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Response surface methodology designation for optimization of lactic acid production by *Streptococcus thermophilus* isolated from industrial yogurt

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Abstract

Using inexpensive sources is a crucial point in the production of valuable compounds such as lactic acid. In the present study, the production of lactic acid by an efficient isolate of *S. thermophilus* was optimized using whey as a carbon source and yeast extract as a nitrogen source. MRS culture medium with 6.5 % NaCl was used for the isolation of streptococci from industrial yogurt samples and the selected isolate was identified using the 16S rRNA gene sequencing. Evaluation of lactic acid production was done by the Randox method. Lactic acid production by the selected isolate was optimized by response surface methodology (RSM) in MRS broth regarding to different concentrations of whey and yeast extract. The isolate that produced the highest amount (8.9 $g.L^{-1}$) of lactic acid within 52 h growth in MRS broth was identified as a strain of *S. thermophilus* by molecular identification. Optimization of concentrations of carbon and nitrogen sources resulted in the induction of lactic acid production by *Streptococcus thermophilus* up to 24.18 $g.L^{-1}$ in the presence of 3.5 % of whey and 5 % of yeast extract as external carbon and nitrogen sources in the MRS medium, which was similar to the value predicted by RSM. Yeast extract was found to be more effective on lactic acid production than whey. Optimization of lactic acid production by low-cost substrates whey and yeast extract resulted in high induction of lactic acid production by *S. thermophilus* compared to some other studies that used other cost-effective substrates and/or bacteria isolated from traditional dairy products.

Introduction

Lactic acid with the formula $C_3H_6O_3$ is a chiral alpha-hydroxy acid with a molar mass of 190.08 $g.mol^{-1}$ (Hujanen *et al.* 2001). The use of lactic acid in industries is widespread due to its low price and bioactivities that make it suitable for use as a preservative, antioxidant, flavoring, prebiotic, cryoprotectant, viscosifier, and acidifier. Also, polylactic acid produced by its polymerization has more applications in the industry than its monomer, especially in the plastics production industry. Lactic acid is a chemical used in the textile industry as a stabilizer and helps with the better fabric absorption of the dye. This organic acid is also involved in the production of varnish and ink and leather tanning. It is also used as a moisturizer and lubricant in cosmetics and added to food to create a sour taste. Lactic acid is an important compound in the production of dairy products including yogurt and cheese and as a starting material in the pharmaceutical industries (Reddy *et al.* 2008). Since the production of pure optical isomers of lactic acid is not possible by chemical methods, the

use of fermentation methods for its production has been more interested nowadays. The biological production of lactic acid accounts for more than half of the world's total production (Kotzamanidis *et al.* 2002). Biological production of lactic acid via cost-effective carbon and nitrogen sources such as whey, milk powder, yogurt powder, and curd powder is expected to provide a suitable market for lactic acid (Reddy *et al.* 2008; Abedi and Hashemi 2020).

Lactic acid bacteria (LABs) that are found in plants, meat, and dairy products are commonly used to produce lactic acid with high production and yield (Ayana *et al.* 2011). *Streptococcus* is one of the most important genera in this group. Most streptococci are catalase and oxidase negative facultative anaerobes. The only species in this genus used in the production of dairy products is *Streptococcus thermophilus*. Lactobacilli are important bacteria in the large group of LABs, are known as starter cultures and key players in the microbial flora of fermented dairy and meat products such as yogurt and sausages, respectively. The synergistic effects between the two bacterial species are the basis of lactose metabolism and high lactic acid production in milk (Brashears *et al.* 2005; Ayana *et al.* 2011).

Due to the fact that the fermentation methods for the production of lactic acid using inexpensive sources, such as whey obtained from dairy factories, is an important phenomenon (Abedi and Hashemi 2020), in this study, the production of lactic acid by a potent strain of *S. thermophilus* isolated from industrial yogurt was optimized using whey as carbon source and yeast extract as nitrogen source.

Experimental

Isolation of bacteria

As in previous study the addition of NaCl salt has been used for stimulation of the growth of lactic acid producing isolates specially *Lactococcus* and *Streptococcus* species (Medveďová *et al.* 2019), in the present study steps for the purification of streptococci was done in 1 – 7 % NaCl in MRS agar (de Man *et al.* 1960) and it was observed that the *Streptococcus* sp. was able to tolerate up to 6.5 % NaCl. Therefore, in order to purify this isolate, industrial yogurt samples were cultured in MRS agar medium containing 6.5 % NaCl followed by incubation at 37 °C for 48 h. Also, as lactose has been proposed for induction of the growth of lactobacilli (Rezvani *et al.* 2017), in the present study the purification steps for lactobacilli was done in 1 – 5 % lactose in MRS agar and it was observed that the *Lactobacillus* sp. grows best in the presence of at least 2 % lactose. Therefore, the purification steps for this isolate from industrial yogurt samples were done in MRS agar medium containing 2 % lactose followed by incubation at 39 °C for 48 h in a candle jar. The microscopic features of the isolated bacteria were analyzed after Gram staining (Taligene Pars, Iran) using the manufacturer instructions. The culture media and chemicals were obtained from Merck Corporation (Germany).

L- lactate assay

The 48 h cultured bacteria were centrifuged at 4,000 × g for 20 min. Then, the amount of lactic acid in the supernatant was measured based on the amount of pyruvate and hydrogen peroxide catalyzed by lactate oxidase enzyme by Randox LC2389 kit (Randox Laboratories LTD, UK) following instructions of the manufacturer. The production of lactic acid was measured every 3 h during 4 days of incubation along with the bacterial growth curve was plotted through assessment of the culture turbidity at a wavelength of 550 nm.

Molecular identification of the selected isolate

Total DNA was extracted using the DN8115C kit (SinaClon, Iran). The extracted DNA quality was determined based on optical density ratio at 260/280 nm wavelengths using a spectrophotometer (Agilent, UV-Vis-NIR), and its quantity was measured by Thermo Scientific™ *NanoDrop* 2000 full-spectrum (Desjardins and Conklin 2010). Then, a fragment in the 16S rDNA gene was amplified by using universal primers, RW01 (5′-AACTGGAGGAAGGTGGGGAT-3′) and DG74 (5′-AGGAGGTGATCCAACCGCA-3′) in a hot started polymerase chain reaction (PCR). The primers were constructed by Bioneer

Corporation (Daejeon, South Korea). The amplification protocol was performed in 4 steps included 1: 95 ºC for 60 s; 2: 94 ºC for 60 s, 58 ºC for 50 s, and 72 ºC for 60 s (repeated 6 times); 3: 94 ºC for 60 s, 56.5 ºC for 60 s, and 72 ºC for 60 s (repeated 37 times); and 4: 72 ºC for 5 min. The PCR mixture involved 1X PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTP (CinnaGen, Tehran, Iran), 0.5 µM of each primer, 1 unit Taq DNA polymerase (CinnaGen, Tehran, Iran), and 1 µg of the extracted DNA in a total volume of 50 µL. The size of PCR products were detected by agarose gel electrophoresis. The amplified fragment was then sequenced and analyzed in the BLAST server (http://www.ncbi.nlm.nih.gov/BLAST; Greisen *et al.* 1994; Fantin *et al.* 2013).

Optimization of lactic acid production by the selected isolate by response surface methodology (RSM)

Based on the results from previous studies conducted by one factor at a time experiments for lactic acid production using low-cost carbon and nitrogen sources (Nancib *et al.* 2001; Schepers *et al.* 2002; Altaf *et al.* 2005; Bernardo *et al.* 2016), in the present study, whey powder (11.5 % protein, 65 % lactose, and 2 % lipid content with 8.5 % ash and 0.15 % acidity) and yeast extract (Merck, Germany) were used in the experiments which designed by RSM methodology, to optimize lactic acid production by the most potent LAB that was isolated from industrial yogurt. For this purpose, the first pre-trials were conducted by supplementing different concentrations of whey powder and yeast extract to MRS medium. Then based on the obtained results for lactic acid production in each concentration, the minimum and maximum levels for each source were entered into the Design Expert software and the software designed the experiments to be done.

Statistical analysis

The data were collected as mean ± standard deviation according to the amount of the produced lactic acid and entered the software for analysis by one-way analysis of variance ANOVA using Qualitek 4 software. F test was used for comparing the results of experiments. Significance levels were considered as $P \leq 0.01$.

Results

Molecular identification of the selected isolates

Among the isolated bacteria, 2 isolates produced high amounts of lactic acid in the MRS medium. These isolates were a Gram-positive rod and a Gram-positive coccus. Fig. 1 shows the 370 bp band resulting from amplification of a fragment in the 16S rRNA coding gene by using universal primers in the genome of 2 initially selected isolates (A and B; Fig. 1).

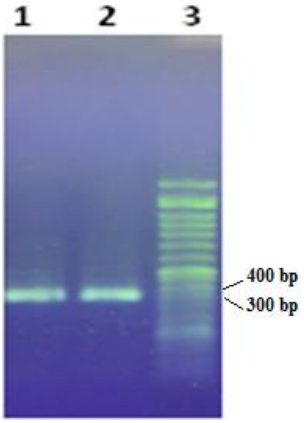

Fig. 1. Amplification of the fragment in the 16S rRNA coding gene resulted in a 370 bp band (**lines 1** and **2**: the isolates A and B, respectively) according to the 50 bp DNA marker (**line 3**).

The alignment of the amplified 16S fragment in the gene bank for 2 initially selected isolates indicated that the bacteria were a strain of *Streptococcus thermophilus* and strains of *Lactobacillus delbrueckii* subsp. *bulgaricus*. The phylogenetic trees in Fig. 2 show the relationships of the isolates with the close strains.

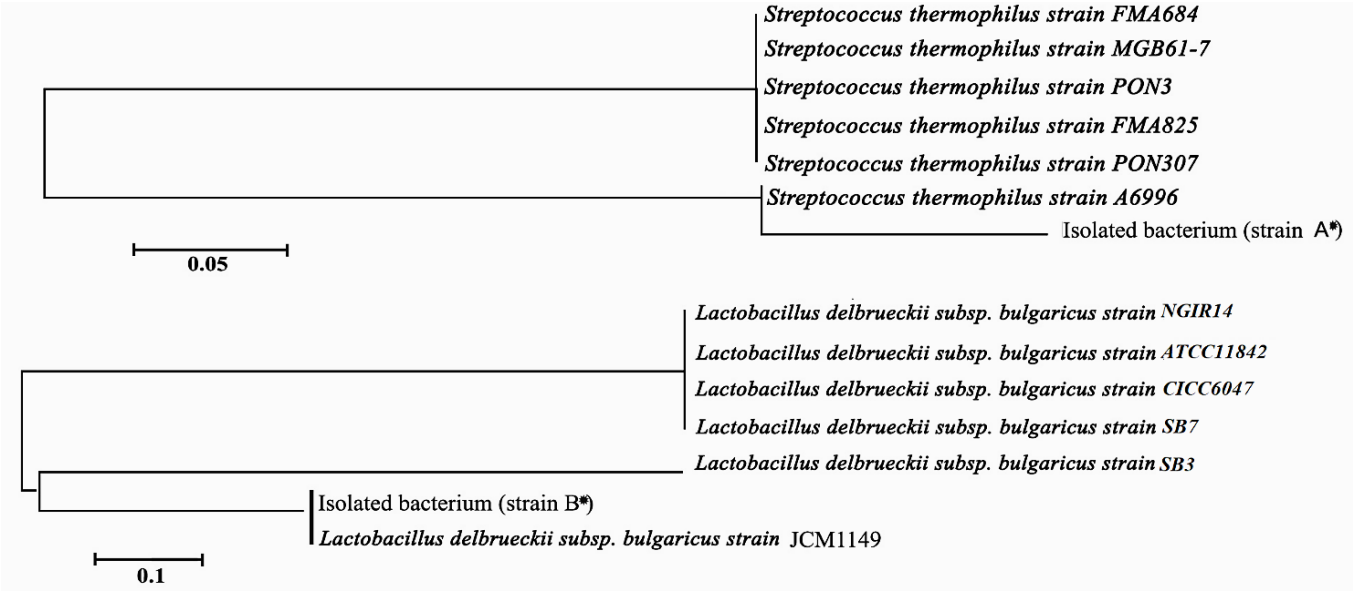

Fig. 2. Phylogenetic trees of the isolated strains: **A*** is related to the strains of *S. thermophilus* with 99.5 % identity and **B*** is related to the strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* with 99.6 % identity.

Growth curve and lactic acid productivity of the isolated bacteria

The growth and lactic acid production curves by the 2 selected isolates during 60 h growth in MRS broth at 37 °C for *S. thermophilus* and 39 °C for *L. delbrueckii* subsp. *bulgaricus* are shown in Fig. 3 and 4. The highest amount of lactic acid (8.9 $g.L^{-1}$) was obtained at 52^{nd} h in MRS broth by *S. thermophilus*. At this point, the bacterium was at the end of the logarithmic growth stage. This productivity was significantly higher than the productivity of *L. delbrueckii* subsp. *bulgaricus* which produced 3.913 $g.L^{-1}$ lactic acid at 57^{th} h in MRS broth ($P < 0.05$). Therefore, the *S. thermophilus* isolate was selected for the production optimization process.

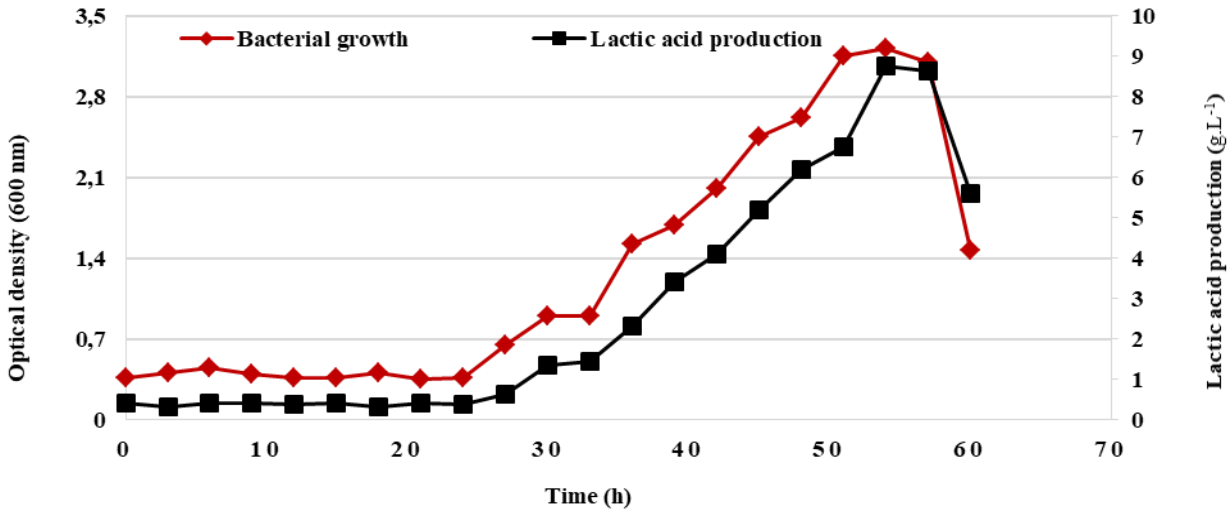

Fig. 3. The growth curve and lactic acid production by *S. thermophiles.*

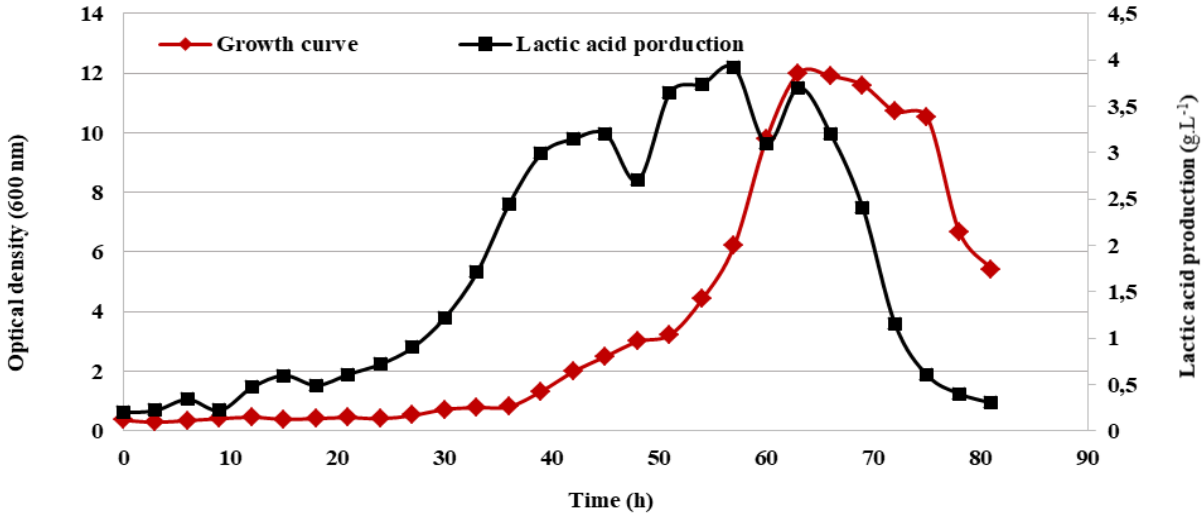

Fig. 4. The growth curve and lactic acid production by *L. delbrueckii* subsp. *bulgaricus.*

Determination of optimal conditions for lactic acid production by Streptococcus thermophiles

The results from one factor at time experiments for initial optimization of lactic acid production by *S. thermophilus* in different concentrations of whey and yeast extract sources are shown in Fig. 5 and 6. The highest amounts of lactic acid were produced at 6 % concentrations of both whey and yeast extract. Therefore, the minimum (3.5 %) and maximum (6.5 %) levels for each source were entered the Design Expert software, and the software designed 13 experiments by RSM to be done (Table 1).

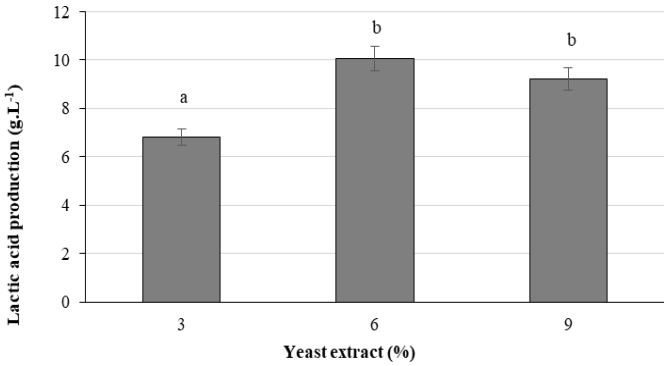

Fig. 5. Lactic acid production by *S. thermophilus* in different concentrations of yeast extract. The isolate represented the most productivity in the concentration of 6 %. Different letters indicate a significant difference between the results ($P < 0.05$).

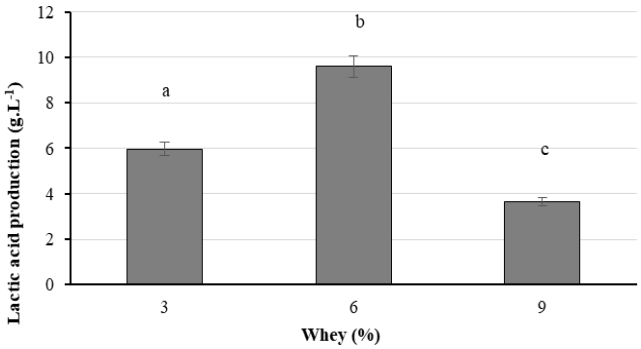

Fig. 6. The lactic acid production by *S. thermophilus* in different concentrations of whey. The isolate represented the most productivity in the concentration of 6 %. Different letters indicate a significant difference between the results ($P < 0.05$).

Table 1. Experiments designed by the Design Expert software.

Experiment no.	Whey [%]	Yeast extract [%]
1	4	4
2	4	6
3	6	4
4	6	6
5	5	3.5
6	5	6.5
7	3.5	5
8	6.5	5
9	5	5
10	5	5
11	5	5
12	5	5
13	5	5

The results obtained from designed experiments are shown in Fig. 7. As seen, the highest lactic acid

production by *S. thermophilus* (24.18 $g.L^{-1}$) was obtained in the presence of 3.5 % whey and 5 % yeast extract as supplementary carbon and nitrogen sources in the MRS medium (experiment no. 7). This result had no significant difference with the result obtained from experiment no. 6 (21.01 $g.L^{-1}$ lactic acid productivity) in which 5 % whey and 6.5 % yeast extract were added as supplementary carbon and nitrogen sources to the MRS medium.

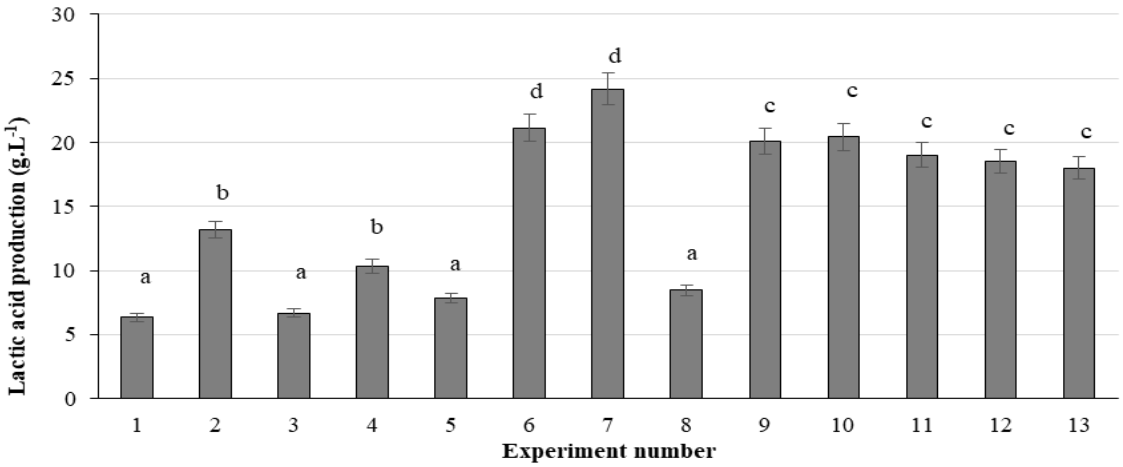

Fig. 7. Comparison of lactic acid production by *S. thermophilus* between 13 experiments designed by Design Expert software. Different letters indicate a significant difference between the results ($P < 0.05$).

Statistical analysis of data

Table 2 shows the results from ANOVA analysis. A quadratic correlation was seen between the effect of examined factors and lactic acid production. Also, a significant correlation was seen between the designed model and the studied factors, and the lack of fit between the results was not significant. The obtained values had normal distribution with no significant difference from the expected values. According to F-test and *P*-values, the effect of yeast extract supplementation on lactic acid production was significantly greater than the effect of whey supplementation ($P = 0.0341$).

Table 2. Results from ANOVA analysis to investigate the quadratic model of lactic acid production.

Source	Sum of squares	Degree of freedom	Mean squares	F-value	P-value	Significance
Model: Quadratic	401.92	5	60.38	5.23	0.0256	Significant
Yeast extract (A)	105.94	1	105.94	6.90	0.0341	
Whey (B)	14.67	1	14.67	0.96	0.3610	
A^2	120.33	1	120.33	7.84	0.0266	
B^2	71.80	1	71.80	4.67	0.0674	
Residual	107.50	7	15.36			
Lack of fit	88.76	3	29.59	6.31	0.0536	Non-significant
Pure error	18.85	4	4.69			
Total	509.42	12				

Normal distribution that observed around the center point by the 5 best results from 13 experiments is shown in Fig. 8. The central red dot is the result of repeated experiments with central amounts of 5% cheese and 5% yeast extract, and the distribution of the best results is shown on the blue lines. Other parts of the blue lines are the values predicted by the software for the results. Based on statistical analysis, the obtained lactic acid production was 24.18 $g.L^{-1}$ under optimal conditions. It was not significantly different from the predicted value that was expected by RSM.

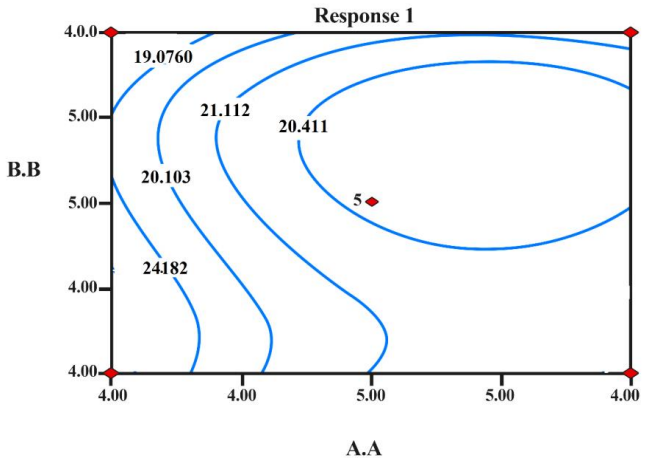

Fig. 8. Two-dimensional distribution of the best test results obtained from design expert software. The red point in the center is the central test, A and B indicate yeast extract and whey factors, respectively.

Discussion

Whey is a by-product of the dairy industry, accounting for 85 – 95 % of milk volume and retaining 55 % of milk nutrients. Lactose is low in whey. All 9 essential amino acids are found in whey protein. The most abundant of these nutrients are lactose, soluble proteins, lipids, and mineral salts. The presence of lactose and other nutrients for the growth of microorganisms makes it one of the potential substrates for the production of various biological products through biotechnological processes (Panesar *et al.* 2007). In the present study, the lactic acid bacteria *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* were purified from industrial yogurt samples with some modifications in traditional MRS medium enriched by 6.5 % NaCl for *Streptococcus* spp. and by 2 % lactose for *Lactobacillus* spp. The isolate was identified by a molecular method. *S. thermophilus* was reported to be more sufficient for lactic acid production than *L. delbrueckii* subsp. *bulgaricus*. Rezvani *et al.* (2017) compared five *Lactobacillus* strains for biomass yield and lactic acid production. The results showed that *L. bulgaricus* was the most efficient strain which produced the highest biomass and lactic acid yield using whey supplemented with lactose and yeast extract as carbon and nitrogen sources. Other species including *L. fermentum*, *L. casei*, and *L. lactis* had less productivity than *L. bulgaricus*. The strain of *L. delbrueckii* had the least biomass and lactic acid production. This result is in coincidence with the present study.

In this study, the lactic acid production by the particular strain of *S. thermophilus* with high lactic acid production was optimized by response surface methodology (RSM) by changing the concentrations of whey powder and yeast extract as low-cost carbon and nitrogen sources. The highest lactic acid production by *Streptococcus thermophilus* (24 $g.L^{-1}$) was obtained in the MRS medium containing 5 % yeast extract and 3.5 % whey powder during 52 hours of incubation. This rate of production was consistent with, or in some cases greater than, the amount of lactic acid produced by other researchers (Nacib *et al.* 2001; Benkun and Yao 2007; Panesar *et al.* 2007; Prachamon and Boonmee 2008; Zhang and Vadlani 2013).

Hujanen *et al.* (2001) optimized the process variables and carbon concentrations for lactic acid production by *Lactobacillus casei* NRRL B-441. The lactic acid yield was proportional to the initial glucose concentration (80 – 160 $g.L^{-1}$) in the medium. The highest concentration of lactic acid that was obtained during fermentation was 118.6 $g.L^{-1}$. RSM was used to optimize the process variables. The optimum temperature and pH for lactic acid production were 35 °C and 6.3, respectively. Malt extract along with yeast extract (4 $g.L^{-1}$) seemed to be an economical alternative to yeast extract alone (22 $g.L^{-1}$), although fermentation time was slightly longer and the usage of glucose as the initial carbon source was not cost-effective. In a similar study, Lu *et al.* (2016) achieved a lactic acid concentration of 83 $g.L^{-1}$ using a combination of high-cost sources, including glucose and xylose, with a 36 h fermentation by *Lactococcus lactis*. Zhang and Vadlani (2013) used a recombinant strain of *L. delbrueckii* to produce lactic acid with a purity of 99 % at the rate of 36.3 $g.L^{-1}$ using a combination of glucose and xylose as the carbon source. Inexpensive substrates have also been used for the production of lactic acid similar the present study. For instance, Prachamon and Boonmee (2008) studied the effect of sugarcane extract as a substrate for lactic acid production. They cultured five strains of lactic acid bacteria with different carbon sources such as glucose, sucrose, and sugarcane

extract. The results showed that when glucose or sucrose was used as the substrate, there was no significant difference in the concentration of lactic acid produced.

The production rates were 18.19 – 20 $g.L^{-1}$ and 17.78 – 20.77 $g.L^{-1}$, respectively. When using sugarcane extract as the substrate, the concentration of produced lactic acid was decreased to 5.56 – 10.3 $g.L^{-1}$. Panesar *et al*. (2007) conducted a study on the fermentation of whey to produce lactic acid using *L. casei*. The effect of various process parameters such as pH, temperature, inoculation size, inoculation age, stirring, and incubation time was investigated to increase the conversion of whey lactose to lactic acid. Process conditions optimization led to the production of 33.73 $g.L^{-1}$ lactic acid after the 36 h of incubation period. Inexpensive sources other than whey also have been used for the production of lactic acid by bacteria. Benkun and Yao (2007) examined the low-cost production of lactic acid using rice husk as the only carbon source in solid-state fermentation and produced 18 $g.L^{-1}$ of lactic acid. Nancib *et al*. (2001) studied the production of lactic acid by fermentation using *L. casei*. Different nitrogen sources were compared for their efficiency in lactic acid production. None of the used nitrogen sources produced as much lactic acid as the yeast extract which resulted in 20 $g.L^{-1}$ lactic acid production. In the present study, the results obtained by the experiments designed by RSM showed that increasing the concentration to more than 6 % for yeast extract and 3.5 % for whey had an inhibitory effect on lactic acid production in RSM designed experiments. Data analysis also showed that lactic acid production is affected more by the concentration of yeast extract compared to that of whey. Similar results have been obtained in the study of Benaissa *et al.* (2017) that used sweet whey as a base medium supplemented with yeast extract, and/or tomato juice for the growth of lactobacilli and lactic acid production. Obtained results showed that the bacteria produced more biomass on whey supplemented with yeast extract than those produced with tomato juice. They concluded that whey is too poor to provide the needs of lactobacilli and optimization of growth medium through the addition of yeast extract (1 %) and/or tomato juice (30 %) as inexpensive substrates can promote lactic acid production by these bacteria similar to the case took place with MRS medium in the current study.

Conclusion

In the present study, high lactic acid production (24.18 $g.L^{-1}$) was obtained by the isolated *S. thermophilus* through supplementation of 3.5 % whey powder and 5 % yeast extract as respective low-cost carbon and nitrogen sources in the production medium. The optimization process by response surface methodology (RSM) resulted in elevated lactic acid production at more levels than some other studies that used other expensive substrates or optimized the production condition by designations in which a single factor has been optimized in each experiment at a time.

Acknowledgments

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Conflict of Interest

The authors declare that they have no conflict of interest.

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