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Response Surface Methodology for Optimization of Glucuronic Acid Production Using Kombucha Layer on Sour Cherry Juice

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Abstract: The optimum conditions for the glucuronic acid production (the important key component for its detoxifying action through conjugation to the xenobiotic metabolism of the substances in liver) using kombucha layer on sweetened sour cherry juice were determined using response surface methodology. Kombucha layer involving a symbiosis of osmophilic yeast species and acetic acid bacteria that convert a very simple substrate to a slightly carbonated, acidic, refreshing beverage with high pharmaceutical and nutritional value. A central composite rotatable design, consisting of seventeen experiments was used to investigate the effects of three independent variables, namely sucrose content, temperature and the cultivation time on five responses: glucuronic acid (g/L), pH value, remained sucrose (g/L), reducing sugar (g/L), and total acidity (g/L). Statistical analysis of the obtained results, depicted that all the factors had a significant effect which leads to glucuronic acid concentration upto maximum 132.5 g/L at 37 °C within two weeks of fermentation process on 8% sucrose-sweetened sour cherry juice.

Key words: kombucha layer; sweetened sour cherry juice; glucuronic acid; response surface methodology.

INTRODUCTION

The sour cherry juice (SCJ) is the most popular beverage not only because of its growth and consumption in about around the world but also because of the neochlorogenic acid, 3-coumaroyl-quinic acid, chlorogenic acid, and epicatechin (Frank *et al.,* 2005) for which is the highest antioxidant activity and anti-inflammatory property prevent various human degenerative diseases (Burkhardt *et al.,* 2001; Pedisic *et al.,* 2007).

Glucuronic acid is normally produced by a healthy liver and can readily be converted into glucosamine, the foundations of our skeletal system, the structures associated with cartilage, collagen and the fluids which lubricate the joints and considered to be one of the important key components found in kombucha beverage due to its detoxifying action through conjugation (Jayabalan *et al.,* 2007; loncar *et al.,* 2000) to the xenobiotic metabolism of substances such as drugs, pollutants, bilirubin, androgens, estrogens, mineral corticoids, glucocorticoids, fatty acid derivatives, retinoid, and bile acids (Wikimedia, 2008).

In recent years, the general population has demonstrated increased awareness and interest in "Functional Foods". However, as a therapeutic substance or functional food, kombucha should be defined and standardized with regard to its microbiological composition and consequently its chemical composition (Wu and Wei, 2002). Kombucha, also named as tea fungus, has been consumed worldwide as a healthy drink for a long time especially in China, Russia and Germany (Dipti *et al.,* 2003) and yet is quite popular today in the West and in Mediterranean region, especially Iran. The tea fungus is a symbiotic culture of acetic acid bacteria *(Acetobacter aceti, Acetobacter pasteurianus, Gluconobacter oxydans)* and yeasts *(Saccharomyces sp., Zygosaccharomyces kombuchaensis., Torulopsis sp., Pichia sp., Brettanomyces sp.)* (Greenwalt *et al.,* 2000; Kurtzman *et al.,* 2001; Liu *et al.,* 1996).

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Kombucha beverage is composed of two portions: a floating cellulose pellicle layer and the sour liquid broth (Chen and Liu, 2000). Typical of such fermentation is the activity of *Acetobacter xylinum* which enables synthesis of a floating cellulose pellicle and converts glucose to glucuronic acid and fructose to acetic acid, in which the embedded cells benefit from the close contact with the atmospheric oxygen (Siever *et al.,* 1995) and yeast cells hydrolyze sucrose into glucose and fructose, producing ethanol (Reiss, 1994). During the fermentation process, bacteria and yeasts metabolize sucrose into a number of organic acids such as acetic acid and glucuronic acid (Chu and Chen, 2006), amino acids, antibiotics and a variety of micronutrients produced during fermentation (Vijayaraghavaity *et al.,* 2000). In 1951, an important population study conducted in Russia by the ''Central Oncological Research Unit'' and the ''Russian academy of Sciences in Moscow'' found that the daily consumption of kombucha was correlated with an extremely high resistance to cancer (Dufresne and Farnworth, 2000). In addition to ethanol and acetic acid, a great number of other compounds emerge as a result of numerous reactions (Balention *et al.,* 1997; Pasha and Reddy, 2005). Important metabolites are organic acids –active ingredients of kombucha tea that may exert beneficial effects (Jayabalan *et al.,* 2007). The US Food and Drug Administration has evaluated the practices of several commercial producers of the kombucha and found no pathogenic organisms or other hygiene violations (CDC, 1996). This beverage has been reported to have medicinal effects against metabolic diseases, arthritis, indigestion and various types of cancer (Sreeramulu *et al.,* 2000).

When many factors and interactions affect desired response, response surface methodology (RSM) is an effective tool for optimizing the process. As the needed information about the shape of the response surface is applied, RSM is an effective statistical method that uses a minimum of resources and quantitative data from an appropriate experimental design to determine and simultaneously solve a multivariate equation.

In present study, we attempt to modify sweetened sour cherry juice using kombucha fermentation with three variables; sucrose content, fermentation times and temperature combinations in order to enhance the glucuronic acid production as a bioactive material by monitoring changes in glucuronic acid, pH value, remained sucrose, reducing sugar, and total acidity. This is the first report of grown kombucha layer on SSCJ in order to produce glucuronic acid.

MATERIALS AND METHODS

2.1. Sour cherry juice and Chemicals:

All SCJ used in this study were produced and packed by Takdaneh Agri & Ind. Co. (P.J.S), Iran. The main characteristics of the SCJ are presented in Table 1.

2.2. Experimental Design and Statistical Analysis:

RSM was used to investigate the influence of the temperature, sucrose content and time treatments of kombucha 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 ($X1$), time ($X2$), and sucrose content ($X3$) used to treat the fermentation process, while the response variable was the glucuronic acid yield ($Y1$), pH ($Y6$), remained sucrose ($Y2$), reducing sugar ($Y3$), total acid ($Y4$) and the biomass of kombucha ($Y5$). 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.

2.3. Kombucha Culture:

Culture pellicle in a minimal volume of liquor was collected from Persian Type Culture Collection, IROST and was preserved in sweetened sour cherry juice (SSCJ) at 2°C temperature. Actively growing kombucha layer grown in above medium was used for inoculation into SSCJ (Pasha and Reddy, 2005).

2.4. Preparation and cultivation of Kombucha:

Different content of sucrose was dissolved in 1L SCJ with characteristics (Table 1), and the preparation was poured into 5L glass jars (each contained 1L medium) under aseptic condition that had been previously sterilized at 121 °C for 20 min. The SSCJs were inoculated with mean 87 g/L of freshly kombucha pellicle that had been cultured in the same medium for 5 days and 10 ml/L of previously fermented liquid SCJ broth aseptically. The fermentation was carried out in an incubator at three constant temperatures on fermentation during. All the medium specifications are presented in Table 2.

2.5. Determination of pH Value and Total Acidity:

The pH value 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).

2.6. Determination of Reducing Sugars and Remaining Sucrose Content:

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

2.8. 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 U.V. 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 2.7. 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 (Jayabalan *et al.,* 2007).

RESULTS AND DISCUSSION

The basic biochemistry of the kombucha metabolite composition and concentration remains largely unknown, but it has been shown (Mayser *et al.,* 1995) to vary due to geographical location, tea type, sugar type, incubation time (Dufresne and Farnworth, 2000) and temperature. Thus, no two solutions ever produce exactly the same final beverage. Although later study on glucuronic acid concentration using kombucha on black tea has been reported that its concentration was reached maximum up to 2.3 g/L on 12th day of fermentation (Jayabalan *et al.,* 2007), until now there have been no reports about the influence of kombucha activity on the component changes on SSCJ using kombucha layer in a fermentation process.

The independent variables were the temperature ($X1$), time ($X2$), and sucrose content ($X3$) used to treat the fermentation process, while the response variable was the glucuronic acid yield ($Y1$), pH value ($Y5$), remained sucrose ($Y2$), reducing sugar ($Y3$), and total acid ($Y4$) (Table 3). Fig.1 and 2, the surface plots based on independent variables fermentation time ($X2$) and temperature ($X1$) when sucrose content ($X3$) was fixed at 8 g/L, was showed.

3.1. pH value:

In present studies, the effect of pH value was studied in SSCJ fermented medium. Initially the pH value of the SSCJs was approximately 3.01, and it dropped to about 2.59 ± 0.8 within14 days of fermentation (Table 3 and fig 1.c). The highest pH values measured at the end of the fermentation was 2.93 whereas the lowest was 2.51 appeared to be rather low when compared with either the results of other authors after the sucrose fermentation on tea (Jayabalan *et al.,* 2007; Loncar *et al.,* 2006) or previous findings for the fermentation on molasses and cheese whey.

3.2. Total acidity:

Content of total acid as a function of fermentation time is presented in Table 3 and Fig 2. Concentration of total acid was changed from 9.2 g/L in SCJ to 292.5 g/L as preparation condition was showed in Fig 2. An analysis of the results presented in Tables 3 shows that the pH values and total acid depend significantly on the process duration, which is in accordance with previous findings (Jayabalan *et al.,* 2007; Loncar *et al.,* 2006). 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.

3.3. Glucuronic acid:

Using SCJ, as a medium for the fermentation process of kombucha layer, the layer's bacteria and yeasts metabolize sucrose into a number of organic acids such as acetic acid and glucuronic acid by different and complementary ways that one of the possible ways of glucose transformation is also its oxidation at C-6 position into glucuronic acid this is what appeared in the results investigated by others. Although Jayabalan *et al.* (2007) reported that green and black tea were found to be the best substrate for glucuronic acid

production (1.73 ± 0.14 g/L and 2.33 ± 0.24 g/L) respectively on 12th day by kombucha culture, its concentration was unstable to pH value and decreased to (1.57 ± 0.14 g/L and 1.5 ± 0.17 g/L) respectively on 15th day. In this work we demonstrated that glucuronic acid content in SSCJ as showed in Table 3 and Fig 1.a was unbelievably very high. Maximum obtained glucuronic acid was 132.5 g/L on 14th day of fermentation period followed by a slow decrease of pH value as revealed in Table 3 and Fig 1.c. They grew kombucha layer on black tea with sucrose substrate as a source of energy (Loncar *et al.,* 2000).

3.4. Reducing sugars:

Glucose is used by the yeasts to yield ethanol, which is initially oxides to acetaldehyde then oxidized by acetic acid bacteria and carbon dioxide. Fructose remains part of the ferment broth and is utilized by the microorganisms to a lesser degree. Glucose was not produced in parallel with fructose but was produced with a lower initial rate (Chen and Liu, 2000). The reducing sugars content 4.5 g/L in SCJ was decreased during fermentation due to increase in total acidity by beverage (Table 3 and Fig 1.b).

3.5. Remained Sucrose:

The SCJ was sweetened by three different concentration of sucrose (Table 2). The sucrose used since it is a traditional carbon source for kombucha fermentation. *Acet. xylinum* was incapable of utilizing sucrose to produce acid (Brown *et al.,* 1976; Greenwalt *et al*., 1998). Therefore, throughout the fermentation, the used carbon source in the cultivation medium is hydrolyzed by the enzyme invertase from tea fungus yeasts in to glucose and fructose. After the 4th day, the sucrose consumption began to accelerate and continued to do so until the 14th day. The reduction of sucrose concentration was temperature dependent and the lowest remained sucrose content (2.1 g/L) monitored on 14th day during fermentation (Table 3 and Fig 1.d).

Table 1: Characteristics of Sour Cherry juice *(SCJ)*

Nutrition value *(per 100 ml)*	Total acid *(g)*	carbohydrate *(g)*		glucuronic acid pH *(g)*	
sour cherry juice *(SCJ)*	9.2	Reducing sugars	Sucrose	3.67	3.01
		4.5	7		

[a]Total of glucose and fructose

Table 2: Relationship between coded and actual values of a variable.

Independent variable	Units	Symbol	Coded levels		
			-1	0	1
Temperature	°C	X_1	18	27	37
Time	day	X_2	4	9	14
Sucrose [a]	*g/L*	X_3	6	8	10

[a]Added to grape juice with initial sucrose concentration

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

Std	Temp °C	Time day	Sucrose[b] *g/L*	Glucuronic acid *g/L*	Remained sucrose [c] *g/L*	Reducing sugar [d] *g/L*	Total acidity *g/L*	pH
16	27	9	8	16.86	5.91	6.94	110.5	2.69
15	27	9	8	15.06	7.22	6.39	98.2	2.66
8	37	9	10	41.31	2.23	10.82	134.9	2.63
9	27	4	6	14.57	18.92	18.56	85.1	2.91
11	27	4	10	14.01	27.61	16.85	98.7	2.93
6	37	9	6	32.6	3.88	15.04	84.1	2.67
14	27	9	8	24.02	7.76	4.11	97.2	2.69
4	37	14	8	132.5	2.1	2.96	292.5	2.54
7	18	9	10	19.44	6.4	4.87	108	2.73
12	27	14	10	35.93	7.68	4.55	138.2	2.51
13	27	9	8	33.92	9.26	6.89	94.3	2.71
1	18	4	8	9.01	32.21	14.44	75.11	2.93
2	37	4	8	15.86	21.14	11.23	92.9	2.88
10	27	14	6	27.07	3.3	5.94	102.4	2.57
3	18	14	8	28.32	6.47	6.24	100.4	2.67
5	18	9	6	20.48	8.28	5.63	96.1	2.78
17	27	9	8	20.18	7.02	4.59	103.5	2.68

[a] Sweetened Sour Cherry juices
[b] Added to Sour Cherry juice within initial sucrose concentration
[c] Means: Unfermented sucrose
[d] Total of glucose and fructose

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

Source	Sum of Squares	df	Mean Square	F Value	P-Value Prob > F	Significant
Model	10.45	5	2.09	46.26	<0.0001	
A[a]	1.62	1	1.62	35.87	<0.0001	
B[b]	0.61	1	0.61	13.49	0.0028	
AB	0.36	1	0.36	7.87	0.0149	
A^2	0.30	1	0.30	6.69	0.0226	
A^2B	0.45	1	0.45	9.91	0.0077	
Residual	0.59	13	0.045			
Lack of Fit	0.18	7	0.026	0.38	0.8866	not significant
Pure Error	0.41	6	0.068			
Total	11.04	18				

[a] A: Temperature
[b] B: Day

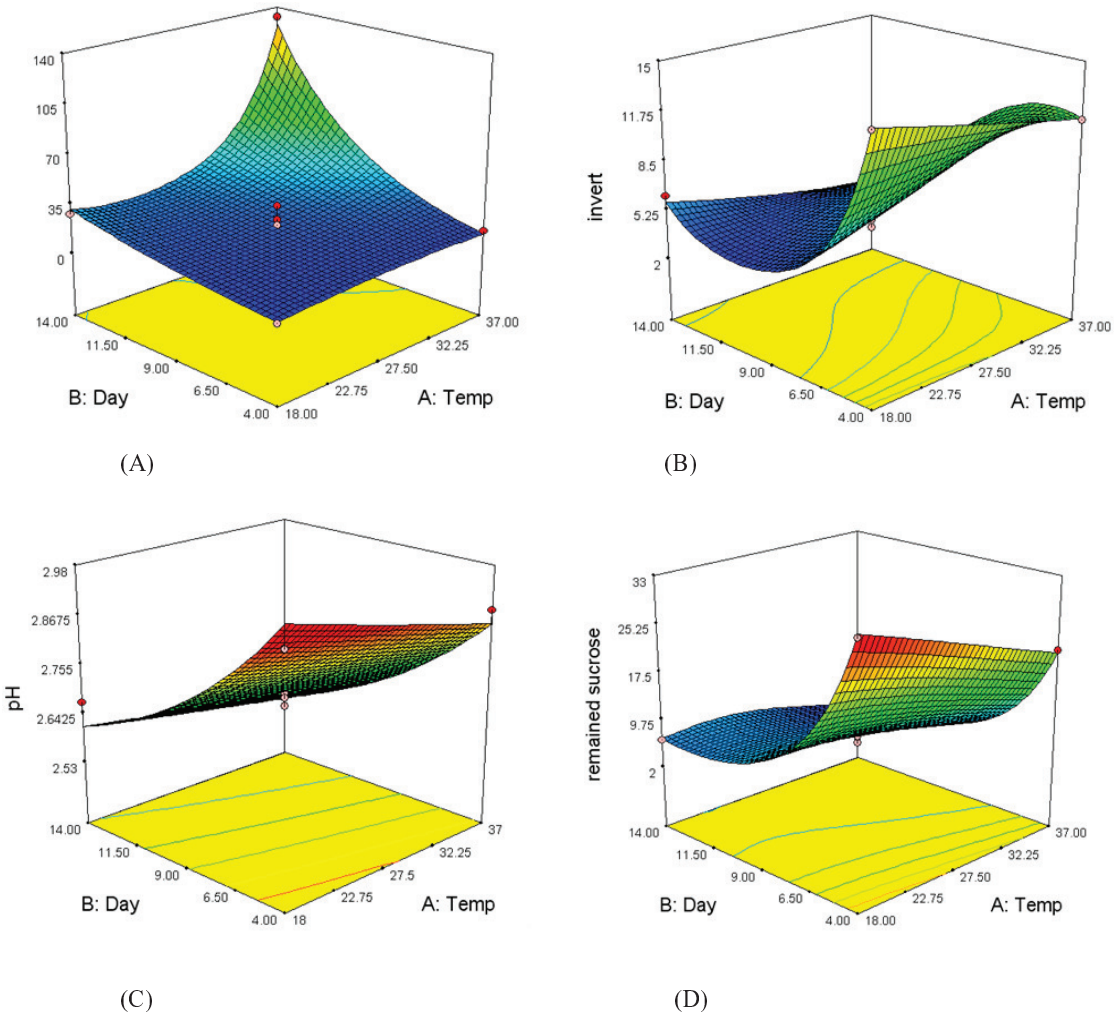

Fig. 1: Surface plot of Glucuronic acid production (a); Reducing sugar (b); Total acidity pH (c) and Remained sucrose (d) of kombucha fermentation on *SGJ* as a function of temperature and fermentation time (in coded values).

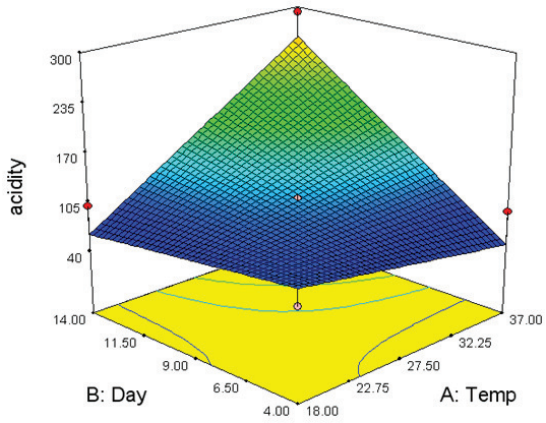

Fig. 2: Surface plot of Total acidity value of kombucha fermentation on *SGJ* as a function of sucrose content and fermentation time (in coded values).

Conclusion:

In the current study, all factors other than origin of culture were changed with this perception; chemical components would also be expected to differ due to these variations. Therefore, we examined the changes in content of glucuronic acid along with pH value, remained sucrose, reducing sugars, and total acid content in SSCJ during kombucha layer fermentation. Amazingly result showed that content of glucuronic acid on 14th day (132.5 g/L) was much higher than the initial concentration 3.67 g/L in SCJ and also revealed the possibility of using SCJ manufacturing the kombucha beverage.

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