Investigation Of Saccharomyces Cerevisiae In Fermented Mulberry Juice

Nguyen Phuoc Minh, Dong Thi Anh Dao

Abstract: Mulberry is grown worldwide. Its leaves are used to feed the silkworms which in turn produce silk fiber. Sericulture is mostly practiced in China, India and Japan. In the rest of the world, mulberry is generally used as forage in animal production, or for other purposes. Besides using the leaves, mulberry bears sweet fruit. The full –bodied flavor of this fruit is a good balance of sweetness and tartness with nutrient elements of vital importance for human metabolism. If these fruits are industrially exploited for various commercially valuable products, mulberry can become an important crop throughout the world. Mulberries are good for health because of their vitamins, minerals and bioactive compounds. In addition, high carotene and organic acids content help to increase the body's resistance. Mulberry can be used for making jam, jelly, pulp, fruit drink, fruit sauce, cake, fruit tea, fruit powder, fruit wine, food colorant, diabetes control agent and as ruminant livestock feed. In order to find the optimal conditions for the fermentation process, the juice was inoculated with *Saccharomyces cerevisiae* isolated from mulberry in various conditions. The results were as follows: inoculum volume 9 %v/v (inoculum concentration 10^7 yeast cells/mL), initial mulberry juice with 24^oBx and pH 3.5, fermentation temperature 18÷20^oC. Fermentation was carried out for 48h and the ethanol content of product was 5 %v/v.

Keywords: Mulberry juice, *Saccharomyces cerevisiae,* optimal condition, fermentation, inoculum volume, temperature, ethanol

1. INTRODUCTION

Ripe mulberry ripe is edible and is widely used in pies, tarts, wines, cordials and tea. The fruit of the black mulberry, native to southwest Asia, and the red mulberry, native to eastern North America, have the strongest flavor. The fruit of the white mulberry, an east Asian species which is extensively naturalized in urban regions of eastern North America, has a different flavor, sometimes characterized as insipid. The mature plant contains significant amounts of resveratrol, particularly in stem bark. The fruit and leaves are sold in various forms as nutritional supplements. Unripe fruit and green parts of the plant have a white sap that may be toxic, stimulating, or mildly hallucinogenic. Three species have been recognized for their economic importance.

- The white mulberry (*Morus alba L*) is native to eastern and central China.
- The red or American mulberry (*Morus rubra L*) is native to eastern United States.
- Black mulberry *(Morus nigra L*) is native to western Asia.

Mulberries are large, deciduous trees native to warm, temperate, and subtropical regions of Asia, Africa, and the Americas. Technically, the mulberry fruit is an aggregation of small fruits arranged longitudinally around the central axis as in blackberry or loganberries. Each fruit measures 2-5cm long. In most species, these berries are purple-red when ripen; however, they can be white, red, purple or multiple colors in the same fruit.

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Table 1. Chemical composition of mulberry fruit (Singhal et al. , 2009) [6]

Chemical constituents	Quantity
Carbohydrates	7.8–9.0%
Protein	0.5–1.4%
Fatty acids (linoleic, stearic and oleic acids in seeds)	0.3–0.5%
Free acid (mainly malic acid)	1.1–1.8%
Fiber	0.9–1.3%
Ash	0.8–1.0%
Moisture	85–88%
Calcium	0.17–0.39%
Potassium	1.00–1.49%
Magnesium	0.09–0.10%
Sodium	0.01–0.02%
Phosphorus	0.18–0.21%
Sulphur	0.05–0.06%
Iron	0.17–0.19%
Carotene	0.16–0.17%
Ascorbic acid	11.0–12.5 mg/100 g
Nicotinic acid	0.7–0.8 mg/100 g
Thiamine	7.0–9.0 µg/100 g
Riboflavin	165–179 µg/100 g

Health benefits of mulberries [7]:

- Delicious, fleshy, succulent mulberries are low in calories (just 43 calories per 100 g). They contain health promoting phyto-nutrient compounds like polyphenol pigment antioxidants, minerals and vitamins that are essential for optimum health.
- Mulberries have significantly high amounts of phenolic flavonoid phytochemicals called anthocyanins. Scientific studies have shown that consumption of berries have potential health effects against cancer, aging and neurological diseases, inflammation, diabetes, and bacterial infections.
- The berries contain resveratrol, another polyphenol flavonoid antioxidant. Resveratrol protects against stroke risk by altering molecular mechanisms in the blood vessels; reducing their susceptibility to damage through decreased activity of angiotensin (a systemic hormone causing blood vessel constriction that would elevate blood pressure) and increased production of the vasodilator hormone, *nitric oxide.*
- In addition, these berries are an excellent source of vitamin-C (36.4 mg per 100, about 61% of RDI), which is also a powerful natural antioxidant. Consumption of foods rich in vitamin-C helps the body develop resistance against infectious agents, counter inflammation and scavenge harmful free radicals.
- Further, the berries also contain small amounts of vitamin A, vitamin E and in addition to the above-mentioned antioxidants. Consumption of mulberry provides another group of health promoting flavonoid polyphenolic antioxidants such as *lutein, zea-xanthin, ß-carotene and α-carotene* in small but notably significant amounts. Altogether, these compounds help act as protect from harmful effects of oxygen-derived free radicals and reactive oxygen species (ROS) that play a role in aging and various disease processes.
- Zea-xanthin, an important dietary carotenoid selectively concentrates into the retinal macula lutea, where it thought to provide antioxidant functions and protects the retina from the harmful ultraviolet rays through light-filtering actions.
- Mulberries are an excellent source of iron, which is a rare feature among berries. Iron, being a component of hemoglobin inside the red blood cells, determines the oxygen-carrying capacity of the blood.
- They also good source of minerals like potassium, manganese, and magnesium. Potassium is an important component of cell and body fluids that helps controlling heart rate and blood pressure. Manganese is used by the body as a co-factor for the antioxidant enzyme, *superoxide dismutase.*
- They are rich in B-complex group of vitamins and vitamin K. Contain very good amounts of vitamin B-6, niacin, riboflavin and folic acid. These vitamins are function as co-factors and help body in the metabolism of carbohydrates, proteins and fats.

In recent years, with considerable work on cultivating mulberry plants under various conditions, mulberry fruit juice has been commercially produced as a health beverage, and it has become very popular in China, Japan and Korea. Without adding preservatives, the original juice of mulberry fruit remains fresh under cold storage for 3 months, while the bottled beverage remains fresh atroom temperature for about 12 months (Dharmananda, 2008). From one acre of mulberry tree cultivation; which has a spacing of 8 x 9' between tree to tree and row to row, about 1993 kg of fruit jam and 2794 liters of fruit pulp can be prepared in sub tropical India. The market survey revealed an income of 1063.72 US $ and 1161.70 US $ from the sale of jam and pulp, respectively in Indian markets (Singhal et al., 2009) [6]. Guo Xia Sun et al. (2013) studied the fermentation process to optimize of mulberry black tea by RSM. Mulberry leaves contained flavonoids, alkaloids and polysaccharides components, which were a dual-purpose scarce resource with drug and food. Fermentation process factors of mulberry black tea were optimized by response surface methodology (RSM). The results indicated that the optimal parameters of fermentation process were as follows: fermentation time 5 h, fermentation temperature 30 °C and amounts of spore suspension (SS) 0.16 %. Compared with mulberry fresh leaf, total flavonoids, total polysaccharides, polyphenols, free amino acids, rutin, quercetin, and 1-DNJ of mulberry black tea processed under optimal conditions increased by 3.06 %, 17.13 %, 33.07 %, 86.46 %, 37.5 % and 6.86 %, respectively [4]. Elisa Alonso Gonzalez et al. (2010) conducted the production and characterization of distilled alcoholic beverages obtained by solid-State fermentation of black mulberry (Morus nigra L.) and black currant (Ribes nigrum L.) to appraise the potential of black mulberry and black currant to be used as fermentation substrates for producing alcoholic beverages obtained by distillation of the fruits previously fermented with Sacchromyces cerevisiae IFI83. In the two distillates obtained, the volatile compounds that can pose health hazards are within the limits of acceptability fixed by the European Council (Regulation 110/2008) for fruit spirits. However, the amount of volatile substances in the black currant distillate (121.1 g/hL absolute alcohol (aa)) was lower than the minimum limit (200 g/hL aa) fixed by the aforementioned regulation [5]. In Chinese markets, mulberry is often provided in the form of a paste called sangshengao. The paste is mixed in hot water to make a tea to improve the liver and kidney and sharpen the hearing and brighten the eyes. For this application, it is combined with the traditional formula Yiqi Congming Tang, which is used for deficiencies in hearing and vision. In Iran, dried mulberries are used as a sweetener in black tea. After a sip of tea, dried mulberry fruits are eaten to sweeten the mouth [6]. The sole use of mulberries in modern medicine is for the preparation of syrup; to add flavors and natural colour in medicines (Singhal et al., 2001, 2003). The mulberry fruit is used for many medical purposes such as for balancing internal secretions and enhancing immunity (Kim and Bonchohak, 1991, Venkatesh Kumar and Chauhan, 2008). It is used to treat urinary incontinence, tinnitus, dizziness, constipation, sore throat, depression and fever. The fruits of M. alba have a cooling and laxative property and are used in throat infection, dyspepsia and melancholia (Jain and De Filipps, 1991). The juice which is refrigerant is used as a drink in febrile diseases. It checks thirst and cools the blood. The fruit juice is commonly used for reducing high fever as febrifuge. This is the first treatment normally given to any patient with symptoms of fever during endemic malaria (Shivakumar et al., 1995). It is mentioned by Singh (1997) that s yrups and recipes prepared from fruits of M. alba are used against hyperlipemia , constipation and insomnia, antiaging, premonitory and apoplexy. Ripened fruit works as a good appetizer and are carminative. Fruits are also used for loss of appetite, flatulence and for controlling intestinal parasites like tapeworm. Fruits can nourish and promote the production of body fluid. As juice has a faint scent and sweet taste, it is suitable for people of all ages [6]. Isolation of lactic acid bacteria (LAB) from fruits and vegetables have frequently been reported [9, 10, 11, 12, 13, 14]. Notablty, Yi-sheng Chen (2010) conducted research to solate, characterize, and identify lactic acid bacteria (LAB) from ripe mulberries collected in Taiwan. Ripe mulberry samples were collected at five mulberry farms, located in different counties of Taiwan. Eighty-eight acid-producing cultures were isolated from these samples, and isolates were divided into classes first by phenotype, then into groups by restriction fragment length polymorphism (RFLP) analysis and sequencing of 16S ribosomal DNA (rDNA). Phenotypic and biochemical characteristics led to identification of four bacterial groups (A to D). *Weissella cibaria* was the most abundant type of LAB distributed in four mulberry farms, and *Lactobacillus plantarum* was the most abundant LAB found in the remaining farm. Ten *W. cibaria* and one *Lactococcus lactis* subsp. *lactis* isolate produced bacteriocins against the indicator strain*Lactobacillus sakei* JCM 1157^T [8]. On the purpose of diversifying mulberry products on the Vietnam market, this research conduct the juice fermentation using *Saccharomyces cerevisae* isolated from mulberry fruits to produce a new alcoholic beverage.

2. MATERIAL AND METHODS

2.1 Classification

Kingdom: Plantae
- Subkingdom: Tracheobionta
  - Supdivision: Spermatophyta
    - Division: Magnoliophyta
      - Class: Magnoliopsida
        - Subclass: Dilleniidae
          - Order: Urticales
            - Family: Moraceae
              - Genus: Morus
                - Species: Morus alba L.

2.2 Raw material

Mulberry fruits are purchased from Da Lat (Lam Dong) at normal ripen stage, soft, intact, specific flavor. Reagent Pectinex Ultra SP-L in liquid status is provided from Novo-Nordisk (Denmark), its activity is 26000 PG/mL.

Figure 1. Mulberry fruit

2.3 Researching method

Protocol of preparation
Mulberry → separation → washing → grinding → incubation with enzyme → extraction → mulberry extract.
Incubation is conducted at 43°C in 4h and added enzyme pectinase with ratio 0.04 mL/100 g pulp.

Isolating and identifying microorganism
Fresh mulberry → propagation → cell extraction → propagation shaking → dilution with Nacl solution → isolation → tameness → species preservation → identification.

Protocol of fermentation
Mulberry extract → fitration → mixing → fermentation → stability → fine filtration → bottle → heating → alcoholic beverage.
Fermentation period is carried out 2÷5 days with the present of *Saccharomyces cerevisae* isolated from mulberry.

2.4 Analytical method
Identify microorganism by gen 28S sequencing.
Determine total yeast by direct counting (ISO6611-1:1992)
Determine ethanol by distillation (TCVN 1273: 1986)
Determin total sugar (TCVN 6958 : 2001)
Determine total acid by titration (ISO 6092: 1980)
Sensory evaluation by giving mark [1].

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of microorganism from mublberry fruit
On our study, we isolate and identify that microorganism species on mulberry fruit is *Saccharomyces cerevisiae*. This is a common yeast popularly used in manufacturing alcoholic beverage. So in our scope, we use this yeast for fermentation.

Figure 2. *Saccharomyces cerevisiae* yeast identification on mulberry.

3.2 Effect of yeast ratio to fermentation

In fermentation, ratio of initial yeast strongly affects to ethanol recovery. Muberry extract is adjusted to 24°Bx, pH 3.5; and vary yeast ratio 3%, 5%, 7%, 9%, 11% to fermenting volume, yeast population 10^7 cells/ml, temperature 28÷30°C in 48 hours. After 24 hour fermentation, at yeast ratio 9% accumulation of ethanol is highest, dry matter is reduced strongest and yeast density is crowded highest. After 2 day fermentation, ethanol concentration in 5 samples are over 5 %v/v. This means at the yeast ratio 3÷11% the fermentation process proceed efficiently. If we add too much yeast, the fermentation slow down negatively.

Table 2. Effect of yeast ratio (%v/v) to pH and ethanol formation (%v/v) during fermentation

Time (h)	Yeast ratio (%v/v)									
	3	5	7	9	11	3	5	7	9	11
	pH					Ethanol (%v/v)				
24	3.45	3.42	3.41	3.34	3.46	0.43	0.43	0.43	1.43	0.43
48	3.32	3.31	3.35	3.14	3.25	6.09	7.11	6.09	7.11	6.09

Table 3. Effect of yeast ratio (%v/v) total acidity (g/L) and total sugar (g/L) during fermentation

Time (h)	Yeast ratio (%v/v)									
	3	5	7	9	11	3	5	7	9	11
	Total acidity (g/L)					Total sugae (g/L)				
24	8.53	9.60	9.60	9.60	9.60	104.00	94.54	99.05	94.54	109.47
48	11.73	13.87	12.80	17.07	14.93	57.78	54.74	61.18	52.00	57.78

We monitor ethanol, pH, total acidity, total sugar and draw out a decision to select yeast ratio 9% (density 10^7 yeasts/ml) to further experiements.

3.3 Effect of initial dry matter

Mulberry extract has a low dry matter 4.0÷4.5^{o}Bx so it's not enough substrate for yeast growth. We adjust the initial dry matter to 22^{o}Bx, 24^{o}Bx, 26^{o}Bx by sucrose.

Table 4. Effect of initial dry matter (^{o}Bx) to pH, total acidity (g/L) and total sugar (g/L) during fermentation

Time (h)	Initial dry matter (^{o}Bx)								
	22	24	26	22	24	26	22	24	26
	pH			Total acidity (g/L)			Total sugar (g/L)		
24	3.97	3.95	3.88	6.40	6.83	5.97	86.67	98.11	120.93
48	3.84	3.80	3.82	8.96	10.67	9.81	67.09	73.24	83.20
72	3.64	3.55	3.62	13.44	14.72	12.80	57.14	61.91	65.82

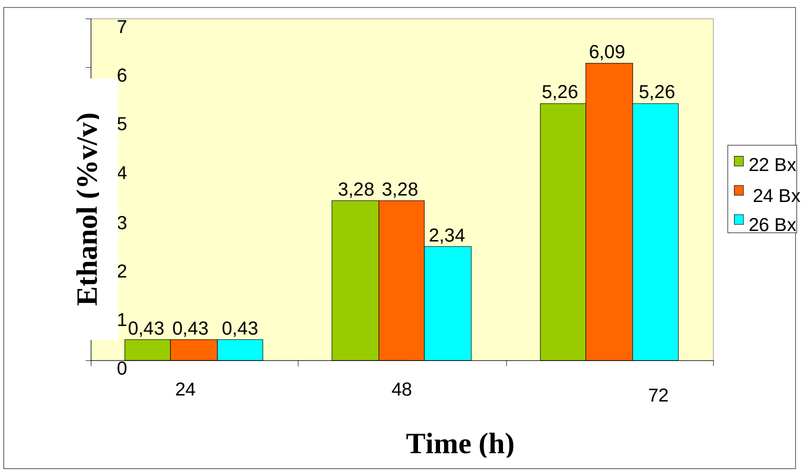

Figure 3. Effect of initial dry matter to ethanol formation during fermentation

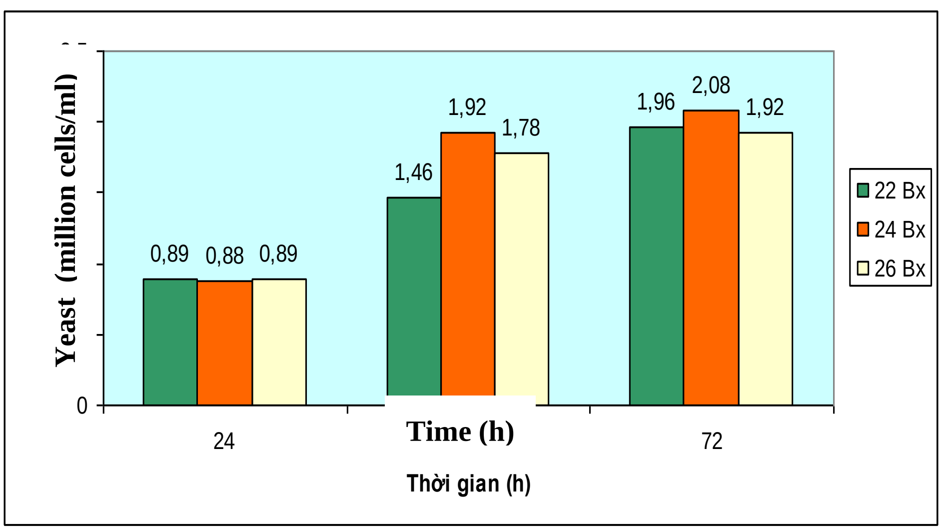

Figure 4. Effect of initial dry matter to biomass formation during fermentation

We demonstrate that at dry matter 24^{o}Bx we receive the highest yeast 1.92 x 10^{9} cells/mL and ethanol formation higher than two other samples. Se we decide to use dyr matter 24^{o}Bx for further experiments.

3.4 Effect of pH in fermentation

pH strongly affect to fermentatioin. pH can inhibit harmful bacteria and create acidity for final product. To survey the effect of pH to fermentation, we use acid citric and Na_2CO_3 to adjust pH value in solution. pH is adjusted at various levels 3.0; 3.5; 4.0.

Table 5. Effect of initial pH to change of pH, total acidity (g/L) and total sugar (g/L) during fermentation period

Time (h)	Initial pH								
	3.0	3.5	4.0	3.0	3.5	4.0	3.0	3.5	4.0
	pH			Total acidity (g/L)			Total sugar (g/L)		
24	2.99	3.43	3.92	7.45	8.53	7.68	106.10	102.00	104.00
48	2.80	3.34	3.54	11.09	13.23	11.52	69.33	61.18	71.23
72	2.68	3.11	3.39	15.79	17.71	16.64	57.78	50.98	56.52

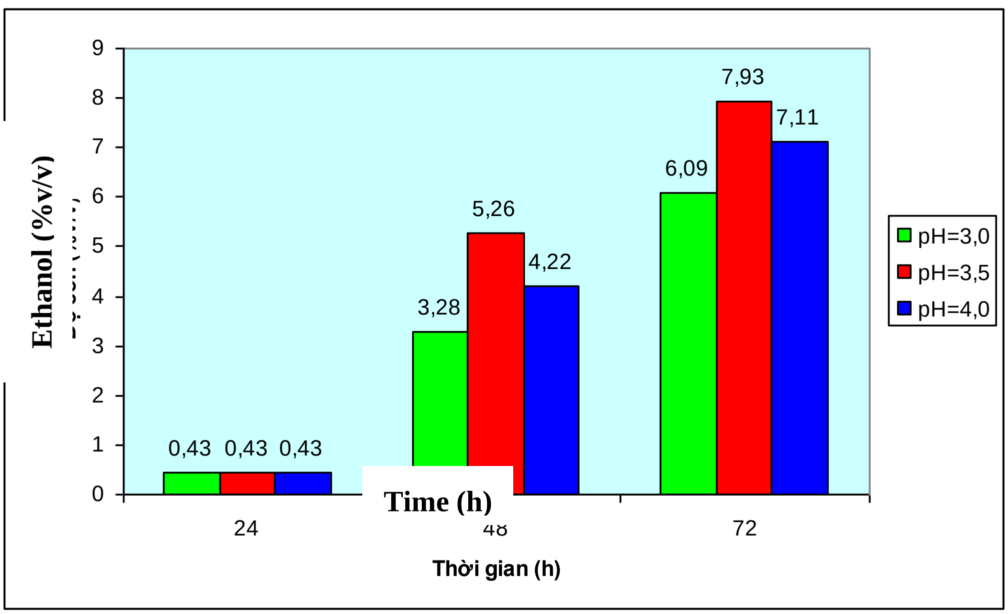

Figure 5. Effect of initial pH to ethanol formation in fermentation

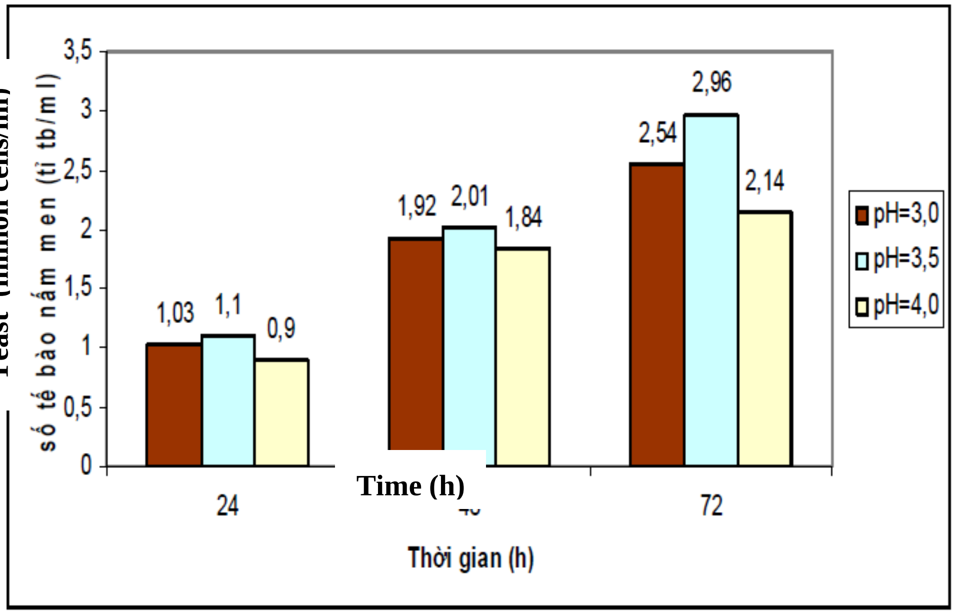

Figure 6. Effect of initial pH to biomass formation in fermentation

We conclude that ethanol and biomass formation in sanple having pH 3.5 increase dramatically and even higher than two other samples so this demonstrates pH 3.5 is an adequate environment for yeast growing. Yeasts consume sugar to develop biomass, reduce pH fermentation batch [1]. So we choose pH 3.5 for further experiments.

3.5 Effect of temperature in fermentation

Temperature of fermentation plays an important role to fermenting speed and product quality. In this experiment, we survey two ranges of temperature: 18÷20°C, cool temperature in Lam Dong province and 28÷30°C normal temperature in the South of Vietnam.

Table 6. Effect of fermenting temperature to change of pH, total acidity (g/L) and total sugar (g/L) during fermentation period

Time (h)	Temperature (°C)					
	18÷20	28÷30	18÷20	28÷30	18÷20	28÷30
	pH		Total acidity (g/L)		Total sugar (g/L)	
24	3.47	3.34	8.11	9.60	98.11	94.54
48	3.32	3.14	14.08	17.07	61.18	52.53

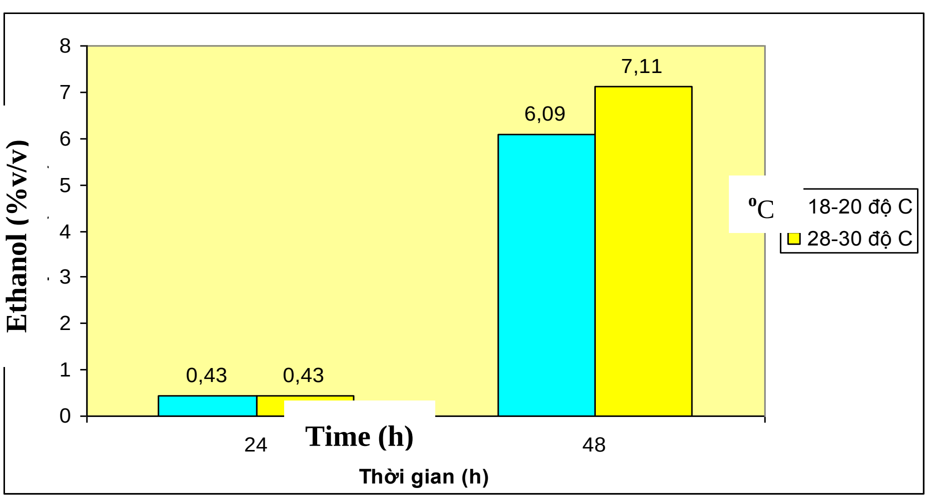

Figure 7. Effect of temprature to ethanol formation in fermentation

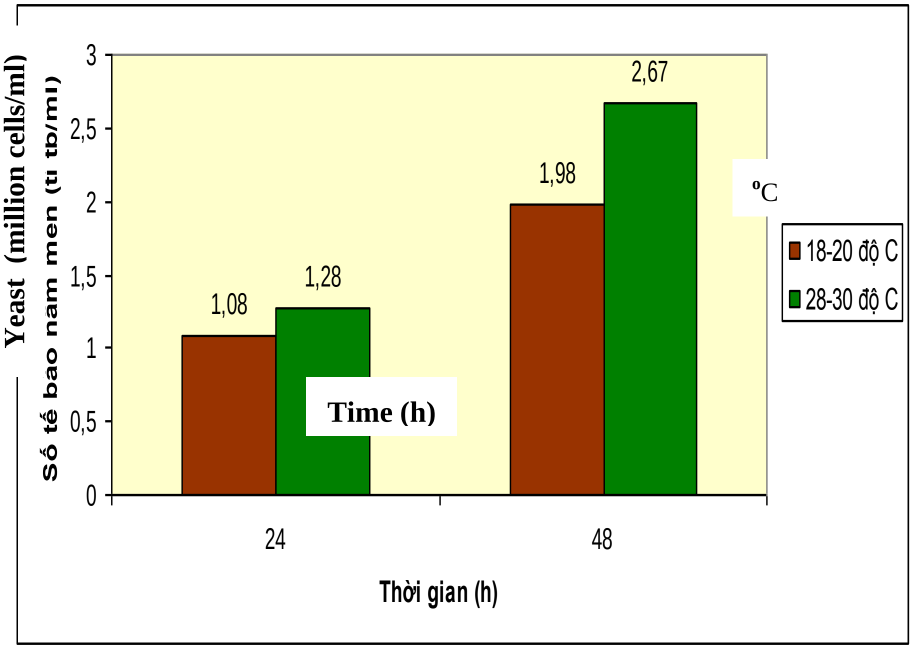

Figure 8. Effect of temprature to biomass formation in fermentation

Ethanol volume in final product fermented at 28÷30°C is higher than ethanol volume in final product fermented at 18-20°C. However, we see that higher temperature the less flavor. So fermention in low temperature prefers to high one [2, 3]. We primarily evaluate the sensory of product fermented at low temperature (18÷20°C), it shows better color, good flavor and taste. So we select temperature là 18÷20°C for further experiment, and fortunately this is also the specific temperature of raw material area (Lam Dong province, Vietnam).

4. CONCLUSION

Alcoholic beverage fermented from *Saccharomyces cerevisae* isolated from mulberry fruit can be valued as a potential trend and applicable to industrial scale owing to simple processing procedure and short-term production (48h).

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