Food Sci. Biotechnol. Vol. 17, No. 5, pp. 899 ~ 903 (2008)

Food Science and Biotechnology

Optimization of Extraction Conditions for the Antibacterial Activity by Clove against *Streptococcus mutans* Using Evolutionary Operation-Factorial Design Technique

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Abstract This study was conducted in order to elucidate the optimum conditions for the extraction of clove that can be used to elicit antibacterial activity against *Streptococcus mutans* using the evolutionary operation (EVOP)-factorial design technique. Higher antibacterial activity was achieved in a higher extraction temperature of 80°C (r=0.7983**) and in a longer extraction time of 26 hr (r=0.6867*). Antibacterial activity was not effected by differentiation of ethanol concentration in the extraction solvent (r= −0.0683). The maximum antibacterial activity of clove against *S. mutans* as determined by the EVOP-factorial design technique was obtained at an extraction temperature of 80°C, an extraction time of 26 hr and a 50% ethanol concentration. Furthermore, the population of *S. mutans* decreased from an initial concentration of 6.850 to 4.195 log CFU/mL in the third set that is more than 2.6 log cycles by EVOP-factorial design technique.

Keywords: clove, *Streptococcus mutans*, evolutionary operation (EVOP)-factorial design technique, antibacterial activity

Introduction

Dental caries found within the oral cavity is a very common disease that afflicts a large proportion of the world's population. Although the prevalence of coronal caries has steadily decreased in many populations in the industrialized world over the last few decades, high-risk groups still exist within such populations (1). Extensive research indicates that dental caries results from a bacterial infection (2) but it is also influenced by the host and dietary factors (3).

Dental caries is an infective condition that is initiated by an acid attack of the tooth enamel, arising largely from the metabolism of sugars by bacteria such as *Streptococcus mutans* (4), *Streptococcus sanguis* (5), *Streptococcus salivarius* (6), and *Lactobacillus* spp. (7). Individuals with high levels of these bacteria in their saliva (>10^6 CFU/mL) are believed to be at high risk for dental caries. *S. mutans* produces strong acids, such as lactic acid and acetic acid that can reduce the pH of the saliva to a level below the critical value of 5.5 for acid demineralization (8).

Traditional uses of clove oil include use in dental care, as an antiseptic and analgesic, where the undiluted oil may be rubbed on the gums to treat toothache (9). It is identfied that clove was not only active against oral bacteria associated with dental caries and periodontitis (10) but also effective against a various kind of food poisoning bacteria such as *Escherichia coli*, *Campylobacter jejuni, Salmonella enteritidis, Staphylococcus aureus,* and *Listeria monocytogenes,* (11-13). Moreover, it was reported that the clove and clove extracts have antifungal (14), anticarcinogenic (15), antiallergic (16), and antimutagenic activity (17). The traditional approach to biological system optimization is based on a single factor search (18), which is not applicable to this system where several factors are simultaneously involved. The response surface methodology (RSM) based on factorial experiments is the statistical approach to the study of the effects of test variables on measured responses (19-21). The mathematical model for the RSM is derived from orthogonal polynomial fitting techniques. The significance of the factorial interaction of variables is considered in order to arrive at the optimum condition. The primary advantage of using the evolutionary operation (EVOP) methodology is its clear-cut decision procedure, which directs the changes of variables towards the maximum objective or minimum values. EVOP was first proposed as a process improvement method for continuous improvement of conditions (22). The methodology can be considered to be a multivariable sequential search technique in which the effects of 2 or 3 factors are studied together and the responses are statistically analyzed in order to arrive at a decision. A trial consists of small factorial designs that are run sequentially until the best alternative is determined (23). This is then followed by another new phase of small repeated changes, and the results of the earlier phase of experiments are essential to the design of the next phase of experiments. Thus, the process evolves to better operating conditions (24).

The objectives of this research were to evaluate combined effects of extraction temperature, extraction time, and the ethanol content on the antibacterial activity of cloves using

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Received August 3, 2007; Revised October 18, 2007;
Accepted November 6, 2007

EVOP-factorial design to maximize the antibacterial activity of clove (*Caryophylli Flos*) against dental caries-causing bacteria, *S. mutans*.

Materials and Methods

Materials and microorganism Clove was obtained from Yakrung market (Daegu, Korea) in January 2005, and was lyophilized for 48-72 hr after storage at –70°C. Freeze-dried samples were pulverized with a blender (HJM-7000; Hanil, Seoul, Korea). Extra pure grade solvents were purchased from Daemyung Scientific Co. (Daegu, Korea) and the chemical reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated. The microorganism used in this study was *Streptococcus mutans* ATCC 27351 (*S. mutans*) which was anaerobically cultured in brain heart infusion (BHI) media at 37°C.

Extraction of clove A 20 g sample was hydrolyzed and extracted in a reflux extraction apparatus by differentiating the extraction temperature (35, 50, 65, 80, and 95°C), extraction time (8, 14, 20, 26, and 32 hr), and ethanol concentration (25, 50, 75, and 100%). The sample was then freeze-dried in order to establish the proper extraction conditions.

Evaluation of the antibacterial activity of clove on the *in vitro* cultured *S. mutans* *S. mutans* cultured as described above were harvested and their density adjusted so that their optical density at 660 nm (OD_{660}) was 0.5. One mL of pre-cultured bacteria were inoculated to 100 mL of BHI medium containing 0.1%(w/v) of clove extract. Each sample was homogenized for 30 sec and serially diluted with peptone water (Difco Lab., Detroit, MI, USA), as needed to determine the microbial populations. The colony-forming unit (CFU) of a particular microbial group was counted in BHI agar at 37°C for 24-72 hr.

Optimization of 3 variables by EVOP-based factorial technique The EVOP-factorial design technique was applied in order to select the optimum conditions of the 3 extraction factors in different experiments. The control or search level experimental conditions (E_{10}, E_{20}) were selected based on the results of earlier investigation into the effect of the individual extraction condition on the antibacterial activity of clove. Furthermore, the new experimental conditions (E_{be}) were selected with higher and lower levels of inducers than in the initial search level (E_{b0}). The antibacterial activities of cloves were estimated following the given assay procedure and were recorded for cycle I and II. Differences in the antibacterial activities were observed between cycle I and II, and the average antibacterial activities were calculated to estimate the effects and error limits. The magnitude of the effects, error limit, and the change in the mean effect were examined as per the decision-making procedure to arrive at the optimum conditions. When the experimental results of the first set did not satisfy the optimum condition requirements, a second set of

Table 1. Experimental design for 3-inducer system and results of Set I

Experimental conditions	E_{10}	E_{11}	E_{12}	E_{13}	E_{14}	E_{20}	E_{21}	E_{22}	E_{23}	E_{24}
Temp. (°C)	50(0)[1)]	35(-)	35(-)	65(+)	65(+)	50(0)	65(+)	35(-)	65(+)	35(-)
Time (hr)	14(0)	8(-)	20(+)	8(-)	20(+)	14(0)	20(+)	8(-)	8(-)	20(+)
Ethanol concen. (%)	50(0)	25(-)	75(+)	75(+)	25(-)	50(0)	75(+)	75(+)	25(-)	25(-)
Antibacterial activity (cycle I)	5.68	6.81	6.11	5.51	5.23	5.78	4.36	6.75	5.78	6.15
Antibacterial activity (cycle II)	5.51	6.89	6.25	5.71	5.08	5.59	4.50	6.59	5.61	6.05
Difference (cycle I-cycle II)	0.17	-0.08	-0.14	-0.20	0.15	0.19	-0.14	0.16	0.17	0.10
Average activity	5.595 (a_{10})	6.850 (a_{11})	6.180 (a_{12})	5.610 (a_{13})	5.155 (a_{14})	5.685 (a_{20})	4.430 (a_{21})	6.670 (a_{22})	5.695 (a_{23})	6.100 (a_{24})

[1)]Numbers in parentheses are the coded symbols of levels of the extraction conditions.

Table 2. Calculation worksheet of effects of 3-variable system, magnitude of effects, and error limits of Set I

Effects		Calculation of effects	
Temp. (°C)		$1/4(a_{13}+a_{14}+a_{21}+a_{23}-a_{11}-a_{12}-a_{22}-a_{24})$	-1.2275
Time (hr)		$1/4(a_{12}+a_{14}+a_{21}+a_{24}-a_{11}-a_{13}-a_{22}-a_{23})$	-0.7400
Ethanol concen. (%)		$1/4(a_{12}+a_{13}+a_{21}+a_{22}-a_{11}-a_{14}-a_{23}-a_{24})$	-0.2275
Temp. × Time		$1/4(a_{11}+a_{14}+a_{21}+a_{22}-a_{12}-a_{13}-a_{23}-a_{24})$	0.1200
Temp. × Ethanol concen.		$1/4(a_{11}+a_{13}+a_{21}+a_{24}-a_{12}-a_{14}-a_{22}-a_{23})$	-0.1775
Time × Ethanol concen.		$1/4(a_{11}+a_{12}+a_{21}+a_{23}-a_{13}-a_{14}-a_{22}-a_{24})$	-0.0950
Temp. × Time × Ethanol concen.		$1/4(a_{21}+a_{22}+a_{23}+a_{24}-a_{11}-a_{12}-a_{13}-a_{14})$	-0.2250
Change in mean effect		$1/10(a_{11}+a_{12}+a_{13}+a_{14}+a_{21}+a_{22}+a_{23}+a_{24}-4a_{10}-4a_{20})$	0.1570
Standard deviation (σ)		$1/2(\sigma_1+\sigma_2) = 1/2(R_1\times f_{k,n}+R_2\times f_{k,n})$[1)]	0.1050
Error limits:	For average	$\pm 1.414\sigma$ ($\pm 2\sigma/n$)	0.1485
	For effects	$\pm 1.004\sigma$ ($\pm 0.71\times 2\sigma/n$)	0.1054
	For change in mean	$\pm 0.891\sigma$ ($\pm 0.63\times 2\sigma/n$)	0.0936

[1)]R_1, (largest difference - smallest difference) in Block 1; R_2, (largest difference - smallest difference) in Block 2; $f_{k,n}$ = constant depending on number of replication (n) and number of experiments (k) per cycle = 0.3 for n = 2 and k = 5.

Table 3. Experimental design for 3-inducer system and results of Set II

Experimental conditions	E_{10}	E_{11}	E_{12}	E_{13}	E_{14}	E_{20}	E_{21}	E_{22}	E_{23}	E_{24}
Temp. (°C)	65(0)[1)]	50(-)	50(-)	80(+)	80(+)	65(0)	80(+)	50(-)	80(+)	50(-)
Time (hr)	20(0)	14(-)	26(+)	14(-)	26(+)	20(0)	26(+)	14(-)	14(-)	26(+)
Ethanol concen. (%)	75(0)	50(-)	100(+)	100(+)	50(-)	75(0)	100(+)	100(+)	50(-)	50(-)
Antibacterial activity (cycle I)	4.38	5.57	4.89	5.18	4.17	4.47	4.68	5.25	4.35	4.95
Antibacterial activity (cycle II)	4.51	5.40	4.75	5.01	4.21	4.32	4.80	5.48	4.16	4.82
Difference (cycle I-cycle II)	-0.13	0.17	0.14	0.17	-0.04	0.15	-0.12	-0.23	0.19	0.13
Average activity	4.445 (a_{10})	5.485 (a_{11})	4.820 (a_{12})	5.095 (a_{13})	4.190 (a_{14})	4.395 (a_{20})	4.740 (a_{21})	5.365 (a_{22})	4.255 (a_{23})	4.885 (a_{24})

[1)]Numbers in parentheses are the coded symbols of levels of the extraction conditions.

experiments was designed, selecting the best condition for the new search level based on the results of the first set of experiments. This procedure was repeated until the optimum condition was obtained.

Results and Discussion

The experimental conditions used in the first set of experiments, the corresponding enzyme activities of cycle and, their differences and average values are presented in Table 1. The error limits, effects, and the change in the mean effect were calculated and the results are shown in Table 2.

According to the decision-making procedure, after calculating the change in the mean effect and error limit, an examination was necessary to determine whether any change in the control (search level) experimental conditions would help to improve the objective function (25). The optimum condition was achieved when the effect was smaller than the error limit while the change in the mean effect was large. Moreover, because the dependent variables are the number of *S. mutans* in which growth is suppressed by the addition of clove, the optimum point was reached when the code of the mean effect was negative.

The determination of the magnitude of the change in the mean effect, which is negative and large compared to the error limit, is a requirement in order to confirm the achievement of the optimum condition. Such a situation where some of the effects are large in comparison to the error limit does not ensure that the condition at the search region (E_{10}, E_{20}) of the first set is the actual optimum condition; therefore, a second set of experiments is called for.

In the second set, the search level (E_{10}, E_{20}) was fixed at the best condition of Set I, at a level of E_{21}, in which the number of *S. mutans* was 4.430 log CFU/mL. The experimental conditions and the results of Set II experiments are presented in Table 3 and the effects and error limits are shown in Table 4. In this case, not all of the effects are smaller than the error limit, even though the change in the mean effect is positive and large compared to the error limit. If all or any of the effects are larger than the error limits, a change in the experimental conditions may yield better results (26).

A third set of experiments was designed in which the best condition of Set II (E_{14}) was selected as the search level (E_{10}, E_{20}) for Set III. The experimental conditions and

Table 4. Calculation worksheet of effects of 3-variable system, magnitude of effects, and error limits of Set II

Effects		Calculation of effects
Temp. (°C)		-0.5688
Time (hr)		-0.3913
Ethanol concen. (%)		0.3913
Temp. × Time		0.1813
Temp. × Ethanol concen.		0.3938
Time × Ethanol concen.		-0.0588
Temp. × Time × Ethanol concen.		-0.0863
Change in mean effect		0.3475
Standard deviation (σ)		0.1080
Error limits:	For average	0.1527
	For effects	0.1084
	For change in mean	0.0962

Table 5. Experimental design for 3-inducer system and results of Set III

Experimental conditions	E_{10}	E_{11}	E_{12}	E_{13}	E_{14}	E_{20}	E_{21}	E_{22}	E_{23}	E_{24}
Temp. (°C)	80(0) [1)]	65(-)	65(-)	95(+)	95(+)	80(0)	95(+)	65(-)	95(+)	65(-)
Time (hr)	26(0)	20(-)	32(+)	20(-)	32(+)	26(0)	32(+)	20(-)	20(-)	32(+)
Ethanol concen. (%)	50(0)	25(-)	75(+)	75(+)	25(-)	50(0)	75(+)	75(+)	25(-)	25(-)
Antibacterial activity (cycle I)	4.11	4.35	4.42	4.32	4.24	4.21	4.36	4.39	4.41	4.29
Antibacterial activity (cycle II)	4.28	4.45	4.28	4.25	4.35	4.26	4.44	4.31	4.27	4.35
Difference (cycle I-cycle II)	-0.17	-0.10	0.04	0.07	-0.11	-0.05	-0.08	0.08	0.14	-0.06
Average activity	4.195 (a_{10})	4.400 (a_{11})	4.350 (a_{12})	4.285 (a_{13})	4.295 (a_{14})	4.235 (a_{20})	4.400 (a_{21})	4.350 (a_{22})	4.340 (a_{23})	4.320 (a_{24})

[1)]Numbers in parentheses are the coded symbols of levels of the extraction conditions.

Table 6. Calculation worksheet of effects of 3-variable system, magnitude of effects, and error limits of Set III

Effects		Calculation of effects
Temp. (°C)		-0.0250
Time (hr)		-0.0025
Ethanol concen. (%)		0.0075
Temp. × Time		0.0375
Temp. × Ethanol concen.		0.0175
Time × Ethanol concen.		0.0600
Temp. × Time × Ethanol concen.		0.0200
Change in mean effect		0.1020
Standard deviation (σ)		0.0690
Error limits:	For average	0.0976
	For effects	0.0693
	For change in mean	0.0615

the results of the Set III are shown in Table 5 and the calculated effects and error limits are presented in Table 6. In the third experiment, we were able to arrive at the proper optimum condition in which the all of the effects were smaller than the error limit and the change in the mean effect was large and positive. Furthermore, the population of *S. mutans* decreased from 6.850 log CFU/mL in the initial set to 4.195 log CFU/mL in the third set as shown in Fig. 1.

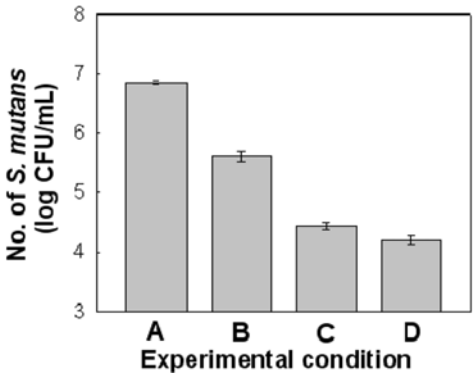

Fig. 1. Comparison of antibacterial activity of clove against *S. mutans* at the central point of each set. A, E_{11} of Set I (extraction temperature; 35°C, extraction time; 8 hr, ethanol concentration; 25%); B, central point of Set I (extraction temperature; 50°C, extraction time; 14 hr, ethanol concentration; 50%); C, central point of Set II (extraction temperature; 65°C, extraction time; 20 hr, ethanol concentration; 75%); D, central point of Set III (extraction temperature; 80°C, extraction time; 26 hr, ethanol concentration; 50%).

And, it was shown that higher antibacterial activity was achieved in a higher extraction temperature of 80°C (r= 0.7983**) and in a longer extraction time of 26 hr (r= 0.6867*). However antibacterial acivity against *S. mutans*

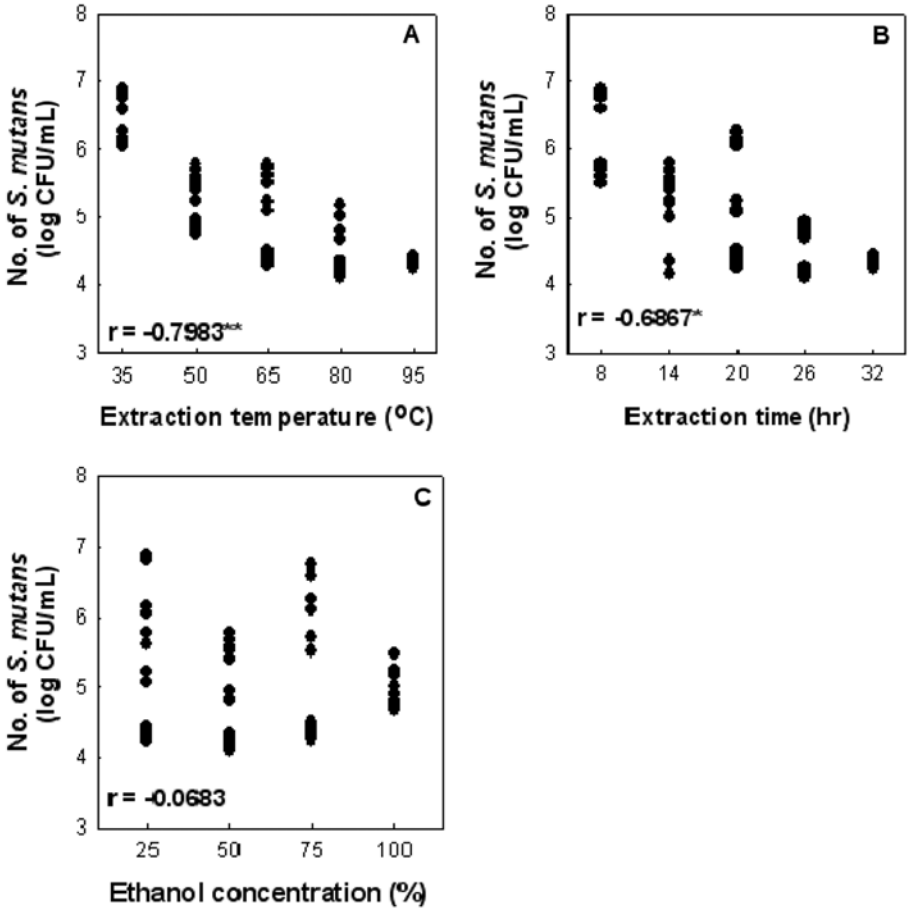

Fig. 2. Main effects plot for responses against independent variables in EVOP. **$p<0.01$; *$p<0.05$. A, extraction temperature; B, extraction time; C, ethanol concentration.

the extraction solvent (r= –0.0683) as shown in Fig. 2.

Therefore, the maximum antibacterial activity of clove against *S. mutans* as determined by the EVOP-factorial technique was obtained at an extraction temperature of 80°C, an extraction time of 26 hr and a 50% ethanol concentration.

The research for the antibacterial activity of clove was mainly accompanied with food spoiling bacteria as target microorganisms. It was reported that the high antibacterial activity of clove was shown against the growth of food spoilage bacteria such as *E. coli, S. aureus, Salmonella typhimurium, Pseudomonas aeruginosa* and the highest antibacterial activity was appeared in the methanol extract followed by water and petroleum ether extract (27). The growth of *L. monocytogenes* was effectively inhibited by 500 ppm addition of clove extract (28,29)

It was expected that not only the growth of food spoiling bacteria but the growth of dental caries bacteria such *as S. mutans* were effectively inhibited by the addition of clove extract. Moreover, the number of *S. mutans* was decreased more than 2.6 log cycles compared to the control by differentiation of 3 extraction conditons (extraction temperature, extraction time, ethanol concentration).

This is the first report of the application of evolutionary operation-factorial design technique to identify antibacterial activity of natural products and their extracts that should be useful for finding optimal extraction condition for the antibacterial activity.

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