Optimization of pH and temperature on the growth of *Streptococcus thermophilus* in whey using response surface methodology

Aghababaie.M[*1], Khanahmadi.M[2], Beheshti.M[3], Mirlohi.M[4], Aghababaie.Z[5]

Abstract

Lactic acid bacteria are employed in different industries such as food and chemical industries. *Streptococcus thermophilus* is one of the traditional starter cultures in dairy industries. This study was designed using response surface methodology in 12 batch pH-controlled cultures of *streptococcus thermophilus* for determining the effect of temperature and pH on the growth and acid lactic production by this strain. Response surface model was significant for maximum specific growth rate of *S.thermophilus*. Maximum biomass concentration and produced lactic acid highly depended on pH. Optimum pH and temperature for the growth and acid production of *S.thermophilus* was 42.8 and 7, respectively.

Key words: *Streptococcus thermophilus*, Whey, RSM, lactic acid production

1- Introduction:

Streptococcus salivarius subsp. *Thermophilus* is a Gram-positive bacteria and a homofermentative facultative anaerobe, of a group lactic acid bacteria which its major product is (L+) lactic acid[1]. Lactic acid is an industrially important product which uses in food, chemical, textile, pharmaceutical and other industries. Nowadays it has also received attention as a biomaterial for the manufacture of biodegradable (polylactic) polymers[2, 3].

S.thermophilus ferments sugars by the Embden -Meyerhoff - Parnas (EMP) pathway to pyruvate, which is converted into lactic acid by lactate dehydrogenase[1]. *S. thermophilus* prefers the disaccharides lactose and sucrose, and its growth on the constituent monosaccharides, glucose, fructose and galactose is slower than the disaccharides[4].

Whey is the major byproduct of dairy industries which is a pollutant and must be treated or disposed. It contains up to 60 g/l lactose and 10 g/l proteins and some minerals[5]. Since lactic acid bacteria have complex nutritional requirements, whey will supplemented with other nutrients[6]. Furthermore for complete utilization of whey lactose, it is necessary to supplement whey with an additional nitrogen source [7]. In other researches whey has been supplemented[7] with yeast extract [8-11], peptone [8], milk powder[12] , corn steep liquor [13],or wheat steep liquor[6]. Beal et al[8] added yeast extract, peptone and lactose to whey for producing *S.thermophilus*.

Temperature and pH are the most important operating factors in fermentation of lactic acid bacteria[14].some researchers[15, 16] has determined optimal temperature for growth and acidification of starter cultures in the milk without pH control. There is a wide range of optimal temperature for *S.thermophilus* (40 - 45°C). Some works has been done [8, 10] to attain optimal pH for growth of lactic acid bacteria in whey medium. Beal et al has recommended[8] pH value of 6.5 and 40°C for growth of *S.thermophilus* 404. Maximum acidification was obtained at pH values and temperatures higher than the optimal growth conditions[8].

In this work effect of pH and temperature on growth and lactic acid production of *Streptococcus thermophilus* ATCC 19258 has determined by the use of response surface methodology. Furthermore optimal pH and temperature has determined for this strain. Data obtained in this study used to kinetic modeling the growth and acid production of *S.thermophilus* in different work.

2- Material and method

2-1-Microorganism

Streptococcus thermophilus PTCC 1738(ATCC 19258) were obtained from microbial collection of Iranian Research Organization for Science and Technology .The pure culture was prepared in M17 medium (Merck). The cultures were kept in deep-frozen stock (-80°C). Working cultures was prepared from frozen cultures 1% (v/v), incubated for18h at 40°C. Ten ml of fresh working culture with the concentration of 10^9 cell ml^{-1} was used as inoculums for biofermentation process.

2-2-Medium

Whey powder was obtained from Isfahan Pegah Dairy products and was dissolved in water to attain the concentration of 10% (W/V). This recombined whey was autoclaved (110°C, 20 min) and filtered. Eight hundred ml of filtrate with 1ml Tween 80(Merck) in 1 lit handmade bioreactor was autoclaved (110°C, 20 min). Solutions of 10g yeast extract (Merck) in 100 milliliter water were prepared and autoclaved (110°C, 20 min) separately, and added to the sterile whey mixture just before the fermentation.

1M $MgSO_4$ * $7H_20$ and 1M $CaCO_3$ were autoclaved (110°C, 20 min) and 1ml of each added to bioreactor just before the fermentation. Mg^{2+} has been shown influence on growth and cell division [17].

Table1. Design of experiments based on RSM, 3 basic responses are maximum biomass concentration (X_{max}), maximum specific growth rate (μ_{max}), lactic acid produced at the end of fermentation (P)

Run	T (°C)	PH	X_s (cell/ml)	$Mu_s(h^{-1})$	P_s(g/l)
1	36	5.7	1E+09	0.827	2.45
2	36	7.3	2E+09	0.946	5.355
3	44	5.7	8E+08	0.827	2.855
4	44	7.3	3.5E+09	0.929	9.955
5	34.34	6.5	1.6E+09	1.015	5.6
6	45.65	6.5	2.87E+09	0.86	9.09
7	40	5.36	1.4E+09	0.859	1.89
8	40	7.63	2.5E+09	1.143	9.36
9	40	6.5	2.3E+09	1.076	5.29
10	40	6.5	3.0E+09	1.28	9.17
11	40	6.5	2.4E+09	1.208	10.15
12	40	6.5	2.8E+09	1.25	6.66

2-3- Analyzes

Batch cultures with controlled pH and constant temperature were carried out in 1 L handmade bioreactor. Lactic acid produced during the fermentation was computed by intermittent addition of 5N sodium hydroxide at appropriate time

Table 2: analysis of variance for maximum biomass concentration

Source	Degree of Freedom	Sum of Squares	Mean of Squares	F value	Pr > F
T	1	1.2E+18	1.2E+18	6.6804	0.041509
PH	1	3.4E+18	3.4E+18	19.2504	0.00463
T*T	1	4.3E+17	4.4E+17	2.4471	0.168779
T*PH	1	7.2E+17	7.2E+17	4.0282	0.091528
PH*PH	1	1.0E+18	1.0E+18	5.8348	0.052179
Model	5	6.6E+18	1.3E+18	7.4023	0.015098
(Linear)	2	4.6E+18	2.3E+18	12.96	0.0066
(Quadratic)	2	1.2E+18	6.3E+17	3.5263	0.097133
(Lack of fit)	3	7.4E+17	2.5E+17	2.2860	0.257373
(Pure Error)	3	3.2E+17	1.0E+17		
Total	11	7.7E+18			

intervals using a Peristaltic pump [18]. In this way pH was controlled in defined value due to the experiments.

Samples were taken every half an hour, and placed in refrigerator. Growth was observed by microscopic counting with a Thoma having 0.02 mm depth.

Biomass specific dry weight was determined by measuring the weight of a dried sample containing a known number of the bacterium cultivated in MRS medium.

2-4-Design of experiments

Response surface methodology central composite: uniform precision has used in order to estimate the effect of pH and temperature on the growth and acid production of S.thermophilus. this design included 12 runs whit 4 factorial, 4 axial and 4 center points. The values centre points were chosen from that of reported by Beal et al[8]: T = 40°C and pH = 6.5 for *S.thermophilus404* . Analysis was done with the use of SAS 9.0 software.

The response surface model for predicting the relationship of responses and two controllable factors will be stated according to equation 1[19]:

$$y = B_0 + \sum_{i=1}^{2} B_i x_i + \sum_{i=1}^{2} B_{ii} x_i^2 + \sum \sum_{i>j=1}^{2} B_{ij} x_i x_j + \varepsilon$$

Where y is the responses (X_{max}, μ_{max} and P) and x_1 and x_2 are temperature and pH. So B is the coefficients of each factor.

3- Results

Maximum biomass concentrations (X_{max}), maximum specific growth rate (μ_{max}), total lactic acid concentration (P) were the responses in the experiments that analyzed with SAS software. Maximum specific growth rate was the slope of ln(X) per time. Total lactic acid concentration was the amount of acid produced after 5.5th hour of fermentation, because all of the fermentations were at the stationary phase at about this time. All of the responses are in table 1.

3-1-Maximum biomass concentration

Linear model was highly significant (Pr<0.01) for maximum biomass concentration and that's because of the significant effect of pH(Pr<0.005).

Table 3: analysis of variance for maximum specific growth rate

Source	Degree of	Sum of	Mean of	F value	Pr > F
T	1	0.006974	0.006974	0.860643	0.389353
PH	1	0.04846	0.04846	5.980458	0.050096
T*T	1	0.153264	0.153264	18.91445	0.004827
T*PH	1	0.000072	0.000072	0.008916	0.927845
PH*PH	1	0.096826	0.096826	11.9493	0.01352
Model	5	0.265258	0.053052	6.547137	0.020273
(Linear)	2	0.055434	0.027717	3.42054	0.102011
(Quadratic)	2	0.209753	0.104876	12.94284	0.006663
(Lack of fit)	3	0.024327	0.008109	1.001491	0.499526
(Pure Error)	3	0.024291	0.008097		
Total	11	0.313877			

On the other hand fitted model was significant for this response ($Pr<0.05$). Temperature and second order of pH was significant, too.

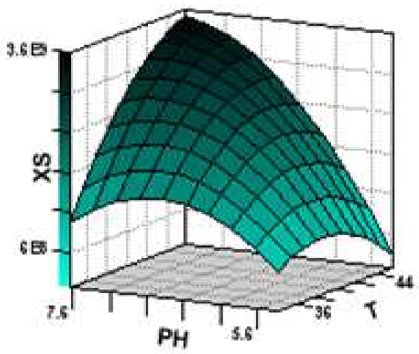

Figure 1: response surface for maximum biomass concentration

3-2-Maximum specific growth rate (μ_{max})

As it has shown in table 3, second order model was highly significant for maximum specific growth rate. Second order of temperature had maximum effect. pH^2 and pH was also significant.

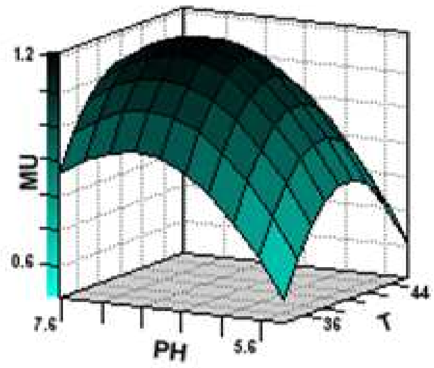

Figure 2: response surface for maximum specific growth rate

3-3-Produced lactic acid

All of the batches were in the stationary phase after 5^{th} hour of fermentation. So lactic acid concentration was measured after 5.5 hours fermentation and analyzed with RSM.

Fitted model for produced lactic acid is similar to maximum biomass concentration. So linear model was highly significant for produced lactic acid and that's because of the significant effect of pH. pH and pH^2 had the significant effect on produced lactic acid.

Table4: analysis of variance for produced lactic acid

Source	Degree of Freedom	Sum of Squares	Mean of Squares	F value	Pr > F
T	1	3.13	3.13	3.7448	0.1011
PH	1	16.26	16.26	19.4302	0.0045
T*T	1	0.77	0.77	0.9154	0.3756
T*PH	1	1.56	1.56	1.8676	0.2207
PH*PH	1	5.15	5.15	6.1575	0.0477
Model	5	26.29	5.26	6.2842	0.0223
(Linear)	2	19.39	9.69	11.5875	0.0087
(Quadratic)	2	5.34	2.67	3.1892	0.1139
(Lack of fit)	3	1.67	0.56	0.4965	0.7101
(Pure Error)	3	3.35	1.12		
Total	11	31.31			

3-4-Optimum pH and temperature

Response surface model for each response has determined for each response and coeficients of fitted model of equattion 1 are in table 5. With the use of predicted model in SAS software optimum pH and temperature for the growth and acid production of *s.thermophilus* was 7 and 42.8ºC, respectively.

Table 5. Coefficients of response surface model for X_{max}, μ_{max} and P and Richards parameters according to equation 1.

B_i Coefficients	X_{max}(cell/ml)	μ_{max}(h^{-1})	P_s(g/l)
B_0	-2.49E+10	-23.07	-55.7
T	5.43E+08	0.775	0.6166
pH	3.72E+09	2.648	12.19
T*T	-1.63E+07	-	-0.0216
pH*pH	-6.32E+08	-0.192	-1. 401
T*pH	-1.33E+08	n.s	-0.195
R^2 (R-square for RSM)	86.05 %	84.51 %	83.97%

n.s: non-significant

4- Conclusion

In this study, effect of pH and temperature, on the growth and lactic acid production of *streptococcus thermophilus* during pH-controlled batch cultures studied with the use of response surface analysis. These experiments used for kinetic analysis of the mix culture of *L.bulgaricus* and *streptococcus thermophilus*. Surface analysis showed that second order model was significant for maximum specific growth rate (μ_{max}) because second order of temperature and pH had the maximum effect on this response. On the other hand response surface model was significant for maximum biomass concentration and produced lactic acid. pH was highly significant for these two responses. Optimum temperature and pH for maximum biomass and specific growth rate and acid production were 42.8°C and 7. Optimum conditions differs from that of reported by Beal et al [8]. Maximum biomass concentration was lower in this study . It is obvious that the low level and high level of the factors is very important, so predicted model highly depend on the considered rage for pH and temperature.

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