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Zygosaccharomyces bailii and *Z. rouxii* induced ethanol formation in apple juice supplemented with different natural preservatives: A response surface methodology approach

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ABSTRACT

In this study, ethanol produced by osmophilic yeasts, *Zygosaccharomyces bailii* and *Z. rouxii,* in apple juice preserved with mint essential oil (MEO), carvacrol and natamycin instead of synthetic preservatives was modeled. Some processing parameters such as sodium benzoate (SB, 0–0.1%) used as a positive control, storage temperature (4–20 °C) and storage time (1–41 days) were selected in the study. Box-Behnken design in response surface methodology was used to evaluate the effects of processing parameters on ethanol levels of apple juice and three models were created for three preservatives for each yeast. Preservative type affected the ethanol formation in apple juice for both yeasts studied. Increase of preservative concentration decreased the ethanol formation during the storage period. The best effective preservative was determined as MEO and *Z. bailii* was found to be quite resistant yeast against to the preserving agents for three models as compared to *Z. rouxii*. Ethanol level increased with the increase of both storage temperature and time for both yeasts. The results showed that apple juice could be preserved by these three preservatives, but the MEO was the most effective agent for apple juice during the storage.

1. Introduction

Foodstuffs generally spoil with the impact of various physical, chemical and microbiological factors after a certain period. Microbiological spoilage is a significant issue in fruits and vegetables and it is closely related to many other physical and chemical changes in fruits and vegetables. Through such changes, significant quality losses occur, taste and structure are lost and ultimately fruit and vegetables turn into inconsumable foodstuffs. Microbiological spoilage also results in significant economic losses and spoilage microorganisms could produce serious toxins (Aksan, 2010). Yeasts and molds constitute the primary natural microbiota of fruits and fruit-originated foodstuffs (Cemeroglu and Acar, 1986). In low pH foods (< 5), generally mold and yeasts cause deterioration since they are more resistant against to the unsuitable conditions namely, they need less water, or they can grow in osmophilic conditions compared to bacteria. Yeasts can benefit from larger molecules like carbohydrates, develop in fruits and produce alcohol. Low molecular weight compounds produced by yeasts can be used by molds. Additionally, yeasts can easily grow in foods having low water activity and cause deterioration (Unluturk and Turantas, 2002). In general, the yeast species cause to the quality problems in the beverage industry belong to the genus of *Zygosaccharomyces*. *Z. rouxii* and *Z. bailii* are the osmophilic yeasts and they are the primary deteriorative microorganisms in fruit juices and concentrates having high sugar levels (Martorell et al., 2007). *Z. rouxii* has haploid cells which are globular, ellipsoid or ovoid structure and it can grow in wide range of pH values (1.8–8) and high salt and sugar media. Also, it can tolerate the ethanol, sulfur dioxide and acetic acid (Kirimli, 2008). As compared to *Z. rouxii*, *Z. bailii* is more resistant against to higher sugar and acid concentrations, ethanol and pasteurization treatments and they may cause serious problems in food industry even at a pH of 1.8 (Kilickaya, 2006).

Many synthetic preservatives are used in food industry to prevent the microbial spoilage thereby extend the shelf life of the final products. In recent years, because of the increasing consumer demand for "healthier" food products having no chemical preservatives, there is a trend for using the natural preservatives and environmentally friendly technologies (Artes et al., *2007*). Suspension of synthetic chemical preservatives leads to harmful effects on human health. Therefore, recent studies have focused on potential use of natural antimicrobial

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Table 1
Box-Behnken experimental design.

Coded values					Uncoded values					
								Model 1	Model 2	Model 3
Runs	A	B	C	D	Time (day)	Temperature (°C)	Sodium benzoate (%)	Mint essential oil (ppm)	Carvacrol (ppm)	Natamycin (ppm)
1	−1	−1	0	0	1	4	0.05	500	375	30
2	−1	0	−1	0	1	12	0	500	375	30
3	−1	0	0	−1	1	12	0.05	0	0	0
4	−1	0	0	1	1	12	0.05	1000	750	60
5	−1	0	1	0	1	12	0.1	500	375	30
6	−1	1	0	0	1	20	0.05	500	375	30
7	0	−1	−1	0	21	4	0	500	375	30
8	0	−1	0	−1	21	4	0.05	0	0	0
9	0	−1	0	1	21	4	0.05	1000	750	60
10	0	−1	1	0	21	4	0.1	500	375	30
11	0	0	−1	−1	21	12	0	0	0	0
12	0	0	−1	1	21	12	0	1000	750	60
13	0	0	0	0	21	12	0.05	500	375	30
14	0	0	0	0	21	12	0.05	500	375	30
15	0	0	0	0	21	12	0.05	500	375	30
16	0	0	1	−1	21	12	0.1	0	0	0
17	0	0	1	1	21	12	0.1	1000	750	60
18	0	1	−1	0	21	20	0	500	375	30
19	0	1	0	−1	21	20	0.05	0	0	0
20	0	1	0	1	21	20	0.05	1000	750	60
21	0	1	1	0	21	20	0.1	500	375	30
22	1	−1	0	0	41	4	0.05	500	375	30
23	1	0	−1	0	41	12	0	500	375	30
24	1	0	0	−1	41	12	0.05	0	0	0
25	1	0	0	1	41	12	0.05	1000	750	60
26	1	0	1	0	41	12	0.1	500	375	30
27	1	1	0	0	41	20	0.05	500	375	30

A, B, C and D were storage time (day), storage temperature (°C), sodium benzoate (%) and mint essential oil (ppm) in model 1,carvacrol (ppm) in model 2 and natamycin (ppm) in model 3, respectively.

substances as food additives (Sagdic et al., 2010).

Natamycin is a preservative having an antifungal activity and it is a polyene macrolide antifungal agent used in the food industry. It is also known as pimarisin produced by aerobic fermentation of *Streptomyces natalensis* (Thomas and Delves-Broughton, *2003*). Natamycin is effective preservative against mold and yeasts. The antifungal effect of natamycin is attributed to its integration of sterols in the cell wall of the yeasts. Bacteria are not sensitive to natamycin because they have no sterols in the structure of the cell wall (Yilmaz and Kurdal, 2005).

Carvacrol is one of the main constituents of common essential oils produced by oregano and thyme which gives a distinctive odor (Coban and Patir, 2010; Gursoy and Gursoy, 2004).

Carvacrol is accepted as a safe food additive and it is used as a flavouring agent in baked goods, sweets, beverages and chewing gum (Fenaroli, 2002). It was reported that the essential oil containing carvacrol shows biostatic and/or biocidal activity against many bacteria, yeast and fungi. Due to its high antimicrobial performance, many researches are performed about its potential food preservative activities (Burt, 2004).

Essential oils extracted from the many medicinal and aromatic plants are natural, complex, multicomponent systems composed mainly of different compounds namely terpenes and some other nonterpene components (Edris, 2007; Fu et al., 2007). Since the ancient times, aromatic plants had been used to extend the shelf life of different foods and they are very popular to care some diseases in folk medicine becasue of their essential oils showing preservative and medicinal properties and imparting aroma and flavor to food. Essential (volatile) oils from aromatic and medicinal plants show biological activity, notably antibacterial, antifungal, and antioxidant properties (Dadalıoglu and Evrendilek, 2004; Irkin and Korukluoglu, 2009).

Number of studies conducted with two strains of *Z. rouxii* and *Z. rouxii* is quite limited in the literature. In this study, ethanol level of the apple juice samples produced by osmophilic yeasts (*Z. bailii* and *Z. rouxii*) and supplemented with MEO, carvacrol and natamycin instead of synthetic preservatives were characterized. Ethanol level of the apple juice samples was determined by chromatographic technique during storage and response surface methodology approach was used to model the levels depending on the selected processing variables namely storage time, storage temperature, sodium benzoate level and natural preservative concentration.

2. Materials and methods

2.1. Materials

Apple juice (Cappy, 100% natural, 12.14 brix) in tetra pack packaged having no food additives was purchased from a local market in Kayseri, Turkey. *Z. bailii* DSM 70492 and *Z. rouxii* DSM 70540 strains were provided from Food Engineering Department of Erciyes University, Turkey. MEO was produced by using Clavenger apparatus in the laboratory. For this purpose, 200 g of powdered mint (*Mentha piperita*) was placed into the bowl of the system and 3 L of distilled water was added. The system was run for 6 h and at the end of the duration, MEO was collected into the tubes and stored at +4 °C for further usage (Sagdic et al., 2009). Carvacrol (5-Isopropyl-2-methylphenol, Merck Chem., Germany) and natamycin (Delvocid®, DSM Co., Holland) were purchased as another two preservative agents.

2.2. Methods

2.2.1. Inoculation of osmotolerant yeasts in apple juice

To prepare the inoculation of the yeasts, firstly apple juice was divided into the 100 mL of glass bottles. And then reactivation of *Z. bailii* and *Z. rouxii* strains was conducted by adding them to the malt extract

Table 2
Ethanol levels[a] of apple juice inoculated with *Z. rouxii* and *Z. bailii* for three different models.

				Ethanol levels (%) in Model 1			Ethanol levels (%) in Model 2			Ethanol levels (%) in Model 3		
Runs	Time (day)	Temperature (°C)	Sodium benzoate (%)	Mint essential oil (ppm)	*Z. rouxii*	*Z. bailii*	Carvacrol (ppm)	*Z.rouxii*	*Z.bailii*	Natamycin (ppm)	*Z.rouxii*	*Z.bailii*
1	1	4	0.05	500	0.28 ± 0.02	–	375	0.26 ± 0.01	0.41 ± 0.04	30	4.63 ± 0.85	7.56 ± 1.11
2	1	12	0	500	–	–	375	0.34 ± 0.06	0.46 ± 0.03	30	5.59 ± 0.98	6.19 ± 0.85
3	1	12	0.05	0	6.05 ± 0.09	5.42 ± 0.07	0	2.98 ± 0.07	3.84 ± 0.09	0	4.18 ± 0.65	5.16 ± 0.32
4	1	12	0.05	1000	0.23 ± 0.01	0.21 ± 0.01	750	0.80 ± 0.02	0.37 ± 0.06	60	4.61 ± 0.52	4.62 ± 0.12
5	1	12	0.1	500	0.28 ± 0.01	0.25 ± 0.00	375	0.40 ± 0.01	0.40 ± 0.01	30	3.72 ± 0.54	3.78 ± 0.14
6	1	20	0.05	500	0.30 ± 0.00	0.27 ± 0.00	375	0.42 ± 0.02	0.42 ± 0.01	30	4.16 ± 0.52	4.31 ± 0.09
7	21	4	0	500	0.25 ± 0.00	0.39 ± 0.00	375	0.52 ± 0.06	0.59 ± 0.03	30	4.69 ± 0.54	8.03 ± 0.41
8	21	4	0.05	0	2.55 ± 0.09	4.85 ± 0.07	0	3.66 ± 0.04	4.79 ± 0.85	0	6.54 ± 0.65	5.62 ± 0.15
9	21	4	0.05	1000	0.14 ± 0.00	0.17 ± 0.00	750	0.43 ± 0.08	0.43 ± 0.06	60	1.75 ± 0.23	3.54 ± 0.21
10	21	4	0.1	500	–	–	375	0.47 ± 0.09	0.52 ± 0.04	30	3.22 ± 0.12	4.94 ± 0.16
11	21	12	0	0	48.33 ± 2.12	60.01 ± 6.22	0	50.58 ± 1.85	57.51 ± 4.45	0	30.5 ± 3.33	63.59 ± 4.44
12	21	12	0	1000	–	–	750	0.41 ± 0.02	0.43 ± 0.06	60	3.36 ± 0.74	7.39 ± 0.96
13	21	12	0.05	500	–	–	375	0.52 ± 0.02	0.53 ± 0.03	30	5.64 ± 0.65	3.2 ± 0.32
14	21	12	0.05	500	–	–	375	0.53 ± 0.06	0.52 ± 0.05	30	5.71 ± 0.55	4.01 ± 0.14
15	21	12	0.05	500	–	–	375	0.54 ± 0.04	0.60 ± 0.05	30	7.35 ± 1.11	3.02 ± 0.54
16	21	12	0.1	0	3.19 ± 0.01	3.90 ± 1.1	0	3.33 ± 0.00	3.95 ± 0.15	0	4.02 ± 0.95	3.87 ± 0.58
17	21	12	0.1	1000	–	–	750	0.40 ± 0.00	0.39 ± 0.03	60	4.32 ± 0.85	2.97 ± 0.06
18	21	20	0	500	8.76 ± 0.06	12.16 ± 0.98	375	0.57 ± 0.08	0.57 ± 0.05	30	9.26 ± 0.62	8.97 ± 0.45
19	21	20	0.05	0	3.99 ± 0.02	43.21 ± 2.85	0	5.54 ± 0.80	35.00 ± 3.65	0	5.73 ± 0.32	61.35 ± 5.21
20	21	20	0.05	1000	–	–	750	0.43 ± 0.02	0.43 ± 0.01	60	5.78 ± 0.62	5.196 ± 0.65
21	21	20	0.1	500	–	–	375	0.49 ± 0.04	0.43 ± 0.01	30	4.48 ± 0.41	3.99 ± 0.52
22	41	4	0.05	500	–	–	375	0.53 ± 0.06	0.62 ± 0.06	30	5.99 ± 0.52	5.32 ± 0.41
23	41	12	0	500	–	15.13 ± 1.14	375	0.48 ± 0.05	0.47 ± 0.05	30	9.06 ± 0.74	10.49 ± 0.69
24	41	12	0.05	0	98.1 ± 2.6	98.9 ± 6.66	0	5.93 ± 0.09	63.65 ± 4.96	0	7.37 ± 0.65	69.75 ± 6.23
25	41	12	0.05	1000	–	–	750	0.48 ± 0.04	0.46 ± 0.02	60	0.42 ± 0.01	6.91 ± 0.52
26	41	12	0.1	500	–	–	375	0.60 ± 0.05	0.48 ± 0.02	30	3.31 ± 0.08	4.6 ± 0.36
27	41	20	0.05	500	–	14.21 ± 1.32	375	0.67 ± 0.01	0.51 ± 0.03	30	5.56 ± 0.12	5.1 ± 0.54

"-" Not detected.
[a] Ethanol level was indicated as "%" of the aromatic compounds detected by GC–MS equipment.

Table 3
Effect of time, temperature, sodium benzoate and mint essential oil (MEO) on ethanol level of apple juice inoculated with *Z. rouxii* and *Z. bailii*.

	Apple juice samples inoculated with *Z. rouxii*		Apple juice samples inoculated with *Z. bailii*	
	Df	Ethanol content (%)	Df	Ethanol content (%)
Model	4	9.4[a]	9	22.6[a]
Linear				
A	1	1.1	1	18.8[a]
B		–	1	12.1[a]
C		–	1	17.3[a]
D	1	20.4[a]	1	88.1[a]
Cross product				
AB		–		–
AC		–	1	5.7[a]
AD	1	6.9[b]	1	19.5[a]
BC		–		–
BD		–	1	6.8[b]
CD		–	1	9.8[a]
Quadratic				
A^2		–		–
B^2		–		–
C^2		–		–
D^2	1	9.2[a]	1	25.1[a]
Residual	22		17	
Lack of fit	20		15	
Pure error	2		2	
Cor error	26		26	
R^2		0.63		0.92

A: Storage time (day), B: Storage temperature (°C), C: Sodium benzoate (%), D: Mint essential oil (ppm).
"-" Not significant parameter removed by stepwise elimination procedure.
[a] $p < 0.01$.
[b] $p < 0.05$.

broth (composed of malt extract, maltose, yeast extract and dextrose, Merck, Germany) and incubated at 25 °C for 20 h. At the end, fresh yeast culture was counted on malt extract agar (Malt extract 30.0 g/L; peptone from soymeal 3.0 g/L; agar-agar 15 g/L, Merck, Germany) and inoculated (10^6–10^7 cfu/mL) into the apple juice samples at 1% (v/v) concentration.

2.2.2. Experimental design by response surface methodology

As a result of the response surface methodology principle, the experimental designs were firstly based on preliminary tests and then the actual trials were performed. Box Behnken design was used with four different processing variables namely storage temperature (4, 12 and 20 °C), storage time (1, 21 and 41 days), preservative concentrations (MEO 0, 500, 1000 ppm; carvacrol 0, 375 and 750 ppm and natamycin 0, 30 and 60 ppm) and a positive control (sodium benzoate, (0, 0.05 and 0.1% w/v). The experimental design was given in Table 1. The effects of MEO, carvacrol and natamycin on the ethanol production performance of *Z. rouxii* and *Z. bailii* were tested at different storage times and temperatures and predictive regression models were constructed based on the findings. For the MEO and carvacrol concentrations, a preliminary sensory analysis was performed and the maximum concentration for the acceptable juice samples in terms of overall preference was determined. For preliminary sensory panel, the aromatic ingredients were incorporated at different concentrations (6 different concentrations) and the samples were tested by the panel members (5 men and 5 women) in terms of taste, aroma and overall preference scores. The maximum preservative agent concentration was decided according to the panel scores (data not shown). For sodium benzoate, the allowed legal limit (0.15%) for the foods was used while there was no limit for natamycin for beverages. Three different models were created for the natural preservatives through 27 experimental runs. According to the models, 27 juice samples in 100 ml glass bottles were prepared and the fresh stock cultures were inoculated in sterile conditions and moved to the appropriate storages according to the design. At the end of each storage duration for each sample, ethanol levels were characterized by instrumental techniques. For three different natural antimicrobial agents, 3 main model designs were created, and the effects of studied natural preservatives on *Z. bailii* and *Z. rouxii* were determined separately in each main model, thus, 6 model designs were studied in total.

2.2.3. Determination of ethanol level in juice sample

Ethanol level of the samples produced by the osmotolerant yeasts was detected using gas chromatography–mass spectrometry (GC–MS) equipment (Agilent 7890 gas chromatograph (Agilent Technologies, Palo Alto, CA) coupled with Agilent 5975 mass selective detector operating in electron impact mode (ionisation voltage, 70 eV). A CP-Wax 52 CB capillary column (50 m length, 0.32 mm inner dia, 1.2 μm film diameter) was used. The temperature program started from 50 °C, then programmed at 3 °C/min to 240 °C, which was maintained for 1 min. Injector, interface, and ion source temperatures were 250, 250, and 230 °C, respectively. Injections were performed in split mode and helium (1 ml/min) was used as the carrier gas. The mass selective detector was operated in the scan mode between 20 and 400 *m/z*. Data acquisition started 4 min after injection. The compounds adsorbed by the fibers (Polydimethylsiloxane/Divinylbenzene (PDMS/DVB)) at a temperature-controlled heater at 50 °C for 25 min were desorbed from the injection port for 20 min at 50 °C and injected into GC–MS in the splitless mode. The ethanol compound was identified through comparisons with the spectra from the libraries (Flavor 2, NIST 05a, and Wiley 7n). All analyses were replicated two times for each sample.

2.2.4. Data analyses, modeling and optimization

In this study, a 4-factor-3-level Box-Behnken experimental design (Box & Behnken, 1960) with three replicates at the center point was used to develop predictive models based on the polynomial equation in Eq.1 for studied parameters. The processing variables, levels and experimental design in terms of coded and uncoded values are presented in Table 1.

$$y = \sum_{i=1}^{4} \beta_{ki} X_i + \sum_{i=1}^{4} \beta_{kii} X_i^2 + \sum_{i,j \le 2}^{4} \beta_{kij} X_i X_j \quad (1)$$

where; Y is the corresponding predicted response value, β_0 is the intercept term, β_i is the linear term, β_{ii} is the quadratic term, β_{ij} is the interaction term, and X_i and X_j are the coded levels of the independent variables. The regression coefficients of linear, quadratic and interaction terms were determined by using Design-Expert® Software (Design Expert 7.1.3, 2007) for each output parameter. A total of 27 juice samples were prepared. To eliminate the insignificant parameters in the fitted equations, stepwise elimination process was applied at the significance level of 0.05. The response values were subjected to the square transformation due to the huge differences among the recorded ethanol content of the sample. The computational work including designation of experimental points, randomization, analysis of variance, fitting of the second-order polynomial models and graphical representations as well as optimization were performed by using Design-Expert® Software (Design Expert 7.1.3, 2007) statistical software.

3. Results and discussion

3.1. Effect of mint essential oil (MEO)

Apple juice samples inoculated with *Z. rouxii* or *Z. bailii* were subjected to GC–MS analysis to determine the ethanol levels based on yeast growth, storage duration, preservative type and concentration. Table 2 shows the ethanol contents (%) of the apple juice samples formed by

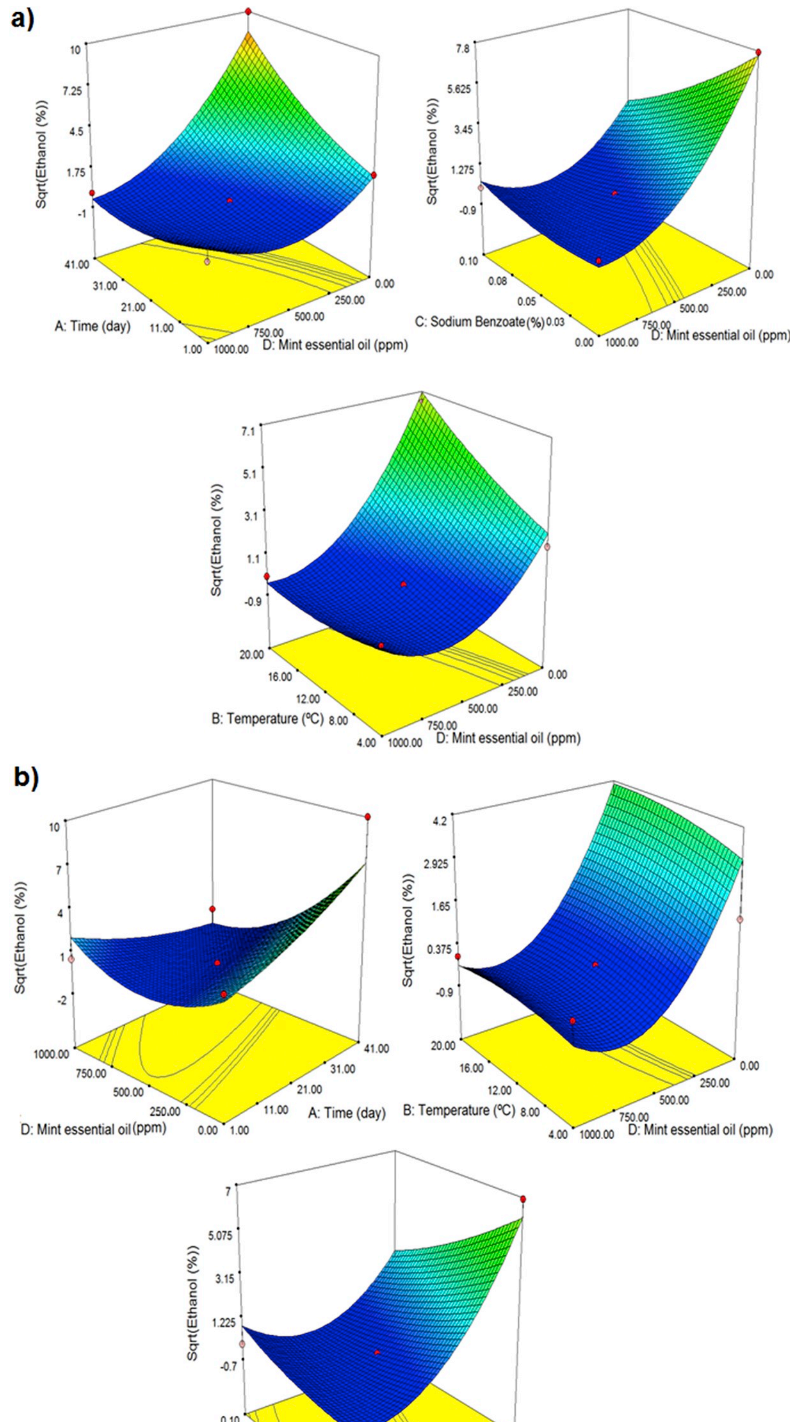

Fig. 1. Effects of processing variables (time, temperature, sodium benzoate and mint essential oil) on ethanol content of apple juice inoculated with *Z. rouxii* (a) *Z. bailii* (b).

the inoculated yeasts during the storage of the samples for all models. It is clear from the table that osmotolerant yeasts (*Z. rouxii* or *Z. bailii*) caused deterioration in some apple juice samples and ethanol formation was observed. As can be seen from the table, different ethanol concentrations were detected in the samples depending on the storage time, temperature, preservative type and their levels. For the samples preserved with MEO, the highest ethanol level (> 98%) for both juice samples inoculated with *Z. rouxii* and *Z. bailii* was determined in the samples having no MEO at the end of the storage at 12 °C. Ethanol was nearly the only major compound in the samples due to the rapid progression of the deterioration. No ethanol was detected in some samples because of the strong inhibition of the yeast due to the preservative agents (MEO). Ethanol levels of the samples showed a significant variation ($p < 0.01$) depending on the processing variables and their

Table 4
Effect of time, temperature, sodium benzoate and carvacrol on ethanol level of apple juice inoculated with *Z. rouxii* and *Z. bailii*.

	Apple juice samples inoculated with *Z. rouxii*		Apple juice samples inoculated with *Z. bailii*	
	Df	Ethanol content (%)	Df	Ethanol content (%)
Model	5	13.9[a]	8	15.5[a]
Linear				
A	1	0.1	1	0.1
B		–	1	0.1
C	1	0.1	1	0.1
D	1	1.6	1	4.1[b]
Cross product				
AB		–		–
AC		–		–
AD		–	1	11.1[a]
BC		–		–
BD		–	1	4.4[b]
CD	1	12.5[a]	1	9.7[a]
Quadratic				
A^2		–		–
B^2		–		–
C^2		–		–
D^2	1	14.1[a]	1	30.7[a]
Residual	22		18	
Lack of fit	20		16	
Pure error	2		2	
Cor error	26		26	
R^2		0.72		0.87

A: Storage time (day), B: Storage temperature (°C), C: Sodium benzoate (%), D: Carvacrol (ppm).
"-" Not significant parameter removed by stepwise elimination procedure.
[a] $p < 0.01$.
[b] $p < 0.05$.

concentrations for both *Z. bailii* and *Z. rouxii* inoculated samples. MEO showed a significant inhibition effect on *Z. rouxii* while the other factors did not have any significant effects ($p > 0.05$). Only the interactive effect of storage time and MEO was found to be significant ($p < 0.05$). As compared to *Z. rouxii,* MEO, time, temperature and sodium benzoate showed significant effects on the ethanol formation of *Z. bailii* ($p < 0.05$) (Table 3). Fig. 1 shows the changes in ethanol levels of yeast-inoculated apple juices during storage based on processing variables. As could be seen from Fig. 1a, increasing MEO levels of the juice decreased the ethanol concentration of apple juice containing *Z.rouxii*. Additionally, ethanol formation increased with the increasing of duration because of the growth of yeasts. Similar effects were determined for the apple juice samples inoculated with *Z.bailii* and MEO showed a significant reduction effect on ethanol formation during the storage (Fig. 1b). As could be seen from Table 2, in some apple juice samples preserved with MEO, a natural antioxidant and antimicrobial agent, ethanol formation was not detected. Mint essential oil is a natural and strong antimicrobial agent. In the literature, it was reported to be a strong inhibitory substance on some microorganisms. Tassou et al. (2000) used MEO in some model foods namely tzatziki (pH 4.9), taramosalata (pH 5.0) and pate (pH 6.8) and they reported that the MEO reduced the total viable counts of *Staphylococcus aureus* about 6–7 logs while it caused a reduction in the count of *Salmonella* Enteritidis only 3 logs. They also stated that the inhibitory effect of MEO was influenced by the incubation temperature as well as by the concentration of essential oil added into the growth medium. In another study conducted by Tassou et al. (1995), MEO inhibited the growth of *Salmonella* Enteritidis and *Listeria monocytogenes* in a culture medium and model foods. Iscan et al. (2002) showed the strong inhibition activity of the peppermint oils on plant pathogenic microorganisms. The major essential compounds of MEO extracted from *Mentha piperita* were reported as methnone and menthol at the level of 44.1 and 29.5%, respectively. The *in vitro* antimicrobial properties of MEO, menthone and menthol showed an inhibition effect on the studied microorganisms at different levels and it was stated that the menthol was the responsible compound for the antimicrobial activity of MEO (Kamatou et al., 2013; Iscan et al., 2002). In another study, a strong antimicrobial activity of MEO and its main constituents, menthol showed strongest inhibitory activity against two antibiotic susceptible and two antibiotic-resistant strains of *Streptococcus pneumoniae* (Sung-Hee and Seung-Won, 2007). Hajlaoui et al. (2008) reported that the *Mentha longifolia* subsp. *longifolia* oil, having menthol (19.4–32.5%) and menthone (20–28%) as major constituents, showed a good antimicrobial activity against some human bacterial pathogens. Trombetta et al. (2005) speculated that the antimicrobial effect of monoterpenes such as (+)-menthol, thymol and linalyl acetate, was attributed to the disruption of the lipid fraction of the plasma membrane by these compounds and these result in altered permeability and leakage of intracellular materials (Kamatou et al., 2013). The main mechanism of the anti-yeast activity of MEO was related to its bioactive compounds. In the literatures, the mechanism of the phenolic substances of MEO on the inhibition of yeasts were not well reported in details, but the inhibition effects of these substance were attributed to inhibition of ATPase activity on cytoplasmic membrane having a vital role on active transport of nutrient for yeast (Cerutti and Alzomora, 1996; Karaman et al., 2016). Conner and Beuchat (1984) suggested that essential oils damaged a variety of enzyme systems of yeasts, which eventually influence structural component synthesis and energy production.

3.2. Effect of carvacrol

Table 2 also shows the ethanol contents of the apple juice samples inoculated with *Z. rouxii* and *Z. bailii* and preserved with carvacrol, a natural aromatic and monoterpenoid phenol present in oregano as active and major compound. As could be seen from the table, the highest ethanol occurrence level for *Z. rouxii* (> 50%) was observed for the sample having no carvacrol and sodium benzoate, a positive control stored at 12 °C for 21 days. Depending on the processing factor levels, ethanol level was determined at different concentrations ranging from 0.34–63.65% for both samples inoculated with both *Z. rouxii* and *Z. bailii*. As compared to both yeasts for the prevention of ethanol occurrence, for the model 2 including carvacrol as preservative, carvacrol preserved the apple juice inoculated with *Z. rouxii* compared to sample inoculated with *Z. bailii*. In most of the samples, ethanol levels were recorded as higher in the samples including *Z. bailii* compared to samples having *Z. rouxii.* For example, ethanol level was determined as 63.65% in the sample including *Z. bailii* prepared for run 24 while it was 5.93% for the sample inoculated with *Z. rouxii.* It could be said that the carvacrol showed a better inhibition activity on *Z. rouxii* as compared to *Z. bailii.* Statistical effects of processing variables for model 2 namely time, temperature, sodium benzoate and carvacrol on the ethanol formation by the yeasts were tabulated in Table 4. It was seen that the model for both yeasts was determined to be significant ($p < 0.01$). The linear effect of carvacrol was found to be significant for *Z.bailii* ($p < 0.05$). Additionally, the interactive effects of sodium benzoate and carvacrol and also the quadratic effect of carvacrol showed significant effects on the ethanol formation of the yeasts. Fig. 2 illustrates the change of ethanol concentration depending on the studied factors. In general, carvacrol increase in the apple juices inoculated with yeast caused a sharp decrement in the ethanol content of the samples (Fig. 2a and b). Arfa et al. (2006) studied the effects of antimicrobial activity of some essential oil compounds like carvacrol, menthol and eugenol on some pathogenic bacteria and eugenol and menthol exhibited a weaker antimicrobial activity as compared to carvacrol and the differences between the activities were attributed to the hydrophobicity of the compounds. As is observed for MEO, a pure compound like carvacrol or menthol shows a weaker antimicrobial

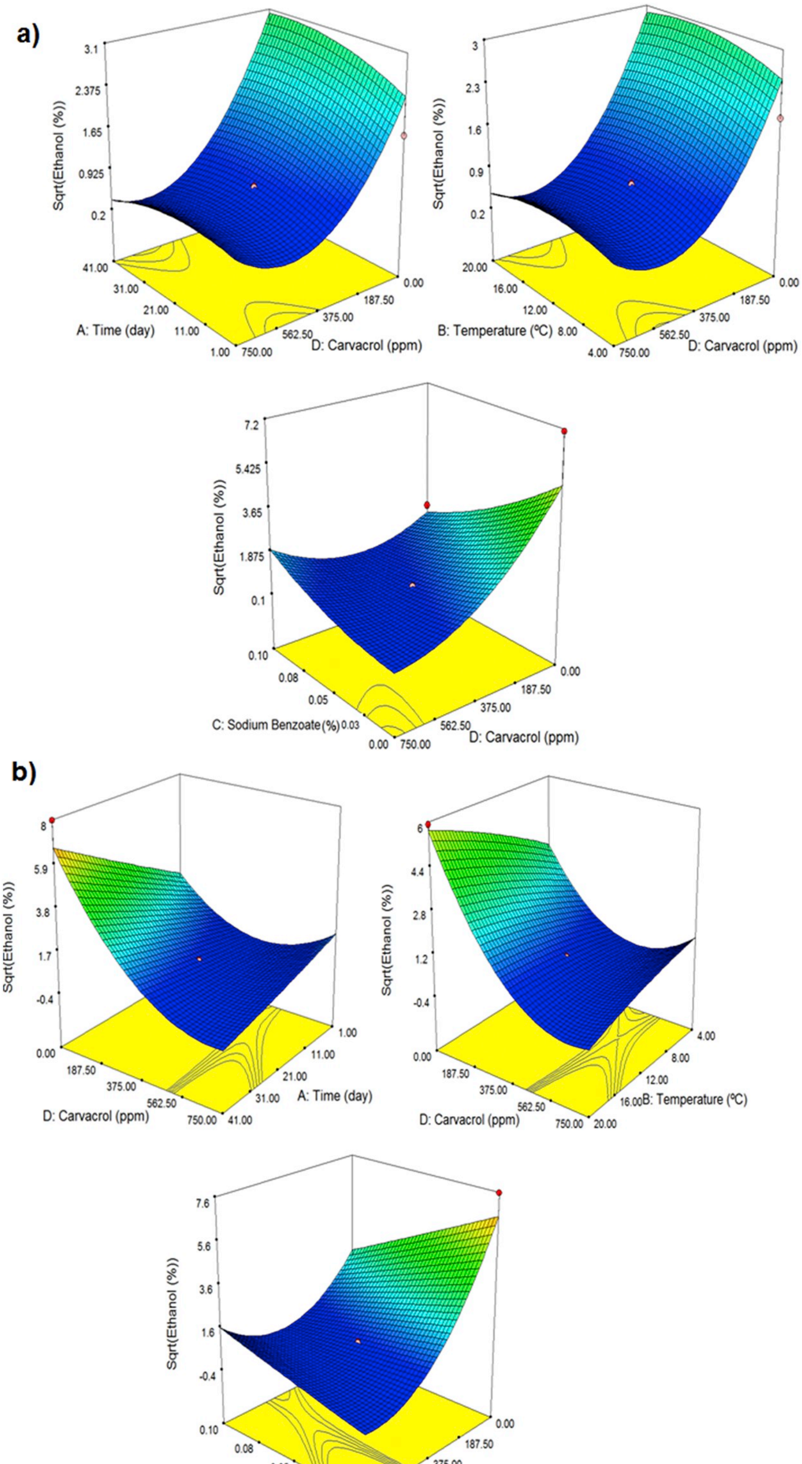

Fig. 2. Effects of processing variables (time, temperature, sodium benzoate and carvacrol) on ethanol content of apple juice inoculated with *Z. rouxii* (a) *Z. bailii* (b).

activity compared to essential oils. Ultee et al. (2000) investigated the antimicrobial activity of carvacrol and it showed a dose-related inhibition on growth of the pathogen. Kiskó and Roller (2012) studied the antimicrobial effects of chitosan, cinnamic acid, carvacrol and cymene as new preservatives against *Zygosaccharomyces lentus* and they reported that the yeast was resistant to the antimicrobial action of chitosan and cinnamic acid but it was sensitive to the biocidal action of cymene and carvacrol.

3.3. Effect of natamycin

Natamycin, one of the most effective natural food preservatives for preventing the growth of yeast and mold, was the other antimicrobial agent tried to prevent the ethanol formation by inhibiting the inoculated yeasts in apple juice during storage. Change in ethanol levels of apple juice samples preserved with natamycin was given in Table 2. As could be seen from the table, the highest ethanol occurrence was

Table 5
Effect of time, temperature, sodium benzoate and natamycin on ethanol level of apple juice inoculated with *Z. rouxii* and *Z. bailii.*

	Apple juice samples inoculated with *Z. rouxii*		Apple juice samples inoculated with *Z. bailii*	
	Df	Ethanol content (%)	Df	Ethanol content (%)
Model	3	13.1[a]	4	7.1[a]
Linear				
A		–		–
B		–		–
C	1	12.2[a]	1	3.2[c]
D	1	14.3[a]	1	6.3[b]
Cross product				
AB		–		–
AC		–		–
AD		–		–
BC		–		–
BD		–		–
CD	1	13.1[a]	1	3.5[c]
Quadratic				
A^2		–		–
B^2		–		–
C^2		–		–
D^2		–	1	6.8[b]
Residual	23		24	
Lack of fit	21		20	
Pure error	2		2	
Cor error	26		26	
R^2		0.64		0.57

A: Storage time (day), B: Storage temperature (°C), C: Sodium benzoate (%), D: Natamycin (ppm).
"-" Not significant parameter removed by stepwise elimination procedure.
[a] $p < 0.01$.
[b] $p < 0.05$.
[c] $p < 0.1$.

observed in the samples having no natamycin (run 11 and run 24). The natamycin inhibition performance was better for *Z.rouxii* compared to *Z. bailii* because the ethanol concentration was 30.5 and 63.59% for the juice sample inoculated with *Z. rouxii* than *Z. bailii*, respectively. In fact, in most of the samples, ethanol levels were higher in the samples including *Z. bailii* due to the weak inhibition performance of natamycin on this yeast. The effects of processing parameters in model 3 namely time, temperature, sodium benzoate and natamycin were given in Table 5. As could be seen, the model was determined to be significant for both yeasts ($p < 0.05$). In addition to this, the linear effects of sodium benzoate (SB) and natamycin influenced the ethanol level significantly for both studied yeasts ($p < 0.05$). For the apple juice samples inoculated with both yeasts, linear effect of natamycin was determined to be significant ($p < 0.05$). For the samples having *Z. rouxii*, interactive effect of sodium benzoate and natamycin was also found to be significant ($p < 0.01$). The change of ethanol concentration in apple juices inoculated with the studied yeasts was illustrated in Fig. 3. Increase of natamycin level decreased the ethanol content of the samples clearly for both yeasts due to the inhibition effect of natamycin on these yeasts. For the sample having *Z. bailii*, natamycin usage prevented the increment in ethanol concentration by showing an inhibition activity on the yeast significantly. Azzouz and Bullerman (1982) investigated the antifungal effects of ground herbs and spices, other plant materials, and some commercial antifungal agents including natamycin and reported that the natamycin produced by *Streptomyces natalensis,* a strong antifungal agent, was effective at very low levels (5 mg/L) and also it was stated that this compound had high potential for future applications as a natural food preservative in the U.S. In the same study, mold growth started after maximum 4 days for the samples preserved with sodium benzoate, calcium propionate and lauricidin, but there was no growth in the samples treated with natamycin showing a strong antifungal activity as compared to others (Azzouz and Bullerman, 1982). Zalazar et al. (2019) performed a research about the effect of natamycin on inhibition of *Z. bailii* and reported that the natamycin was effective on the yeast depending on the concentration and indicated that 12 mg/L natamycin treatment inhibited the growth of the yeast in model medium. In a different study, Gallo and Jagus (2006) reported that 12.5 mg/L was necessary to inhibit the growth of *Saccharomyces cerevisiae* in cheese whey. Arroyo-López et al. (2012) also reported that the natamycin was an effective antifungal agent and 10–22 mg/L was needed to inhibit *S. cerevisiae, Candida boidinii* and *Wickerhamomyces anomalus* in liquid culture. Ollé Resa et al. (2013) studied the effect of natamycin on the physical properties of starch edible films and their effect on *Saccharomyces cerevisiae* activity and reported that the films containing 1.85 mg natamycin/dm^2 of natamycin developed a fungistatic effect until 72 h of storage.

3.4. Regression modeling and optimization

Table 6 shows the regression models constructed for the estimation of the ethanol level produced by the inoculated yeasts during the storage. As could be seen from the table, some models were accepted due to higher coefficient of determination than 0.70. The best estimation performance was found for the MEO in the sample inoculated by *Z. bailii* ($R^2 = 0.92$, Table 3). An optimization study was conducted to determine the best conditions for the apple juice samples. For this purpose, minimization procedure was applied to detect the limits for the processing variables. For model 1 used MEO as preservative in apple juice inoculated with *Z. rouxii*, the lowest ethanol content would be at in sample having 1000 ppm MEO and no sodium benzoate for 41 days storage at 20 °C. For the highest sodium benzoate usage (0.01%), MEO level was determined to be 763 ppm. For the sample having *Z. rouxii*, the lowest level would be at the level of MEO as 834 and 610 ppm for 0.00 and 0.01% sodium benzoate usage at the same storage conditions, respectively. For the model 2, the lowest ethanol level would be in sample having 556 ppm carvacrol and 0.00% sodium benzoate for 41 days storage at 20 °C. It was calculated as 534.9 ppm for the sample having 0.1% benzoate for the sample inoculated with *Z. bailii.* These values were calculated as 568.1 and 381.2 ppm for the sample inoculated with *Z. rouxii* and added with 0.00% and 0.1% sodium benzoate, respectively for 41 days storage and 20 °C. For the model 3 used natamycin as preservative, the lowest ethanol concentration would be at 87.9 or 63.45 ppm for the sample inoculated with *Z. bailii* and added with 0.00% and 0.10% sodium benzoate, respectively. For the apple juice samples inoculated with *Z. rouxii*, they were calculated as 111.6 and 213 ppm, respectively. As could be seen, for three natural preservatives, *Z. rouxii* inhibition was achieved by the use of the lower concentrations of the related preserving agents as compared to *Z. bailii.* In other words, to keep the ethanol level low in the apple juice during storage, all preservative levels were determined to be lower in the sample inoculated with *Z. rouxii* as compared to *Z. bailii*. Stratford and Capell (2003) reported that *Z. bailii* was the most preservative-resistant organism known as able to resist high concentrations of acetic acid and ethanol. Strains of *Z. bailii* can tolerate sorbic and benzoic acids at pH 4.0 at up to 1000 ppm. *Z. bailii* forms ascospores and these are not heat-resistant and it grows well in sugar syrups and juice concentrates, containing 50–60% sugar. In different studies, highly resistance of *Z. bailii* was reported to sorbic, benzoic, acetic and propionic acids (Ingram, 1960; Neves et al., 1994; Malfeito-Ferreira et al., 1997) and to sulphite (Hammond and Carr, 1976; Goto, 1980; Goswell, 1986). The causes of resistance in *Z. bailii* have been investigated on several occasions and the overall results can be circumscribed by two possible hypotheses; 1. Degradation and metabolism of the preservatives, and 2. Efflux pumps removing preservatives (Stratford et al., 2013).

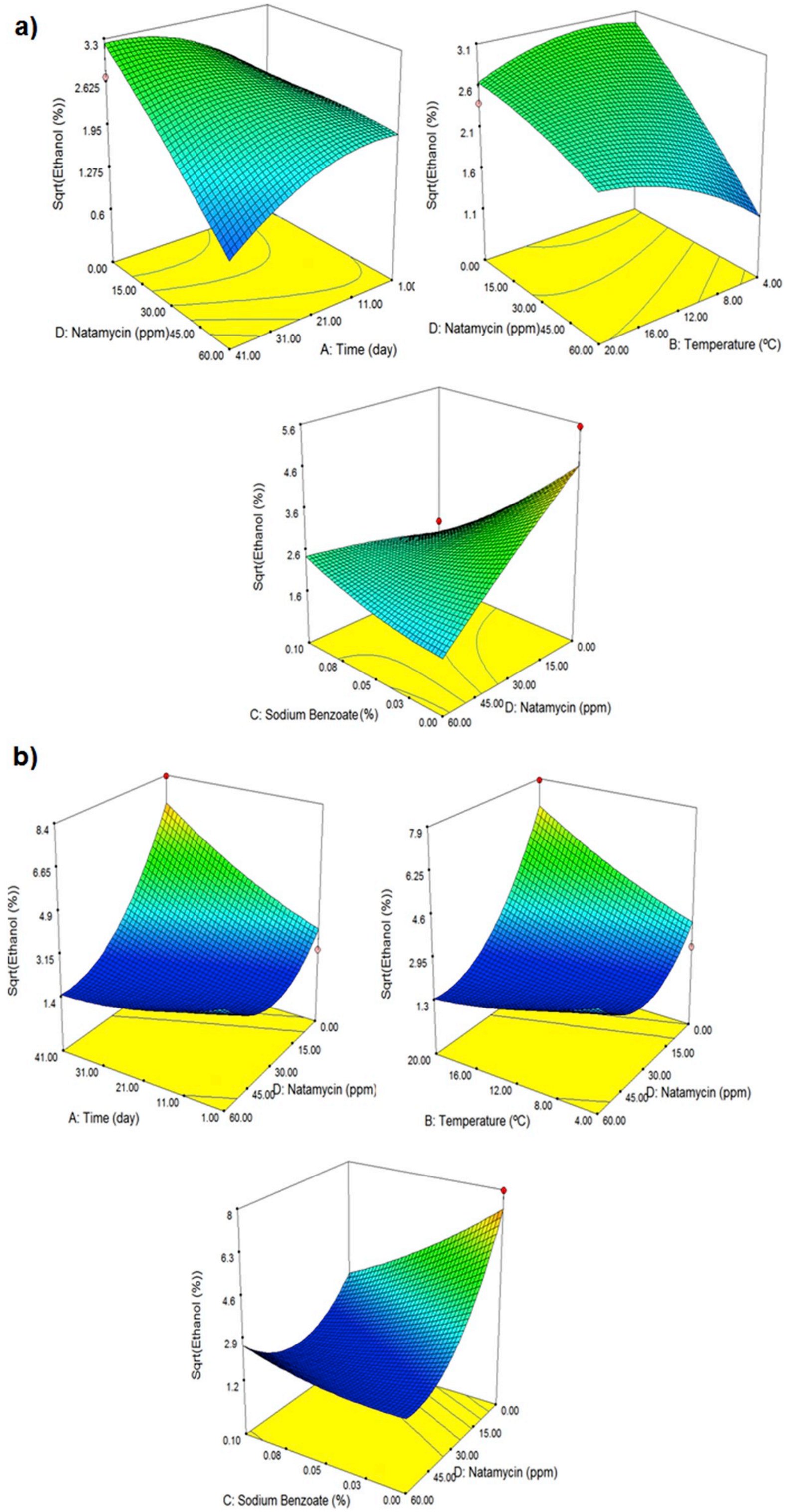

Fig. 3. Effects of processing variables (time, temperature, sodium benzoate and natamycin) on ethanol content of apple juice inoculated with *Z. rouxii* (a) *Z. bailii* (b).

4. Conclusion

The research results showed that ethanol produced by *Z. bailii* and *Z. rouxii*, which are osmotolerant yeasts in sugar rich or low water activity foods like apple juice or apple juice concentrate could be prevented or limited by using some natural preservatives such as mint essential oil, carvacrol or natamycin. Increase of these preserving agent concentrations clearly provided a decrement in the ethanol levels of apple juice samples during the storage due to their inhibition activity on the yeasts. In general, ethanol levels were determined as to be lower in the samples inoculated with *Z. rouxii* as compared to *Z. bailii* because of its strong resistance activity. Optimization results indicated that ethanol production of the yeasts could be limited using these preserving agents with sodium benzoate or without depending on the processing variable levels. Because the growth of osmotolerant yeasts is an important problem for fruit juice industry, the results of this study suggest that the

Table 6
Fitted equations indicating effect of each processing variables on ethanol content of apple juice samples inoculated with *Z. rouxii* and *Z. bailii*.

Models	Fitted model equations[a]	R^2
	Equations for apple juice samples inoculated with *Z. rouxii*	
Model 1 (mint essential oil)	$Y_{ethanol} = 0.34 + 0.45A\text{-}1.99D\text{-}2.1AD + 1.8D^2$	0.63
Model 2 (carvacrol)	$Y_{ethanol} = 0.45 + 0.1C\text{-}0.49D + 1.76CD + 1.95D^2$	0.72
Model 3 (natamycin)	$Y_{ethanol} = -6.57 + 14.2C\text{-}9.45D + 15.7CD$	0.64
	Equations for apple juice samples inoculated with *Z. bailii*	
Model 1 (mint essential oil)	$Y_{ethanol} = 0.85 + 1.15A + 0.93B\text{-}1.11C\text{-}2.5D\text{-}1.1\,AC\text{-}2.1AD\text{-}1.2BD + 1.44CD + 1.8D^2$	0.92
Model 2 (carvacrol)	$Y_{ethanol} = 0.26 + 0.1A\text{-}0.1B\text{-}0.1C\text{-}0.9D\text{-}1.98AD\text{-}1.24BD + 1.85CD + 3.41D^2$	0.87
Model 3 (natamycin)	$Y_{ethanol} = 313.5 + 18.C + 686D + 20.9CD + 377D^2$	0.57

A, B, C and D were storage time (day), storage temperature (°C), sodium benzoate (%) and mint essential oil (ppm) in model 1, carvacrol (ppm) in model 2 and natamycin (ppm) in model 3, respectively.
[a] Final equations in terms of coded factors after stepwise elimination process and square transformation was applied.

natural preservatives could be used in prevention of ethanol occurrence by inhibiting the growth of osmotolerant yeasts in apple juice during the storage period as an alternative to synthetic preservatives.

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