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Optimizing Glucuronic Acid Production Using Tea Fungus on Grape Juice by Response Surface Methodology

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Abstract: Grape juice phytochemicals such as resveratrol and polyphenol antioxidants have been positively linked to inhibit cancer heart disease degenerative nerve disease viral infections and mechanisms of Alzheimer's disease. Glucuronic acid is the key component in human health due to its detoxifying action through conjugation to the xenobiotic metabolisms in liver and associated with cartilage, shown substantial benefit in the treatment of osteoarthritis. Here we report first analysis of evaluate the effect of treatment variables sucrose content temperature and cultivation time on glucuronic acid production (g/L) as well as monitored changes in pH, remained sucrose (g/L), reducing sugar (g/L) and total acidity (g/L) by sing Kombucha layer on sweetened grape juice. Kombucha is a refreshing beverage obtained through the fermentation of sugared grape juice with a symbiotic culture of acetic bacteria and fungi consumed for its distinct antibiotic effects against the number of disease organisms and several therapeutic purposes in human medicine. Response surface methodology using Box–Behnken design showed that all the factors had a significant effect on glucuronic acid production. The optimum medium composition for predicted maximum glucuronic acid production was appeared on 7% sucrose-sweetened grape juice within two weeks of fermentation process at 37°C.

Key words: *Kombucha; sweetened grape juice, glucuronic acid, response surface methodology, therapeutic purposes.*

INTRODUCTION

Kombucha layer has been claimed to be a prophylactic and therapeutic agent to human health from weight loss to metabolic diseases, arthritis, indigestion, curing cancer, and AIDS (C. Dufresne and E. Farnworth, 2000). Kombucha is a traditional beverage prepared by fermenting sweetened black tea with the tea fungus which is a symbiosis of Acetobacter, including *Acetobacter xylinum* as a characteristic spieces, and various yeasts, such as the genera of *Brettanomyces, Zygosaccharomyces, Saccharomyces,* and *Pichia* depending on the source (P. Mayser, *et al.,* 1995). It is consumed all around the world, but historically in China, Russia, Germany (P. Dipti, *et al.,* 2003) and yet is quite popular in the West and Mediterranean region especially in Iran.

One of the main metabolites identified in the kombucha beverage is a glucuronic acid (C.H. Liu, *et al*., 1996). Glucuronic acid is a highly water-soluble carboxylic acid, that normally produced by a healthy liver, that can be converted into glucosamine and related chondroitin-sulfate are associated with cartilage, collagen and the fluid which lubricate the joints (Frank and Günther, 1991). Acetic acid bacteria take up the monosaccharides (glucose and fructose) that resulting from sucrose hydrolysis as carbon source in the cultivation medium by yeast invertase due to their hydrolases and kinases shortage (D. Cvetkovic, *et al.,* 2008). Kombucha layer researchers believe that, its detoxifying property is presumably due to the capacity of glucuronic acid binding to toxin molecules and increasing their excretion from the organism by the kidneys or the intestines (R. Jayabalan, *et al.*, 2007). Butyric acid, also found in kombucha beverage protects human cellular membranes and combined with glucuronic acid strengthens the walls of the gut and also protects against parasites as a result of its bond to glucuronic acid (U. Mann, 1988).

Comparing diets among western countries, researchers have discovered that although they tend to eat higher levels of animal fat, surprisingly the incidence of heart disease remains low, a phenomenon, suggest occurring from protective benefits of regularly consuming grape wine (Wikimedia Foundation, Inc, 2008). Grape juice (GJ) phytochemicals such as resveratrol, bears a significant transcriptional overlap with the beneficial effects of calorie restriction in heart, skeletal muscle and brain (S. Das and D.K. Das, 2007), whereas anthocyanins tend to be the main polyphenolics that are attracting the efforts of scientists to define their properties for human health (E. Cantos, *et al*., 2002).

During the fermentation process, bacteria and yeasts metabolize number of organic acids such as acetic acid and glucuronic acid (S.C. Chu and C. Chen, 2006), amino acids, antibiotics and a variety of micronutrients (R. Vijayaraghavan, *et al*., 2000). However, as a therapeutic substance or functional food, kombucha should be

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defined and standardised with regard to its microbiological composition and consequently its chemical composition (C.D. Wu and G.X. Wei, 2002).

Response surface methodology (RSM) is a powerful technique for testing multiple process variables because fewer experimental trials are needed and also, interactions between variables can be identified and quantified (R. Myers and D.C. Montgomery, 2002). In this study, we applied RSM, especially Box-Behnken design (BBD) (G.P. Box and D.W. Behnken, 1960), to evaluate the influence of treatment variables; sucrose content, temperature and the cultivation time on the glucuronic acid production as a bioactive material as well as monitored changes in pH, remained sucrose, reducing sugar and total acid. This is the first report of grown Kombucha layer on SGJ in order to produce glucuronic acid and, to our knowledge, no other reports on this matter has appeared in the literature so far.

MATERIALS AND METHODS

Grape juice and Chemicals:

All the grape juice (GJ) used in this study were produced and packed by Takdaneh Agri & Ind. Co. (PJS), Iran. The main characteristics of the GJ are presented in Table 1. Glucuronic acid was purchased from the Fluka Chemical AG. (Industriestrasse 25, CH-9741 Buchs Switzerland). All the other chemicals and solvents were high-analytical grade ones.

Kombucha Layer and Cultivation:

Culture pellicle in a minimal volume of liquor was collected from Persian Type Culture Collection, IROST. Preserved in GJ at 2°C temperature. Actively growing Kombucha layer, grown in above medium were used for inoculation into Sweeten Grape Juice (SGJ) (C. Pasha and G. Reddy, 2005). Sucrose content was dissolved in 1L GJ with specific characteristics (Table 1) and then was poured into 5L glass jar under aseptic condition that had been previously sterilized at 121°C for 20 min. The SGJs were inoculated with mean 95 g/L of freshly pellicle that had been cultured in the same medium for 7 days and 30 ml/L of previously fermented liquid SGJ broth aseptically. The fermentation was carried out in an incubator at three constant temperatures. All the medium specifications are presented in Table 2.

Table 1: Characteristics of grape juice (*GJ*).

Nutrition value *(per 100 ml)*	Total acid *(g)*	Carbohydrates *(g)*		Glucuronic acid *(g)*	pH
		Reducing sugars [a]	Sucrose		
Grape juice *(GJ)*	9.5	9.5	< 1	3.49	3.6

[a] Total of glucose and fructose

Table 2: Independent variables and their coded and actual values in the experimental design.

Independent variable					Coded levels	
		Units	Symbol	-1	0	1
Temperature		°C	X_1	18	27	37
Time		*day*	X_2	4	9	14
Sucrose [a]		*g/L*	X_3	5	7	9

[a]Added to grape juice with initial sucrose concentration

Experimental Design and Statistical Analysis:

RSM was used to investigate the influence of temperature, sucrose content and time treatments of Kombucha layer fermentation on the glucuronic acid production. A Box–Behnken factorial design with 3 factors and 3 levels was used for fitting a 2nd-order response surface. The independent variables were the temperature (*X*1), time (*X*2), and sucrose content (*X*3) used to treat the fermentation process, while the response variable was the glucuronic acid yield (*Y1*), remained sucrose (*Y2*), reducing sugar (*Y3*), total acidity (*Y4*), the biomass of Kombucha layer (*Y5*) and pH (Y6),. The factors, their values, and the experimental design are presented in Table 2. The surface plot based on two independent variables was generated by keeping the 3rd independent variable at a constant level. Data from the Box–Behnken factorial design shown in Table 3.

Determination of pH and Total Acidity:

The pH of the samples was measured with an electronic pH meter (Metrohm model 827) calibrated at pH 4 and 7 at 25 °C. Total acidity was determined using the volumetric method by titration with a standard solution of sodium hydroxide and phenolphthalein as indicator (AOAC, 1980).

Table 3: Box–Behnken design matrix and six responses of the tea fungus fermentation on *SGJs* [a].

Std	Temp	Time	Sucrose [b]	Glucuronic acid	Remained sucrose [c]	Reducing sugar [d]	Total acidity	Biomass	pH
_	°C	day	g/L	g/L	g/L	g/L	g/L	g	_
16	27	9	7	36.79	3.51	18.15	109	249.5	3.05
15	27	9	7	29.1	1.22	19.5	108	255.31	3.06
8	37	9	9	136.84	4.15	21.89	319	245.74	2.66
9	27	4	5	63.36	2.88	15.17	156	133.58	2.9
11	27	4	9	71.16	2.23	19.5	168	174.02	2.87
6	37	9	5	135.06	1.38	17.07	300	324.36	2.71
14	27	9	7	27.82	2.45	22.43	96	233.56	3.07
4	37	14	7	178	1.02	19.32	348	478.15	2.64
7	18	9	9	64.47	3.64	14.37	156	174.07	2.87
12	27	14	9	160.48	1.12	9.17	271	413.11	2.63
13	27	9	7	30.36	2.34	21.96	102	257.99	3.06
1	18	4	7	40.03	2.37	16.16	107	116.23	3.18
2	37	4	7	62.14	2.04	16.06	144	112.43	2.88
10	27	14	5	88.32	0.92	15.01	164	421.07	2.84
3	18	14	7	76.99	1.43	13.42	176	395.21	2.62
5	18	9	5	65.59	1.44	15.17	180	158.34	2.89
17	27	9	7	45.47	2.36	18.82	118	241.52	3.05

[a] Sweetened Grape Juice
[b] Added to Grape Juice within initial sucrose concentration
[c] Mean: Unfermented sucrose
[d] Total of glucose and fructose

Determination of Reducing Sugars and Remained Sucrose Content:

Reducing sugars and the remained sucrose content after the fermentation during were determined using the Lane-Eynon general volumetric method (AOAC, 2002).

High Performance Liquid Chromatography Analysis of Glucuronic Acid:

Diluted sample (1:10) was passed through Millipore filter (0.45 µ) into HPLC vials. The filtrate obtained was subjected to analysis of glucuronic acid by Reverse Phase (RP)-HPLC. A 20 µl sample of filtrate was injected to a HPLC system equipped with a UV detector. Using the Nucleocil C-18 column (4 mm ID ×250 mm, 5 µm) by a single pump Bischoff HPLC system for the analysis. The mobile phase was a 50 mM sodium dihydrogen phosphate, pH 2.58. The flow rate was maintained as 1.0 ml/min and column was at room temperature. Detection was carried out at 210 nm. The resolution peaks were recorded on the HPLC chart according to the retention time of glucuronic acid as standards. The concentrations were quantified from standard curves and multiplied dilution factor (R. Jayabalan, *et al.*, 2007).

Yield of Kombucha layer Biomass:

Yield of the obtained biomass was determined by mass measurement; the cellulose floating pellicle layer was removed from the fermented liquid surface, rinsed with distilled water and dried with filter paper.

RESULTS AND DISCUSSION

In present study production of glucuronic acid performed, using Kombucha layer on SGJ with 10 ml/L of previously grown fermentation broth, which converts this very simple substrate to a slightly carbonated, acidic, refreshing beverage with high pharmaceutical and nutritional value. This is may be due to the basic biochemistry of the kombucha metabolite composition and concentration remains largely unknown (P. Mayser, *et al.,* 1995), and it has been shown to vary due to geographical location, tea type, sugar type, incubation time (C. Dufresne and E. Farnworth, 2000), and temperature (A. Holmes, 1997). Thus, no two solutions ever produce exactly the same final beverage.

PH:

In present study, the effect of pH was investigated on SGJ fermented medium. Initially the pH value of the GJs was approximately 3.6, and it dropped to about 2.9 ± 0.3 (Table 3). Increase in acidity as a consequence of the physiological activity of the Kombucha layer and synthesis of organic acids and glucuronic acid are shown in (Table 3). As shown in Figure 1, the surface plot that depicting, based on independent variables fermentation time ($X2$) and temperature ($X1$) when sucrose content ($X3$) was fixed at 7 g/L, the highest pH values measured at the end of fermentation was 3.18, whereas the lowest was 2.62. This appeared to be rather low when compared with either the results of other authors after the sucrose fermentation on tea (R. Jayabalan, *et al.*, 2007) or previous findings for the fermentation on molasses (R.V. Malbasa, *et al.,* 2007) and cheese whey (G., Belloso-Morales and H. Hernndez-Snchez, 2003).

Table 4: ANOVA table for Glucuronic Acid of the kombucha fermentation on SGJs[a].

Source	Sum of Squares	df	Mean Square	F Value	Prob >F	
Model	35085.67	10	3508.57	102.47	≤ 0.0001	significant
A	5029.65	1	5029.65	146.90	≤ 0.0001	
B	8917.80	1	8917.80	260.46	≤ 0.0001	
C	0.11	1	0.11	3.181E-003	0.9569	
A^2	2633.90	1	2633.90	76.93	0.0001	
B^2	2575.25	1	2575.25	75.21	0.0001	
C^2	6842.01	1	6842.01	199.83	≤ 0.0001	
AB	941.88	1	941.88	27.51	0.0019	
BC	1676.08	1	1676.08	48.95	0.0004	
AB^2	164.17	1	164.17	4.79	0.0711	
B^2C	1171.76	1	1171.76	34.22	0.0011	
Residual	205.44	6	34.24			
Lack of Fit	3.63	2	1.82	0.036	0.9649	not significant
Pure Error	201.80	4	50.45			
Cor Total	35291.10	16				

A: Temperature
B: Day
C: Sucrose

Total Acidity:

Content of total acid as a function of fermentation time is presented in Table 3. Concentration of total acid was changed from 95 g/L in GJ (Table 1) to 348 g/L. An analysis of the surface plot presented in Fig 1, based on independent variables fermentation time (*X*2) and temperature (*X*1) when sucrose content (*X*3) was fixed at 7 g/L, showd that the pH values and total acid content depend on the process duration, which is in accordance with previous findings (R. Jayabalan, *et al.*, 2007). The differences between the total acid and the concentrations of glucuronic acid in the different substrates can be attributed to the presence of other acid metabolites such as gluconic, lactic, acetic acid.

Glucuronic Acid:

Bacteria and yeast, which presented in Kombucha layer, metabolize sucrose into the number of organic acids such as acetic acid and glucuronic acid similar results published by Chu and Chen, 2006. Although later study on glucuronic acid concentration using tea fungus on black tea have been reported that its concentration was reached maximum up to 2.3 g/L on 12th day of fermentation (R. Jayabalan, *et al.*, 2007), until now there have been no reports about the influence of Kombucha layer activity on the component changes in SGJ. Considerable variations in the glucuronic acid production were observed when the fermentation process was subjected to various temperature, time, and sucrose content (Table 3). Glucuronic acid yields ranged from 34 g/L (Table 1) to 178 g/L (Table 3). Results from the surface plot, which indicating the glucuronic acid content based on independent variables fermentation time (*X*2) and temperature (*X*1) when sucrose content (*X*3) was fixed at 7 g/L (Figure 1), showed that glucuronic acid production was attributed to the independent variables and also, the accuracy and general ability of the polynomial model was good, and the analysis of the response trends using the model was considered to be reasonable.

Reducing Sugars:

Table 3 showed that reducing sugars level changes within fermentation. *Acet. xylinum* was incapable of utilizing sucrose to produce acid, this is similar to the findings of Greenwalt CJ, Ledford RA, and Steinkraus KH, 1998, therefore, throughout the fermentation, the carbon source in the cultivation medium is hydrolyzed by the enzyme invertase from Kombucha layer yeasts in to glucose and fructose (21). The surface plot, based on independent variables fermentation time (*X*2) and sucrose content (*X*3) when temperature (*X*1) was fixed at 27 °C (Figure 2), showed that the the reducing sugars content 95 g/L in GJ (Table 1) was decreased during fermentation due to increase in total acidity.

Remained Sucrose:

All the GJs were sweetened by three different concentration of sucrose (Table 2). The sucrose is used by the yeasts to produce ethanol, which is initially oxides to acetaldehyde and then oxidized by acetic acid bacteria. The surface plot, based on independent variables fermentation time (*X*2) and temperature (*X*1) when sucrose content (*X*3) was fixed at 7 g/L (Figure 1), showed reduction of sucrose concentration was temperature dependent and the lowest remained sucrose content (0.92 g/L) monitored on 14th day during fermentation.

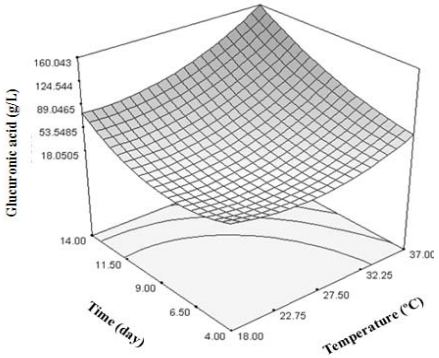
Yield of Biomass:

The symbiotic culture of acetic acid bacteria and yeasts produces a dark red cellulose mat that resembles a surface mold and which helps in the aeration process of the fermentation due to increased oxygen transfer

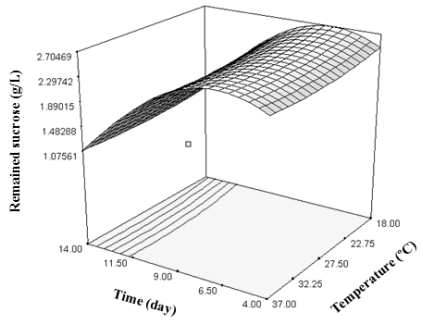
surface from the air to the liquid phase, which is especially important for the activity of acetic acid bacteria. Analyzed results in Table 3 appeared that the smallest quantity of fungus mass was 112.43 g and the largest was 478.15 g after 14-day fermentation. The yield of biomass was increased during the fermentation process.

Conclusion:

In the current study, all factors except origin of culture were changed, with this perception; chemical components would also be differed due to these variations, and therefore we examined the changes in content of glucuronic acid along with pH, remained sucrose, reducing sugars, Kombucha layer biomass and total acid content in SGJ within Kombucha layer fermentation. Glucuronic acid was reached maximum upto 178 g/L on 14th day of fermentation. Amazingly results showed that content of glucuronic acid on 14th day were much higher than the initial concentration in GJ and also revealed the possibility of using GJ manufacture the kombucha beverage. The characteristics of the SGJ kombucha were compared and following conclusions were drawn: the pH values were lowered to 2.62 on 14th day; the highest content of both total acid 348 g/L and glucuronic acid 178 g/L were observed in the same substrate; the yield of biomass was significantly increased to 478.15 g after 14 days. The products obtained on these substrates were rich in glucuronic acid between 34 g/L to 178 g/L, which may be considered as an advantage compared to the product on tea 2.3 g/L or other substrates.



(a)



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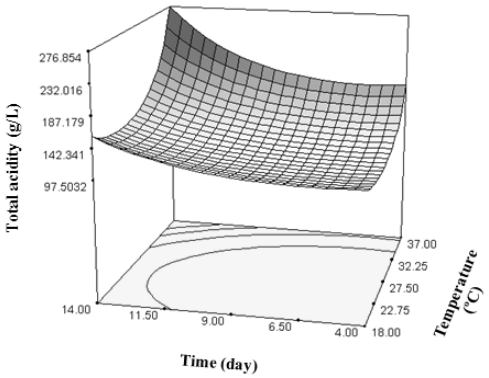
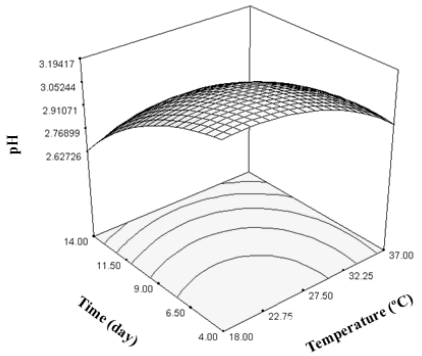


(c)



(d)

Fig. 1: Surface plot of Glucuronic acid production (a); Remained sucrose (b); Total acidity (c) and pH (d) of tea fungus fermentation on SGJ as a function of temperature and fermentation time (in coded values).

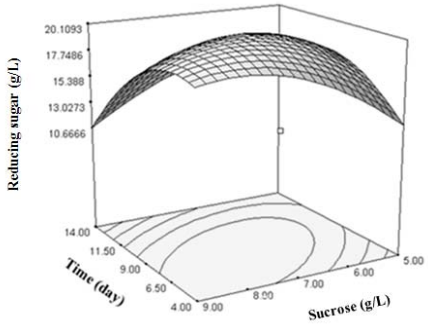


Fig. 2: Surface plot of Reducing sugar value of tea fungus fermentation on SGJ as a function of sucrose content and fermentation time (in coded values).

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