3 Experiment Methods

We used the deficiency medium to test which trace element is the important factor in *R.oryzae* growth according to the difference of hyphae growth weight to estimate the main trace elements.

2.3.1 The Culture Medium Preparation

In order to test out the important trace elements of growth for the *R.oryzae* ATCC2809. We used the rich medium

which was composed of sucrose 100g, MgSO₄•7H₂O 0.5g, NH₄NO₃ 3g, FeSO₄ 0.1g, KH₂PO₄ 2g and distilled water 1000ml compared with the deficiency medium such as Phosphorus deficiency medium, Sulfur deficiency medium, Potassium deficiency medium, Magnesium deficiency medium, Ferrum deficiency medium in turn (All of these experiments were carried out in triplicate).

In the Phosphorus deficiency medium: We used the equal mol KCl instead of KH₂PO₄, Sulfur deficiency medium: used the equal mol MgCl₂ instead of MgSO₄•7H₂O, Phosphorous deficiency medium: used equal amount NaH₂PO₄ instead of KH₂PO₄. Magnesium deficiency medium: used equal amount Na₂SO₄ instead of MgSO₄•7H₂O. Ferrum deficiency medium: used equal amount Na₂SO₄ instead of FeSO₄.

2.3.2 Biomass Measurement to Test the Amount of Mycelium Growth Productivity

Biomass is a fundamental parameter in the characterization of microbial growth. Its measurement is essential for kinetic studies on hyphae growth. Direct determination of biomass in liquid fermentation medium is very difficult due to the problem of separating the microbial biomass from the substrate. On the other hand, for the calculation of growth rates and yields, amount of biomass is important. Methods that have been used for biomass estimation in culture process belong to Direct evaluation of biomass (Mitchell et al, 1991). The experimental data were statistically analysed to find out the important facts that effects the *R. oryzae* ATCC2809 growth.

2.3.3 Different Plate Culture Medium's Effect on *Rhizopus Oryzae* ATCC2809 Growth

R. oryzae ATCC2809 was inoculated on Potato Dextrose Agar (PDA), Malt Extraction medium (ME), Bean Sprouts medium (BS), Czapek's medium, their growth status and morphology was observed.

2.3.4 High Temperature Tolerance Test of *Rhizopus Oryzae* ATCC2809 Spores

In order to obtain a higher accuracy of identification and to investigate the thermotolerance of each fungal isolate, the diameters of *R.oryzae* fungal colonies were measured after incubation at 50°C, 55°C, 60°C, 65°C, 70°C, respectively. Because we used *R. oryzae* ATCC2809 as the koji applied for the industrial production, we always face such problem is that the temperature is always nearly arising to 60°C, especially in summer. So we must test its property in resisting to the high temperature if it can keep high activity at such condition. *R. oryzae* ATCC2809 spores were inoculated on Potato Dextrose Agar (PDA) and incubated for 24 h at 50°C, 55°C, 60°C, 65°C, 70°C. Then observe the mycelium growth situation in order to get out the temperature effect to the *R. oryzae* ATCC2809's growth.

2.3.5 The Saccharification Power Test

Use the steam rice as the substrate, *R.oryzae* ATCC2809 was incubated and successively fermented for 2 days. Then saccharification power was measured by fermented liquid

measuring, according to Nelson’s modification of Somogi’s method (Somogi 1937; Nelson1944). This is very sensitive and reasonably quick method for quantitative estimation of reducing sugars. Carbohydrates with free reducing sugars undergo isomerization, oxidation and cleavage, while the oxidizing agent copper is reduced. After reduction copper reacts with an arsenomolybdate color forming reagent and produce blue color.Reducing sugars were estimated in 1mL of plant extract in separate test tube. Each tube received 1 mL of copper reagent. After thorough mixing the tubes they were placed in boiling water bath for 20min. and quickly cooled by dipping them in cold water for five min. 1 mL of arsenomolybdate reagent was added to each tube and contents were shaken rapidly until the evolution of CO2 was completed. The tubes were left for 15 min. for the development of blue color. The absorbance was recorded at 500 nm against reagent blank, using Photic-100 spectrophotometer. The amount of reducing sugars was estimated as µg glucose g-1 fresh weight.

2.3.5 HPLC-UV analysis

Amino acid analysis was carried out by HPLC using phase column (Waters, 1525) and a gradient with acetonitrile in 0.1 mol/L monopotassium phosphate (a flow rate of 1 ml/min). The injection volume was 10 ul. The detection was carried out at the wave length of 254 nm. Detection and spectral characterization of peaks were accomplished with a dual λ UV absorbance detector (Waters 2478) and Breeze software (Waters).

3. Results and Discussion

3.1 Effect of Metal Ion on Rhizopus Oryzae ATCC2809 Growth of Mycelium

The result of Biomass Measurement of strain grown in different deficiency medium and full nutrition medium was shown in figure 1

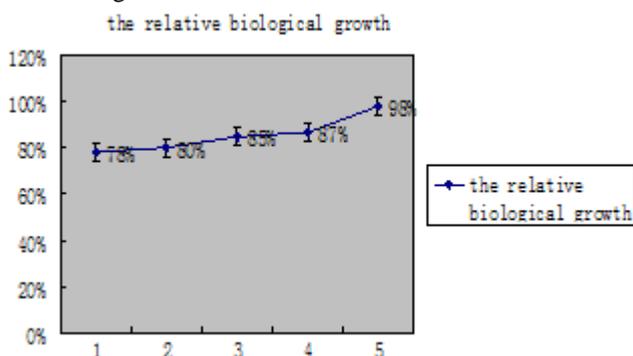


Figure 1: The relative growth ± stand deviation of triplicate determinations. point1 stand for phosphorus deficiency, point2 stand for magnesium deficiency, point3 stand for potassium deficiency, point4 stand for sulphur deficiency, point5 stand for ferrum deficiency

Trace elements were chosen among the culture medium in regular manufacturing practice and their effects on the growth of mycelium were analyzed. The activity, when examined with the addition of different metal ions, showed that phosphate (P) and magnesium (Mg) , stimulated

Rhizopus Oryzae ATCC2809 growth of mycelium, whereas sulphur (S), potassium (P) and ferrum(Fe) had an little effect on Rhizopus Oryzae ATCC2809 growth of mycelium (Figure 2). These results were in accordance with the results of Li et al. (28). From figure1 we can see that the most important elements is phosphorus (P) and magnesium(Mg). Compared with the complete culture medium production, the next influence factors are sulfur (S), potassium (P) and ferrum (Fe), the effected result is 90%, 89%, 95%, 99% , respectively.

3.2 The Strain Morphology in Different Medium Culture

Table 1: Characters of ATCC2809 on 5 culture mediums for 22 hours

The kind of medium	Wort medium	Bean sprouts medium	PDA	Czapek’s medium	Czapek’s, Bran medium
The size of colonies	8.1cm	6.8cm	6.7cm	4.3cm	5.0cm
The shape of colonies	Near round, wrinkles, thick	Near round, thick	Near round, thick	Near round, sparsely	Near round, sparsely
Aerial hyphae	sparsely and the width is13.1um	very abundant aerial mycelia the width is 14.1um	very abundant aerial mycelia the width is 17.6um	A little The width is 15.4um	sparsely the width is 22.5um
The color of hyphae	Yellowish-white	Gray-white	White and bright	White and bright	White and bright
Sporangiospore	A little, length is 4.83um	A little, length is 5.34um	A lot, length is 6.81um	A little, length is7.96	A little, length is 8.18um

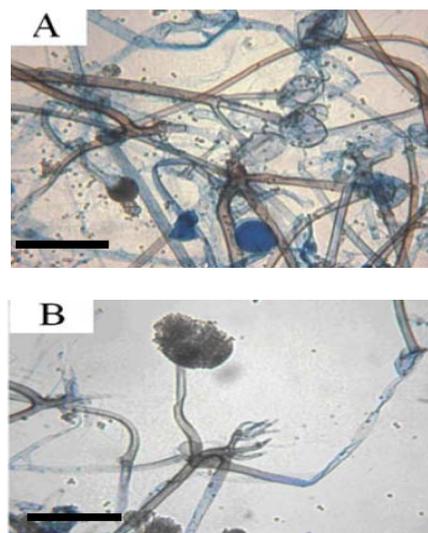


Figure 2: Morphological observation of Rhizopus oryzae ATCC2809 grown in PDA medium under optical microscopy. (A) Sporangium and sporangiospores and (B) Rhizoids. (Scale bars represent 20 mm.)

According to table1. and Figure 2. we can see that the best colony in size and morphology was cultured in PDA medium, which had many wrinkles in its surface, fast-

growing; their spores is big, the mycelium of the *R. oryzae* ATCC2809 were robust, bright, luster. So we select the PDA as the generation and preservation medium.

3.3 The result of saccharogenic power test

Table 2: The saccharogenic power of ATCC2809 on different culture mediums

Culture Medium / Saccharification	Wort medium	Bean sprout medium	Czapek's medium	PDA	Czapek's bran medium
Mash volume(ml)	153	162	165	159	196
sugar degree (Bx)	28.1	31	33.4	35.1	30.3
Total sugar amount = Mash volume * sugar degree(Bx)	4299	5022	5511	5581	5933

In the table 2 the mash volume is instead of the volume after fermentation in this type culture medium by squeezed the liquor from the mash, then used it to multiple the sugar degree of the mash volume. We can get the total sugar amount even is the saccharogenic power in this culture medium. Then we can used the total sugar amount to determine the saccharifying ability by in order. From table 2 the ATCC2809's saccharifying ability is highest after cultured in the Czapek's medium compared with others. So the Czapek's bran medium was found to be the most suitable medium for the rejuvenation and production.

3.4 Temperature effect on growth and saccharification activity

The production of ethanol by *Rhizopus oryzae* fermentation in tropical countries (such as the south of china) , especially during the summer season, is not economically feasible because of the high energy input required to cool the fermenters. Thermotolerant *Rhizopus oryzae* offer potential advantages in the alcohol industry by reducing cooling costs and by having faster fermentation rates, thereby making the process more economical (Kiransree et al. , 2000; Pasha et al., 2007).

Table 3: Comparing of characters of *Rhizopus* ATCC2809 at 4 temperatures for 22 hours

The different temperature	24°C	28°C	32°C	36°C	40°C
Colonies size	4.5cm	7.0cm	7.4cm	6.7cm	6.0cm
Stolon mycelium	Sparsely and growing slowly	Thick & growing fast	Thick, denser & fast growing	Thick & grow fast	Thick and hyphae growing well
Aerial mycelium	Seldom or never, have gloss	A little, have gloss	Many, have gloss	many, obscure	
Spores	A little	Many, grey	Many, grey	A few, grew	

Fig.2 The saccharogenic power of F6 at different temperatures (g/l)

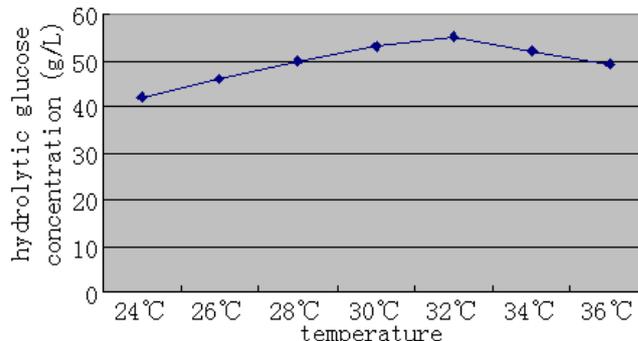


Figure 2: The saccharogenic power of ATCC2809 at different temperatures

According to the colony size, mycelium growing morphology, spores' number, the saccharogenic power and the saccharification power, the most optimum growing temperature was obtained at 32°C.

3.5 Spore's tolerance to the high temperature

Table 4: The growth of high temperature treating for 30 minutes

Different Temperature	50°C	55°C	60°C	65°C	70°C
Growth situation	Grown better	Grown better	Grown normal	Grown normal	Grown poorly

3.6 Content of Amino Acids

Amino acids, not only nutrient components but also precursors for aroma compounds in Chinese rice wine, are an important quality parameter of Chinese rice wine [24]. Figure 2. showed the chromatogram graph of 18 amino acids for (a) *R.oryzae* strain ATCC2809-processed rice wine mask, (b) *R.oryzae* strain F14-processed rice wine mask and (c) *R.oryzae* strain Q303-processed rice wine mask .The content of amino acid in rice wine masks was summarized in Table 6. The data were the mean values of three replicates. Of all the 18 amino acids determined, the content of 16 amino acids in *R.oryzae* strain ATCC2809-processed rice wine mask (1.7g/100ml) was 43.7 % higher than the two others (b) *R.oryzae* strain F14-processed rice wine mask (1.1g/100ml)and (c) *R.oryzae* strain Q303-processed rice wine mask (1.5g/100ml). This may be due to the new *R.oryzae* strain ATCC2809 enzymatic hydrolysis is stronger than the the current widely used industrial strains, which improved the digestion of protein. De Mesa-Stonestreet et al. [25] . Wang et al. [26, 27]

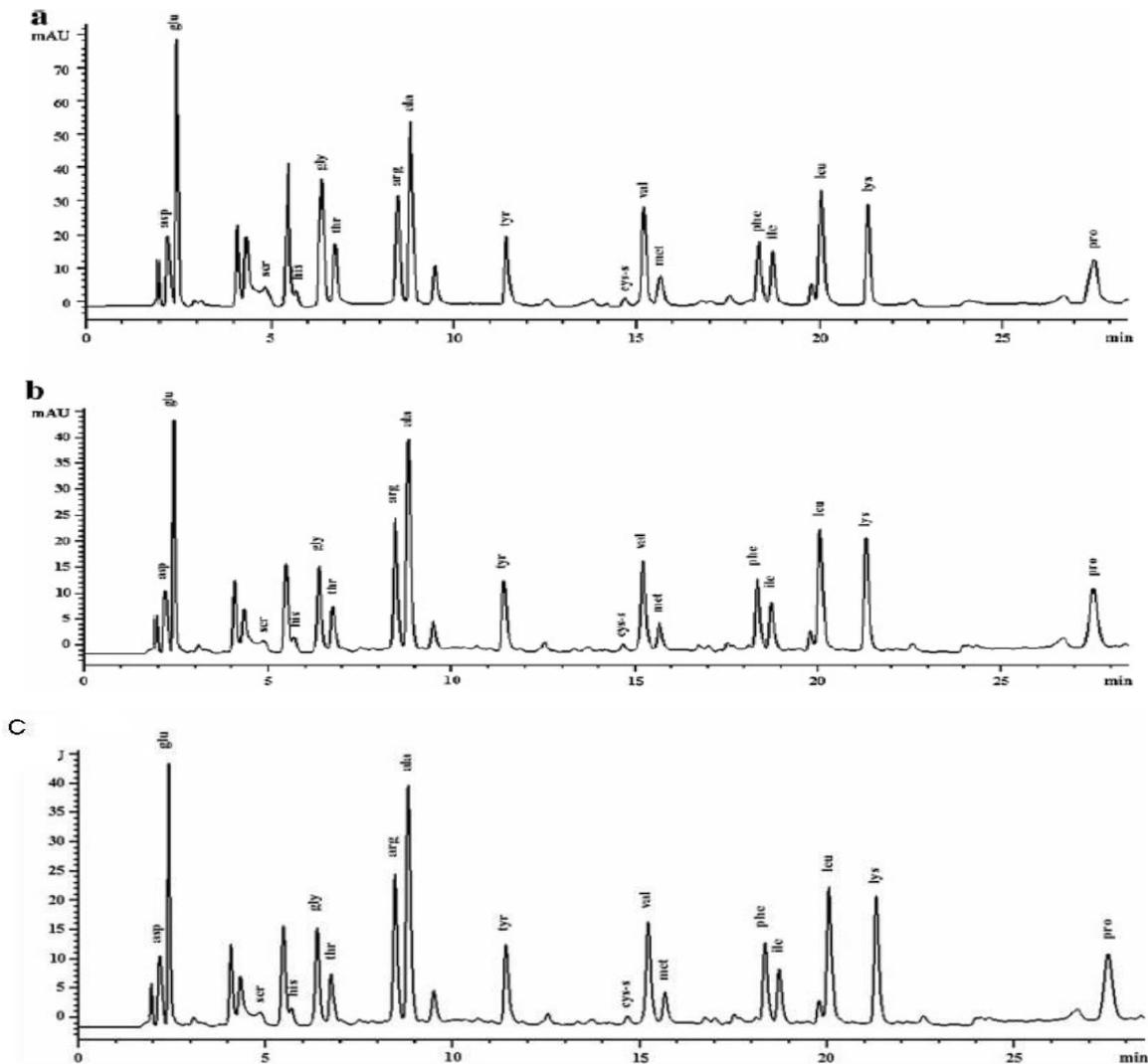


Figure 4: Amino acid Chromatogram profiles of (a) R.oryzae strain ATCC2809-processed rice wine mask, (b) R.oryzae strain F14-processed rice wine mask and (c) R.oryzae strain Q303-processed rice wine mask

Table 6: Contents of amino-acid in sweet fermented-glutinous made from different strains (g/100ml)

Test items	ATCC2809	F14	Q303
Asparagine (Asp)	0.2	0.14	0.18
glutamate(Glu)	0.32	0.19	0.28
Serine(Ser)	0.1	0.07	0.1
Arginine(Arg)	0.14	0.08	0.14
Glycine(Gly)	0.1	0.06	0.08
Threonine(Thr)	0.08	0.05	0.06
Proline(Pro)	0.12	0.12	0.12
Alanine(Ala)	0.13	0.07	0.1
Valine(Val)	0.1	0.06	0.08
Methionine (Met)	<0.01	0.01	<0.01
Isoleucine(Ile)	0.07	0.04	0.05
Leucine(Leu)	0.12	0.08	0.1
Phenylalanine(Phe)	0.08	0.06	0.08
Histidine (His)	0.04	0.04	0.04
Lysine (Lys)	0.08	0.05	0.07
Tyrosine (TYR)	0.01	0.01	0.01
Cysteine(CYS)	<0.01	0.01	0.01
Tryptophan (TRP)	<0.01	<0.01	<0.01
Total	1.7	1.1	1.5

The amino acid is very important in sensory science judgement affected the quality and flavory of rice wine. Amino acids are found in wine (Kato et al. 2011), and take great effect to the flavor of the rice wine. We reported that rice wine contain the amino acid forms of Ala, Asn, Asp, Arg, Glu, Gln, His, Ile, Leu, Lys, Ser, Tyr, Val, Phe and Pro; These amino acids were found at concentration in the rang of 0.07-0.13 g/100ml (Ala), 0.14-0.20 g/100ml(Asp), 0.19-0.32 g/100ml, the three amino acid is the mainly flavor matter in the rice wine (Xu, Y., et al, 2010) . From above table, we can see clearly that between the three strains R. oryzae ATCC2809 is highest between the three strains.

These amino acid increased the sensory score and produced a strong taste (Nojun) (K.Okada et al.2013) . When Ala is relatively higher in the wine, the value for the umami taste in the sensory evaluation increased, without effect on the aroma of the wine at all. (K.Okada et al. 2013)

4. Conclusion

According to the result of the study, The trace elements of P(phosphorus) and Mg(manganese) have especially significant effect on *R. Oryzae* ATCC2809 growth, The optimal culture medium for conserving ATCC2809 is PDA, whereas Czapek's bran medium is the premium medium used as culture medium for the rejuvenation of *Rhizopus Oryzae* ATCC2809. The most premium temperature is at 32 C. The fermented wine using *R. oryzae* ATCC2809 contains 18 amino-acid, methionine and tryptophan's contents are less, whereas rich content in asparagine and glutamine, which helps getting a distinct flavor. Contrasting with two traditional strains of F14 and Q303, the sweet fermented-glutinous of ATCC2809 got the highest content of total amino acids and 8 essential amino acids, reached 1.7g/100ml and 0.53g/100ml. To satisfy the human body's requirement in essential amino acid is important. Strain ATCC2809 has a potential for commercial amino acid production. Improvement of microbial strains for the overproduction of industrial products has been the hallmark of all commercial fermentation processes. Such improved strains can reduce the cost of process with increased productivity and may process some specialized desirable characteristics.

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Author Profile

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