

An Exploration of Parameters of The Fermentation Process of Honey Riched in Gluconic Acid – Oriented in Cosmetics Applications

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Abstract: Honey was fermented by *Gluconacetobacter xylinus* for the purpose of obtaining honey that is rich in gluconic acid, oriented in cosmetic applications. We study parameters of honey fermentation process, which resulted in the following factors: The dilution ratio of honey and old coconut milk was 1: 4, the proportion of *Gluconacetobacter xylinus* bacteria at the beginning was 4%, the fermented pH was 4.5, the fermentation was set at 30°C and the duration lasted for 5 days. From there, we study the changes in the fermentation process on the BioFlo fermenter system. The gluconic acid value reached its maximum when fermented on the BioFlo fermenter system at 128.91 mg / L.

Keywords: honey, fermentation, gluconic acid, cosmetic applications, *Gluconacetobacter xylinus*.

I. Introduction

Honey is a natural sweet substance produced by honey bees from the nectar of plants or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature [1].

Gluconic acid is a component of polyhydroxy acids (PHAs) [2]. Gluconic acid is also an important ingredient in many cosmetic products today, loosening the bonds between dead corneum cells, thereby peeling them off the skin surface (exfoliating), stimulate collagen production to regenerate and increase elasticity, reduce wrinkles and give the skin a smoothed and younger appearance [3]. The market is now moving towards natural cosmetic products and replacing the addition of gluconic acid to the product by using gluconic acid fermentation products. Fermented honey riched in gluconic acid is a cosmetic product for the purpose of makeup removal (gluconic acid) and skin care that exploit the nutrients found in honey and products formed during the honey fermentation process [4].

Currently, skin care products from fermented honey have been presented in several countries around the world such as Japan and Korea which are trustingly used by many people. However, this product line from fermented honey is not available in Vietnam yet. In addition, no article has been published to inform people about the parameters and the honey fermentation process to create this product line of cosmetics. Therefore, in this study, we conducted a survey of parameters of the honey fermentation process with gluconic acid as the objective function.

II. Materials And Methodology

2.1 Materials, chemicals, research medium

Source of microorganism strain: The microorganism strains which have the ability of gluconic acid biosynthesis used in this study were selected in the variety collection of Department of Biotechnology, Ho Chi Minh City University of Technology includes: *Gluconacetobacter aceti*, *Gluconacetobacter xylinus*, *Gluconacetobacter intermedius*, *Gluconacetobacter xylinum*.

Materials: This study used Vina Ong honey, a product of Vina Ong Joint Stock Company, lot B2-28, road no. 04, Industrial Zone Tan Dong Hiep B, Tan Dong Hiep Ward, Di An, Binh Duong.

Medium: The components of the old coconut juice environment used for bacteria strains keeping and propagation were as follows: Old coconut juice: 1 liter, glucose: 20g, (NH₄)₂SO₄: 8g, (NH₄)₂HPO₄: 2g, peptone: 5g, yeast glue: 5g, agar: 20g, acetic acid: 5ml.

2.2 Methodology

2.2.1 Prior experiments

- Breeding

Fermented four strains of *Gluconacetobacter* sp in a medium of diluted honey, in which there is the homogeneity of cell density, nutrient content, and fermentation conditions. After 7 days of fermentation, collected the fermented liquid, centrifuged and measured the content of gluconic acid. Selected high-gluconic

acid biosynthetic breeds for the following steps of the experiment, conducted a thorough and microscopic observation of selected strains.

- Researched the biological characteristics of the selected breeds

Used the technique of streaking the selected breed of bacteria in the nutrient agar plates, and combined with brewing at room temperature for 48 hours to observe the bacteria colony's characteristics. Performed bacteria's biomass Gram dying after 48 hours of culturing in the agar plate medium to observe bacterial cells' characteristics under optical microscope in 100x optical glass lens which has glass oil.

- Constructed the growth curve of the selected strain

The growth curve of the bacteria is based on the cell density at each time point. The experiment was limited to different timelines, with 3 replicates.

- The main components of honey

Evaluated some basic criteria of the original honey materials of Vina Ong to compare with the honey after fermentation, used the enzyme method to quantify gluconic acid and the acid-dinitro-salicylic (DNS) method to quantify inverted sugar.

2.2.2 *Researching the fermentation process*

There are 5 factors affecting the ability to produce gluconic acid in the medium of honey diluted with old coconut juice: the ratio of honey diluted with old coconut juice, the rate of the initial bacteria strain, the initial pH, fermentation temperature, and fermentation duration. All of these factors were researched sequentially in the condition of static fermentation. The results of the preceding survey will be the premise for the following factor survey. The scope of the survey is shown in Table 1.

Table 1: Scope of research factors on the ability of achieving gluconic acid

Research factors	Ratio of honey diluted with old coconut juice	Ratio of the initial bacteria strain (%)	Initial pH	Fermentation temperature	Fermentation duration (days)
Research scope	1:2	2	4	25	3
	1:4	4	4.5	2.5	4
	1:6	6	5	30	5
	1:8	8	5.5	32.5	6
	1:10		6	35	

2.2.3 *Researching the changes during the fermentation on the BioFlo fermenter system*

The selected bacteria were fermented in the BIO FLO 110 fermentation system with a volume of honey and of fermented coconut water by 1 liter. The initial rate of bacteria was prepared in the incubator was 4% before being injected into the fermenter. The fermentation conditions on fermenter BIO FLO 110 was at 100 rpm, temperature 30°C.

We study the changes in the fermentation time from 1 to 7 days, sampling 12 hours apart.

2.2.4 *Method for quantitative determination of gluconic acid*

Principle of measurement: D-Gluconic acid (D-gluconate) is phosphorylated to D-gluconate-6-phosphate by ATP in the presence of the enzyme gluconate kinase with the simultaneous formation of ADP. In the reaction catalyzed by 6-PGDH, D-gluconate-6-phosphate is oxidatively decarboxylated by nicotinamideadenine dinucleotide phosphate (NADP) to ribulose-5- phosphate with the formation of reduced NADPH. The amount of NADPH formed in the above reaction is stoichiometrically related to the amount of D-gluconate. The increase in NADPH is measured at 340nm.

III. RESULTS AND DISCUSSION

3.1 *Prior experiments of selecting microorganism strain*

- Selection of strain

After 6 days of fermentation under the same fermentation condition, the gluconic acid content of each strain is shown in Chart 1 as follows:

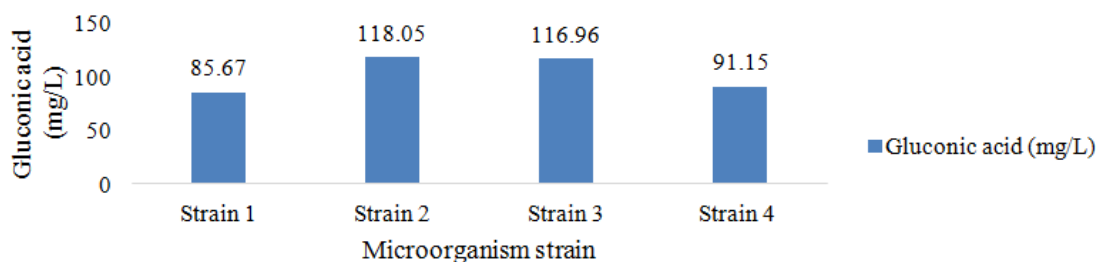


Chart 1: Gluconic acid content achieved from each strain

Chart 1 shows that in the same fermentation condition (honey dilution ratio with old coconut juice is 1:6, 6% breed rate, pH 4.5, 30°C), strain 2 (*Gluconacetobacter xylinus*) resulted in the highest gluconic acid in the four strains (118.05 mg/L). Meanwhile, the amount of acid obtained from the fermentation using the other three strains was much lower than that of strain 2. Therefore, *Gluconacetobacter xylinus* was selected as a microorganism variety for the next steps of the study.

- Researching the biological characteristics of the selected breed:

The colonies of *Gluconacetobacter xylinus* are milky white, thin, about 1 to 1.5 mm in size. *Gluconacetobacter xylinus* Gram-negative, straight, single or in pairs, forced aerobic, no cilia, no ability to mobile.

3.2 Researching results of factors affected the ability to produce gluconic acid.

The fermentation process of gluconic acid in the medium of honey diluted with old coconut juice was directly influenced by objective factors, which were presented in Table 1.

The results of the influence of the dilution ratio on the fermentation process are shown in Figure 2

- Influence of the dilution ratio of honey and old coconut juice to the ability to produce gluconic acid

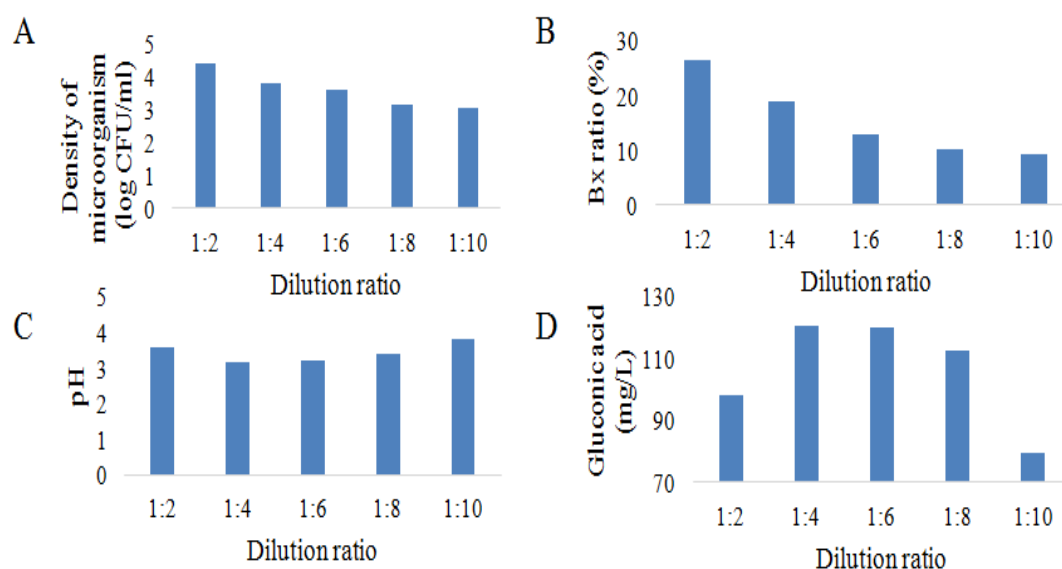


Chart 2: A, Density of microorganism (log CFU/ml); B, Brix rate (%); C, pH; D, Gluconic acid content (mg/L) of the fermented honey liquid at different dilution ratio of honey and old coconut juice.

The results show that at the initial dilution ratio between honey and coconut juice was 1: 4, the highest gluconic acid content was formed (120.54 mg / L). The content of gluconic acid produced when fermented at the lower dilution ratios is lower. Specifically, at a dilution ratio of 1: 2, the acid content was 98.05 mg / L. The cause of low acid content may due to the excessive dilution rate, excessive supply of glucose from honey compared to the growth and development needs of *Gluconacetobacter xylinus*. It should have inhibited the producing process of gluconic acid. In contrast, when the dilution ratio is higher than 1: 4, the amount of gluconic acid produced decreased. At a 1:10 dilution rate, the gluconic acid content was lowest (79.25 mg / L). The reason may be that this dilution does not provide enough nutrients for well-developed microorganisms.

The results were inversely proportional to the amount of gluconic acid obtained at different dilution ratios. This shows that the bigger amount of acid produced leads to the decrease of pH. Thus, in the 5 levels of dilution ratio between honey and old coconut juice examined, the 1: 4 dilution ratio is most suitable for honey fermentation because this dilution ratio produces bigger content of gluconic acid than the remaining ratios. Therefore, the 1: 4

dilution ratio was chosen as a fixed factor for the subsequent research experiments of factors affecting gluconic acid production.

- Influence of the initial breed ratio on the ability to produce gluconic acid

Fermentation breed rate plays an important role in the fermentation process, as this is a key factor in the efficiency of the fermentation. In addition, the rate of fermentation breed also determines the time of fermentation to be fast or slow. Research results are shown in Chart 3.

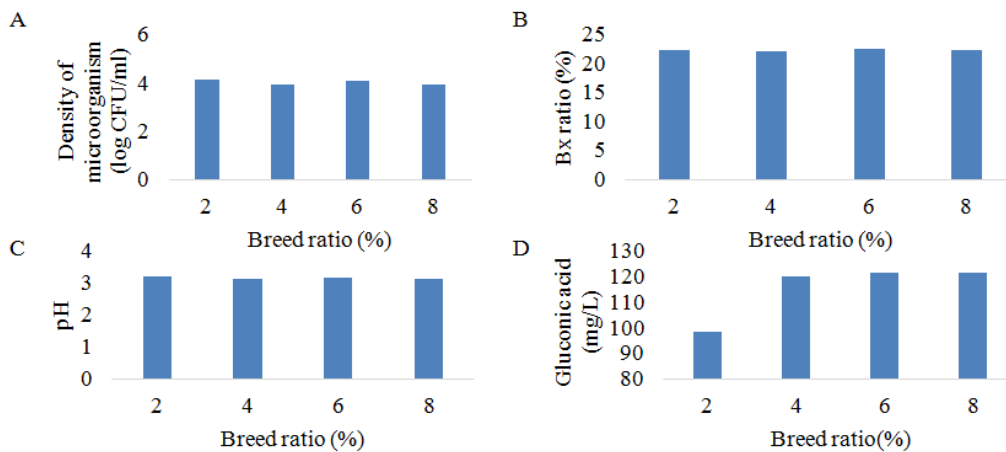


Chart 3:A, Density of microorganism (log CFU/ml); B, Brix rate (%); C, pH; D, Content of gluconic acid (mg/L) of fermented honey liquid at different initial breed ratios.

The results from chart 3 show that at the initial breed rate of 6%, the highest gluconic acid content was formed (121.66 mg / L). The level of gluconic acid produced when fermented at the lower breeding rates. At 2%, the acid content was 98.54 mg / L. The cause of low acid content may due to the low initial breed rate which caused not enough bacterial cells to perform the metabolism process, from which the acid production level is low. At the breed rate of 8%, the result was higher than the 2% breed rate but still lower than the 6% breed rate. The cause may be due to too much initial bacterial density which caused fermentation substrate does not meet their growth and development needs. After a certain fermentation time, they will use their own products to go through the catabolism process.

Although the amount of gluconic acid obtained at the initial breed rate of 6% was the highest, however, the comparison of the remaining factors, such as OD, pH, Brix between the breed ratios of 6% and 4% shows slight differences (chart 3 A, 3 B, 3 C). On the other hand, the 4% breed rate is not too high so it is economically significant. The 4% breed rate was appropriately selected for subsequent experiments.

- Influence of pH on the ability to produce gluconic acid

During the growing and development process, microorganisms are affected by many external factors, including pH. Each microbial species has a certain pH range and its growth, development depends largely on the pH of the habitat. Examination of the influence of pH on the ability to produce gluconic acid is necessary to determine the pH value at which the ability to form gluconic acid is most effective. The results of the pH research are shown in Figure 4.

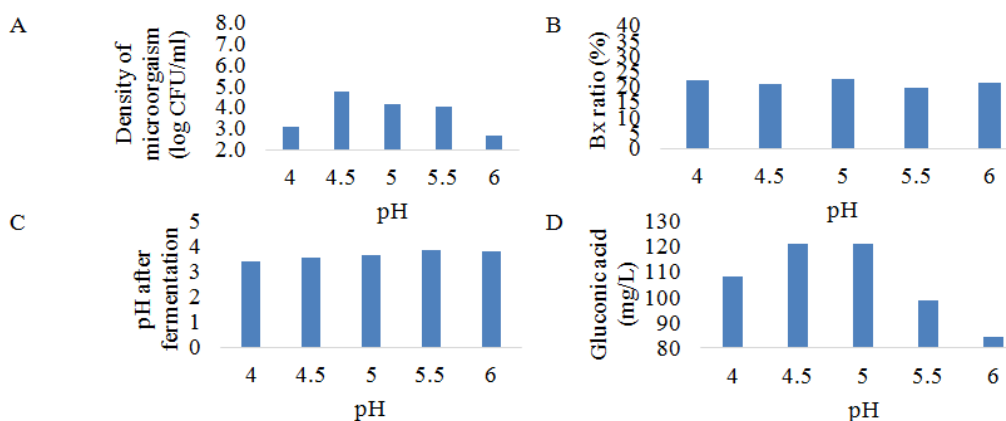


Chart 4: A, Density of microorganism (log CFU/ml); B, Brix rate (%); C, pH after fermentation; D, Content of gluconic acid (mg/L) of honey liquid fermented at different pH value.

The results from chart 4 indicate that pH 4-5 is the appropriate pH for the formation fermentation of the gluconic acid of honey because the amount of acid produced in this pH range is quite high compared to the amount of acid produced at the pH 6 value. Gluconic acid reached the highest value at pH 4.5 (121.48 mg / L) and *Gluconacetobacter xylinus* did not grow well when the pH exceeded 5. Thus, at pH 5.5, the gluconic acid content was not high (98.82 mg/L), the content of gluconic acid at pH 6 is lowest (84.25 mg / L). At the lower pH of 4, the content of acid produced was 108.5 mg / L, which is consistent with the study of Pederson and his partner in 1995. In their study, it was pointed out that acetic acid develops well at a low pH of 4 to 5. pH 4.5 is therefore chosen as a fixed element to continue serving the next steps of this research.

- Influence of temperature on the ability to produce gluconic acid

Fermentation temperature is an important factor affecting the growth and development of microorganisms. During the honey fermentation process, the fermentation temperature needs to be taken into consideration to determine the appropriate temperature limit for the development of the fermentation bacteria to obtain the highest gluconic acid content. The influence of temperature on the ability to produce gluconic acid is shown in chart 5.

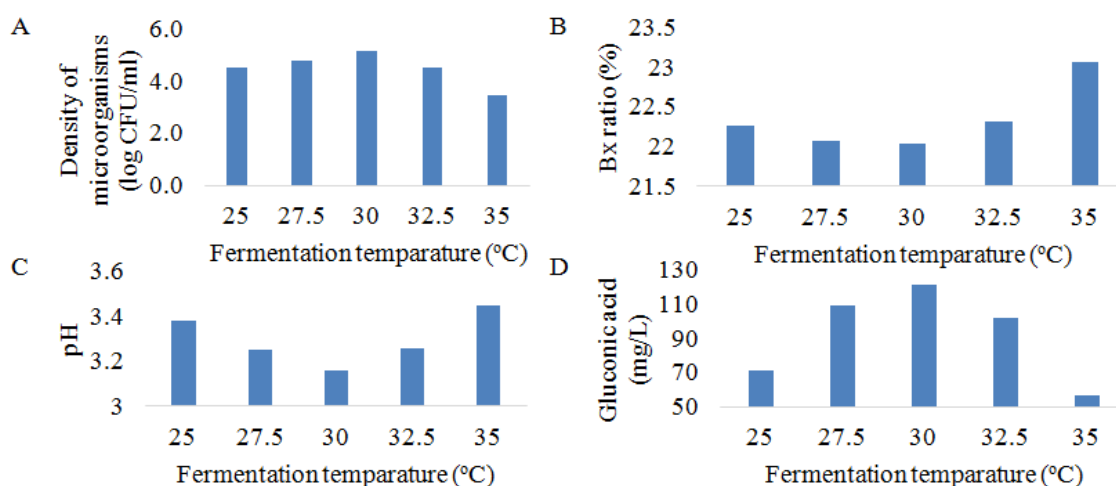


Chart 5: A, Density of microorganisms (log CFU/ml); B, Brix rate (%); C, pH; D, Content of gluconic acid (mg/L) of honey fermented at different fermentation temperatures.

According to Hestrin (1947), the appropriate growth temperature of *Gluconacetobacter xylinus* from 12°C to 35°C, they do not grow at elevated temperatures even in optimum nutrient medium [5]. The results showed that 25°C - 35°C was about the temperature range in which *Gluconacetobacter xylinus* could survive, indicating that gluconic acid was formed at all five research temperatures (25, 27.5, 30, 32.5, 35°C). Results from chart 5 show that *Gluconacetobacter xylinus* grows best at 30°C for the production of the highest gluconic acid content (121.47 mg / L). At temperatures lower than 30°C, the amount of gluconic acid produced is also lower (less than 110 mg / L). Higher temperatures than 30°C inhibit the growth and development of *Gluconacetobacter xylinus*, thus resulting in low levels of gluconic acid. At 35°C, the gluconic acid content was lowest (56.87 mg / L).

The temperature which produces the highest gluconic acid content selected in this survey is 30°C. This temperature is kept fixed to serve the subsequent steps in this research of factors affecting the ability to produce gluconic acid.

- Influence of fermentation duration on the ability to form gluconic acid

Fermentation duration is important, especially economically. If the fermentation time is too short, microorganisms do not have enough time to grow and develop, thus not creating products to be acquired. The longer the fermentation time will be, the longer it will affect the content of the product that is neither economically significant. In this study, the fermentation time study was conducted to select the appropriate fermentation time for honey fermentation to obtain the highest gluconic acid content.

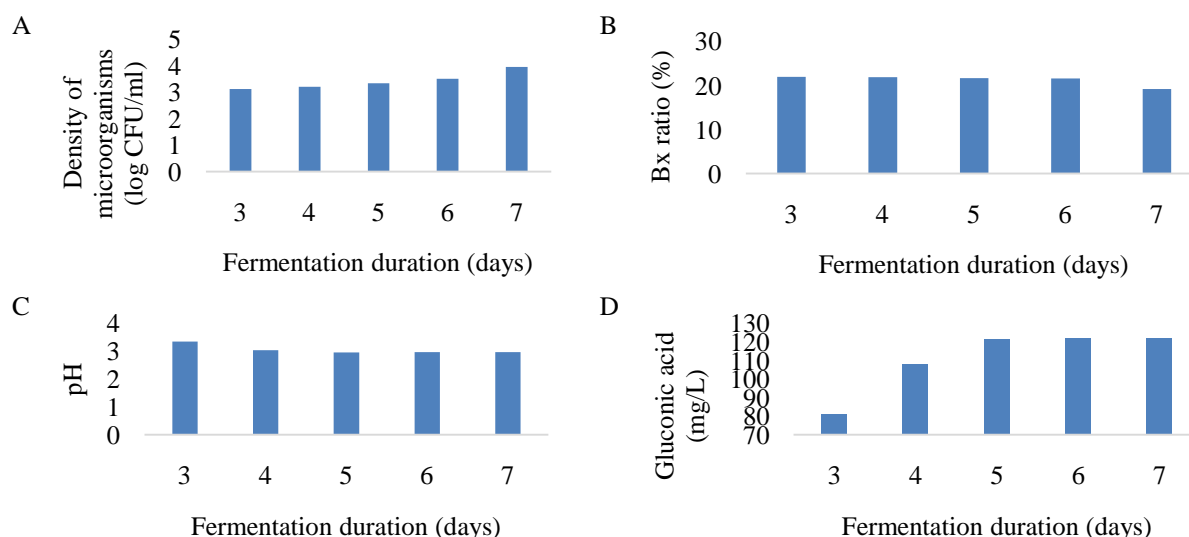


Chart 6: A, Density of Microorganisms (log CFU/ml); B, Brix rate (%); C, pH; D, Content of gluconic acid (mg/L) of honey liquid fermented in different fermentation durations.

Results from chart 6 show that after 3 days of fermentation, gluconic acid content has started to form (81.25 mg / L). Then, the amount of acid increased steadily and reached the highest value at the time of fermentation 6 days (121.90 mg / L). After 6 days of fermentation, particularly on the seventh fermentation day, gluconic acid content decreased but not significantly (121.88 mg / L).

Thus, the highest gluconic acid content was at 6 days fermentation time. However, when considering and comparing three pH, Bx and density of microorganisms, we found no significant difference between 5 and 6 days fermentation (Chart 6 A, 6 B 6 C). On the other hand, short fermentation time will reduce the costs as well as the risks that may occur during the fermentation process. Therefore, 5 days is the time chosen to be a fixed factor for the research of factors affecting the ability of gluconic acid formation.

Results of single factor affecting the ability to produce gluconic acid are shown in table 2.

Table 2: Results of single factor affecting the ability to produce gluconic acid

Factors	Appropriate point	Content of gluconic acid (mg/L)
Dilution ration of honey and old coconut juice	1:4	120.54
Initial bacteria breed ratio (%)	4%	120.09
Initial pH	4.5	121.48
Fermentation temperature (°C)	30°C	121.47
Fermentation duration (days)	5 days	121.51

3.3 Reseached changes in the fermentation process on BIO FLO fermenter system

Gluconacetobacter xylinus was fermented in a BIO FLO 110 fermentation system with a1 liter volume of fermentation medium. The initial breed ratio was stocked in an incubator following the ratio of 4% before being injected into the fermenter.

Fermentation conditions on fermentor BIO FLO 110 at 100 rpm, at 30°C.

The variations in OD, pH, Brix values measured over time are shown in Table 3.

Table 3: Changes in the fermentation process on the fermenter system

Fermentation time (hours)	Gluconic acid (mg/L)	pH	Brix Rate (%)	OD (600nm)	Density of microorganisms (log CFU/ml)	Content of inverted sugar (mg/mL)
0 (initially)	22.95	4.39	22.33	0.561	6.709	269.371
12	25.11	4.05	22.27	0.62	6.877	258.527
24	28.92	4.04	22.13	0.632	6.911	244.431
36	50.15	4.01	22.13	0.649	6.960	237.925
48	61.89	4.01	22.07	0.705	7.119	229.250
60	70.16	4.01	22	0.761	7.279	181.540
72	82.64	4.01	22	0.769	7.302	178.287

84	94.45	3.99	21.93	0.777	7.324	171.781
96	110.86	3.98	21.93	0.788	7.356	160.937
108	118.67	3.98	21.87	0.778	7.327	157.684
120	125.87	3.96	21.73	0.771	7.307	149.010
132	126.91	3.86	21.67	0.788	7.356	147.925
144	128.90	3.87	21.60	0.784	7.344	145.757
156	128.91	3.86	21.53	0.772	7.310	144.672
168	128.85	3.87	19.2	0.774	7.316	141.419

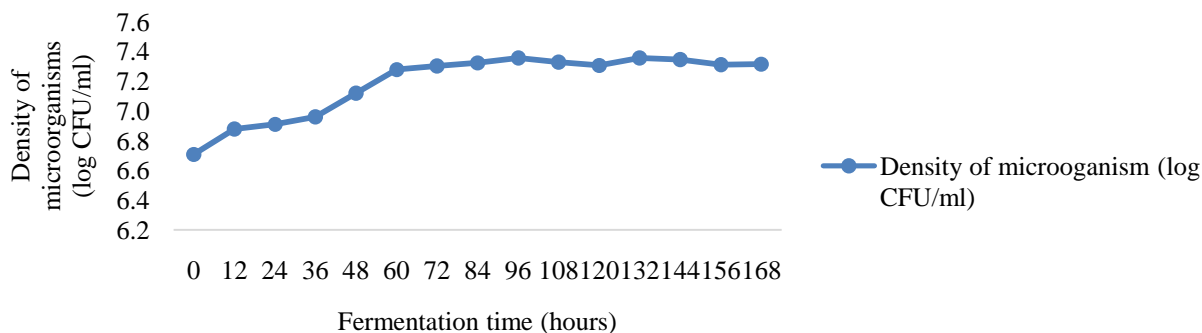


Chart 7: Changes of microbial population during fermentation on fermenter system

Chart 7 shows the increasing microbial density during the fermentation process. Specifically, cell density increased from 6.709 log CFU/ml at the beginning of the fermentation process (0 hours) to 7.316 log CFU/ml (168 hours) - also the highest value of microbial population.

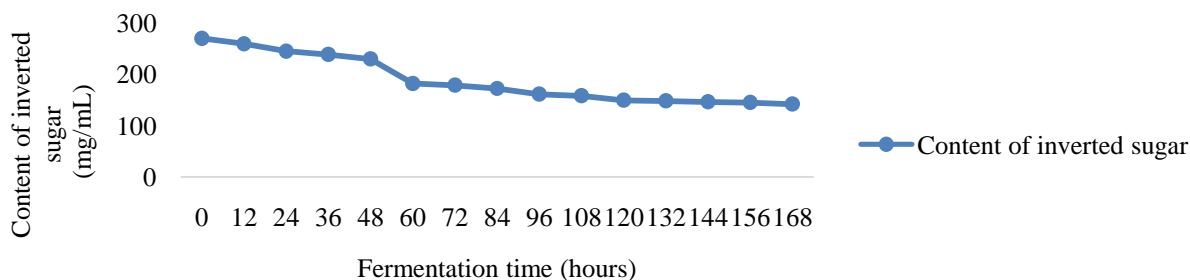


Chart 8: Changes in inverted sugar content during the fermentation process on fermenter.

During the fermentation process from 0 hours to 168 hours, the inverted sugar content was reduced to the lowest value on the last day at 141.419 mg / mL (168 hours). The change of cell density is consistent with changes of the inverted sugar during the fermentation process. The more microbial cells are, the lower the inverted sugar content. This indicates that *Gluconacetobacter xylinus* uses glucose substrates to produce gluconic acid synthesis.

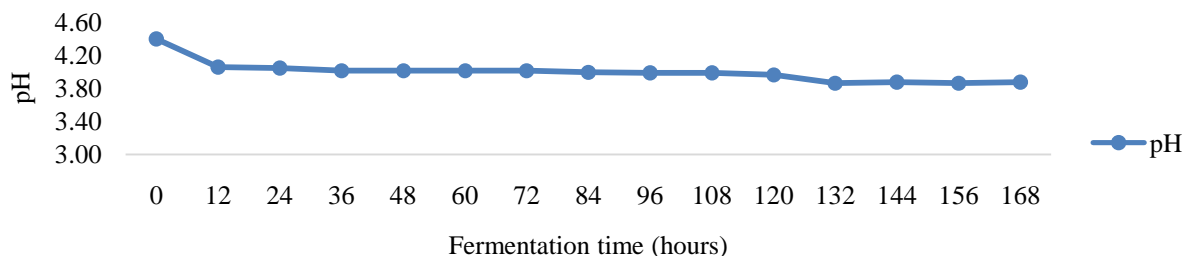


Chart 9: Changes of pH in the fermentation process on the fermenter system

According to the results shown in chart 9, the pH value of the fermented liquid decreased during the fermentation process, decreasing from 4.39 (at the beginning of the process) to 3.87 (168 hours).

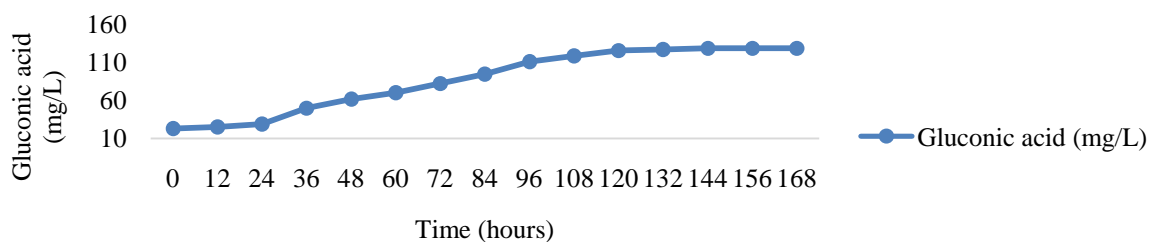


Chart 10: Changes of the content of gluconic acid during the fermentation process on the fermentation.

The results shown in chart 10 indicate that the gluconic acid content increases with time of fermentation. From 24 hours to 120 hours, the gluconic acid content increased sharply. After 120 hours, the process of biosynthesis of gluconic acid gradually stabilizes but tends to increase gradually. At 156 hours fermentation, gluconic acid content was highest (128.91 mg / L). After 156 hours, i.e. at 168 hours, the biosynthesis of gluconic acid decreased slightly but not significantly (128.85 mg / L).

IV. Conclusion

With the *Gluconacetobacter xylinus* strain, the study was initially to research the basic parameters of honey fermentation process for the purpose of obtaining honey that is rich in gluconic acid. The results of parameters for 5 factors are the dilution ratio of honey and coconut milk (1: 4), the rate of seed (4%), initial pH (4.5), fermentation temperature (30⁰C) and fermentation time (5 days). These parameters are applied to the fermentation process on the BioFlo fermenter system in order to orient in cosmetic applications. The gluconic acid value reached its maximum point when fermented on the BioFlo fermenter system at 128.91 mg / L.

Acknowledgements

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