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Growth Optimization of *Lactobacillus plantarum* T5jq301796.1, an Iranian Indigenous Probiotic in Lab Scale Fermenter

Faranak Noori[1], Maryam Tajabadi Ebrahimi[2], Parvaneh Jafari[3]*

1. Department of Microbiology, College of Sciences, Saveh Science and Research Branch, Islamic Azad University, Saveh, Iran.
2. Assistant Professor, Department of Biology, Faculty of Sciences, Islamic Azad University Central Tehran Branch, Tehran, Iran.
3. Assistant Professor, Department of Microbiology, Faculty of Science, Islamic Azad University Arak Branch, Arak, Iran.

Abstract

Background and Objective: *Lactobacillus plantarum* is one of the probiotics species used in functional food products. These bacteria or their purified bacteriocins are used as biological preservatives in the food industry. The first step in production of an array of probiotic products is optimizing production in fermentors. This study aimed to examine factors affecting the in vitro growth optimization of *Lactobacillus plantarum* T5JQ301796.1 in a lab scale fermentor.

Materials and Methods: Following 24 hours of anaerobic culture of the lactobacillus at 37°C, the pre-culture was ready and was inoculated to a 5 liter fermentor at 37°C and stirred at 40 rpm. Then factors affecting lactobacillus growth including carbon and nitrogen sources and pH were studied. The results were interpreted using response surface methodology (RSM), and optimal conditions for the equipment were determined.

Results and Conclusion: For optimal growth of *Lactobacillus plantarum* T5JQ301796.1 in lab scale fermentor, the optimal conditions were 25.96 gl^{-1} of glucose, 1.82% of yeast extract, pH of 7.26, and stirring at 40 rpm at optimum temperature between 37-40°C. In this condition, maximum viable cell in the batch fermentation was 1.25×10^{10} CFU ml^{-1}. Application of central composite design for the growth optimization of this bacterium led to maximum viable cells equal to 1.25×10^{10} CFU ml^{-1}. So the mentioned features can lead to optimum industrial scale production and usage of this probiotic strain in probiotic products.

Conflict of interest: The authors declare that there is no conflict of interest.

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Correspondence to
Maryam Tajabadi Ebrahimi
Department of Biology, Faculty of Sciences, Islamic Azad University Central Tehran Branch, Tehran, Iran.
P O. Box: 14155-8189
Tel: +98-21-88983647
Fax: +98-21-88983647
E-mail: m.tajabadi@iauctb.ac.ir

1. Introduction

Probiotics are rather new products, which are usually consumed in fermented foods [1-3]. Probiotics are usually defined as living microorganisms, which their sufficient consumption improves the host's health [4]. Lactic acid bacteria (LAB) are the most common microorganisms used as probiotics [5]. There has been a rising demand for probiotic products in recent years. Thus, industrial and semi-industrial fermentation of probiotics can underlie the importance of these products as generally recognized as safe (GRAS) products [6,7].

LAB are gram-positive, acid tolerant, nonsporogenic, anaerobic bacilli or cocci [8]. Lactic acid production is considered as the most common activity of these bacteria [5,9]. Members of this extensive group are used as probiotics because they prevent stable attachment, settlement and proliferation of pathogens in the gastrointestinal sy-

stem, and suppress precarcinogenic tumors [10,11]. LAB can regulate the immune system, and eliminate harmful effects of stress [12,13]. Accordingly, production of these bacteria has increased considerably [14].

LAB grow hardly, and many elements like carbohydrates, amino acids, peptides, nucleic acids, and vitamins are required for their optimal growth [15]. Fermentation conditions including the pH, temperature, aeration, standardization and compounds of culture medium are the most important and influential factors in their growth [16]. Microbial growth optimization can be performed in different methods, such as Response Surface Method (RSM) [17,18]. RSM can be used for assessing the relative importance of various factors in the presence of complex interactions [19]. It is a powerful test technique for multi level factors, as it requires fewer practical laboratory tasks compared to studying one factor at a time [20]. With RSM, different levels of functional factors can be used to achieve maximum efficiency with minimum tests.

The most common practical method in RSM is application of Central Composite Design (CCD), as coefficients of the second degree model can be estimated easily [13]. In this study, CCD was used in RSM. Variables of glucose concentration, yeast extract, and pH were studied for designing tests in two levels.

The ultimate aim of this study is to find optimal growth conditions to maximize biomass production.

2. Materials and Methods

2.1. Microorganisms and culture conditions

In the present study, *Lactobacillus* (*L.*) *plantarum* T5JQ301796.1 stored in glycerol 25% at -80°C was used. This bacterium was previously isolated from Tarkhineh (a traditional Iranian fermented food stuff) by Ebrahimi et al. [21,22]. Inoculation and culture media comprised 20 gl^{-1} of glucose, 10 gl^{-1} of yeast extract, 0.2 gl^{-1} of sodium phosphate, 0.2 of gl^{-1} di-potassium phosphate, 0.01 of mgl^{-1} magnesium sulfate, 0.01 gl^{-1} of iron sulfate, 1 mll^{-1} of Twin 80 (all ingredients from Merck, Germany), and 1 mll^{-1} of vitamin solution [23]. The vitamin solution included 2 g of pyridoxine hydrochloride, 1 gl^{-1} of pantothenic acid, 1 gl^{-1} of niacin, 1 gl^{-1} of riboflavin, and 1 gl^{-1} of folic acid. It was first filtered and then added to sterile culture medium before inoculation. Five ml of stored culture was inoculated into 200 ml of liquid MRS culture medium, and incubated at 37°C for 48 hours to prepare inoculum. The resulting cells were centrifuged at 3000 ×g for 15 minutes, rinsed twice with PBS buffer, and then used as inoculum. To determine the effects of carbon, nitrogen, and pH on the growth of lactobacilli, all other factors and compounds were kept constant, and only the level of testing factor was considered variable. Optical density of the samples was determined after performing tests and fermentation process. Thus, based on previews experiments, the range of the factors for optimal growth was identified. Accordingly, the best range was 20 gl^{-1} for glucose, 0.7 gl^{-1} for nitrogen source, and 7 for pH. CCD test was designed for optimization of the fermentor according to the data obtained from the range tests.

2.2. Experimental design and statistical analysis

Optimization of biomass production was designed based on 2-level, 3-factor RSM method using CCD, which resulted in 15 tests. Independent variables included initial pH, glucose and yeast extract concentrations.

Table 1. Factors (and their levels) affecting growth of *L. plantarum* T5jq301796.1 in lab scale fermenter.

Factor	Code	Unit	Lower level	Upper level
pH	A	-	4.0	8.0
Glucose	B	gl^{-1}	15.0	30
Yeast extract	C	gl^{-1}	0.7	2.0

Dependent variable of optical density was measured by spectrophotometer. Fifteen designed tests were performed randomly. The amount of yeast extract and lactose added varied according to the requirements of each experiment. The initial pH of culture medium was adjusted by HCl (N=5) or NaOH (N=5).

Table 2. Responses of the central composite design for evaluation of impact of three factors on growth of *L. plantarum* T5jq301796.1 in lab scale fermenter.

Run	Glucose (gl^{-1})	Yeast extract (gl^{-1})	pH	OD
1	10.00	0.20	3.00	0.42
2	25.00	2.61	5.50	0.90
3	40.00	2.20	3.00	0.60
4	25.00	1.20	5.50	1.54
5	25.00	1.20	5.50	1.30
6	25.00	1.20	9.04	1.02
7	3.79	1.20	5.50	1.20
8	25.00	0.10	5.50	1.56
9	25.00	1.20	5.50	0.43
10	46.21	1.20	5.50	0.61
11	10.00	2.20	8.00	1.55
12	25.00	1.20	5.50	0.52
13	40.00	0.20	8.00	0.19
14	25.00	1.20	1.96	1.58
15	25.00	1.20	5.50	0.80

2.3. Fermenter

Biomass production of *L. plantarum* T5JQ301796.1 was performed in a fermentor, model Electrolab, Fermac 360-UK, with working volume of 5 litters. For each fermentation run, 500 ml preculture was inoculated to 4500 ml of the culture medium prepared with desired composition according to RSM runs. The fermentor was set up at 37°C and 40 rpm. Aeration was not required because *L. plantarum* is an anaerobic bacterium. This process lasted 24 hours, and periodical sampling was performed every 2 hours. After each expe-

riment, the resulting cell mass was measured at 600 nm using spectrophotometer (Vis-Genus, UV mini-1240 UV). Colony forming unit was evaluated by the serial dilutions method on MRS agar culture medium. Before cell count, the samples were incubated at 37°C for 48 hours. Each sample was cultured in plates in two replicates.

2.4. Analytical methods

After every experiment, the resulting cell mass was measured at 600nm using spectrophotometer (Vis-Genus, UV mini-1240 UV).

Viable bacterial cells were produced using serial dilutions and culture method in MRS agar culture medium. Each sample was cultured in plates in two replicates. Before cell count, the samples were incubated at 37°C for 48 hours. In this experiment, pH, glucose and yeast extract concentrations were considered as influential factors in growth optimization using CCD method. Parameters were benchmarked according to the initial experiments. ANOVA test for Quadratic model and analysis of variance was performed; in other words, ($p \leq 0.05$) indicate the significance of the results of this test.

3. Results and Discussion

In recent years, the world has witnessed extensive advances in the role of biotechnology in qualitative and quantitative improvement of food and consumer health, especially through the use of beneficial bacterial strains at industrial and commercial levels. Probiotic bacteria play an effective and significant role in human health through control of pathogenic strains in the digestive system and improving the immune system. Effectiveness of these bacteria depends on their stability during production, formulation and storage conditions [24].

Modern laboratory techniques and methods are being studied for optimizing production and extending use of these health promoting microbial agents [4]. Beukes et al. and Lim et al. isolated and identified, *L. plantarum*, *L. delbrukii* and *L. Salivarius* from samples of a traditional dairy product called *amasi* in South Africa and Namibia [25].

Sathyanarayanan et al. conducted a study on riboflavin produced by *L. fermentum* isolated from yoghurt samples. In their study, riboflavin was produced by lactic acid bacteria separated from fermented milk samples. *L. fermentum* MTCC8711 produced large amounts of riboflavin. RSM was used for optimization of fermentation and lactobacillus bacterial growth, and consequently, riboflavin production [26].

RSM is a powerful tool in optimizing the fermentation medium for Lactobacilli. However, Ghasemi and Ahmadzadeh used RSM for optimization of culture medium for mass production of *bacillus subtilis* UTB96 with laboratory and semi industrial fermenters [27].

In the current study, pH, glucose and yeast extract concentrations were considered as influential factors in growth optimization. We selected the effective parameters based on the initial experiments (Table 1). Conditions of influential factors and optical density results are presented in Table 2, and the ANOVA test results for the second degree model and variance analysis are shown in Table 3.

Data processing with resulted the below fitted equation based in coded value: Eq 1.

Optical Density= 1.64+0.067 A+0.17 B+ 0.18 C+ 0.47 AB+ 0.38 AC+ 0.16 BC- 0.33 A2- 0.35 B2- 0.29 C2

Where, A: glucose (gl^{-1}), B: yeast extracts (gl^{-1}), and C: pH

Based on the result, the fitted model was calculated and given by software. It means that biomass production by this bacterium follow this model and it can predict the biomass production in different conditions. The results of this study showed that the model used here was appropriate because, according to the p-value, there was only probability of 1.16% ($p \leq 0.016$) for error appearance in these results. According to the p-values presented in Table 3, AB, AC, A2, B2 and C2 are also significant in this model. Whatever the number of factors used in the model is more significant in the case of the proposed model is more appropriate.

Three dimensional surface plots exhibited the optical density of *L. plantarum* T5 JQ301796.1 growth for different combinations of variables. Plots are depicted as a function of two variables while the others are fixed at zero level. Examining the interaction of influential factors showed that at constant glucose concentration of 15 gl^{-1}, the growth level reduces with increasing the yeast extract concentration from 0.7 gl^{-1} to 2 gl^{-1}. At constant yeast extract concentration of 0.7 gl^{-1}, the growth level dramatically reduces with increasing carbon from 15 gl^{-1} to 30 gl^{-1}, and when carbon density is kept constant at 30 gl^{-1}, the growth level significantly increases with increasing the yeast extract from 0.7 gl^{-1} to 2 gl^{-1} (Figure 1). Furthermore, at constant glucose concentration of 15 gl^{-1}, the growth level slightly reduces with increasing the pH from 4 to 8, and at constant pH value of 4, it significantly reduces with increasing the carbon concentration from 15 gl^{-1} to 30 gl^{-1}. Yet, the growth significantly increases with increasing the pH from 4 to 8 at constant carbon concentration of 30 gl^{-1} (Figure 2). The effects of nitrogen concentration and pH on growth are shown in Figure 3. Accordingly, at constant pH of 8, the growth rate significantly

Table 3. Analysis of variance for evaluation of impact of three factors on growth of *L. plantarum* T5jq301796.1 in lab scale fermenter.

	Sum of squares	DF	Mean square	F-value	p-value	
Source model	3.065	9	0.341	9.515	0.0116	Significant
A-C source	0.018	1	0.018	0.504	0.5093	
B-N source	0.115	1	0.115	3.219	0.1328	
C-pH	0.125	1	0.125	3.493	0.1206	
AB	0.445	1	0.445	12.438	0.0168	
AC	0.288	1	0.288	8.057	0.0363	
BC	0.049	1	0.049	1.381	0.2929	
A^2	0.816	1	0.816	22.794	0.005	
B^2	0.933	1	0.933	26.057	0.0038	
C^2	0.661	1	0.661	18.465	0.0077	
Residual	0.179	5	0.036			
Lack of Fit	0.125	1	0.125	9.275	0. 382	Not significant
Pure Error	0.054	4	0.013			
Total	3.244	14				

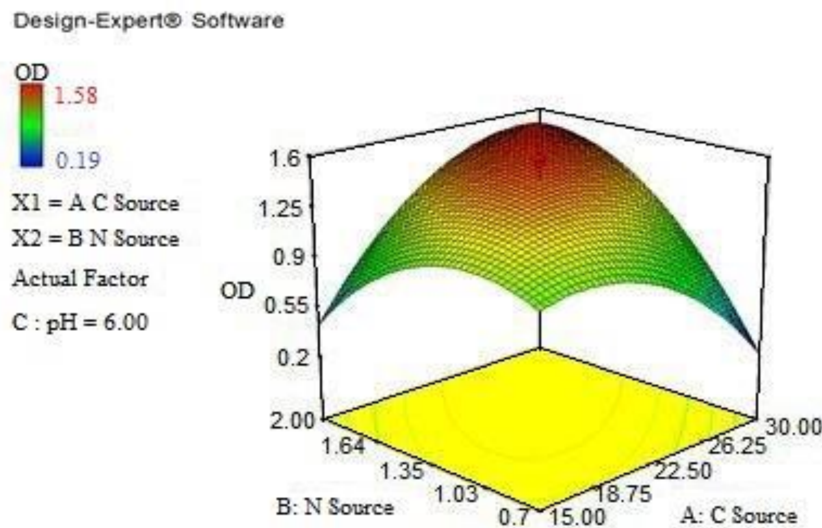

Figure 1. Interaction effect of yeast extract and glucose on growth rate of *L. plantarum* T5jq301796.

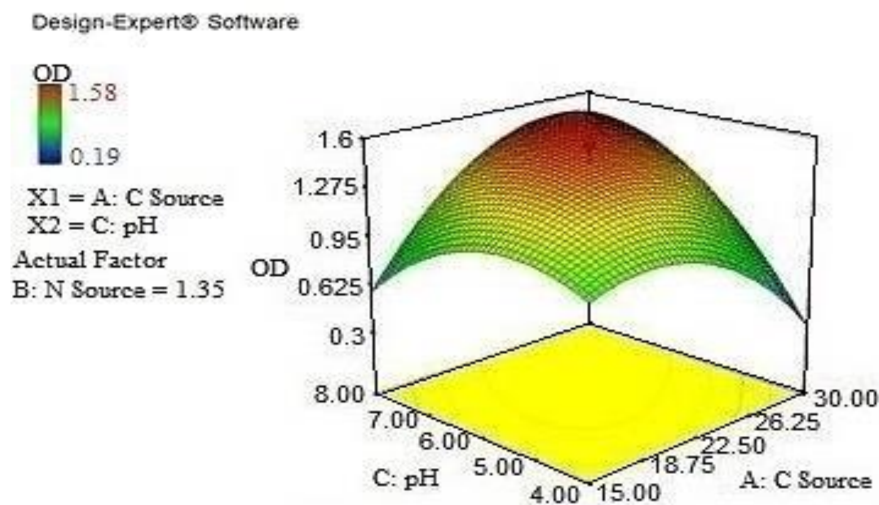

Figure 2. Main effects of carbon density and pH on growth rate of *L. plantarum* T5jq301796.

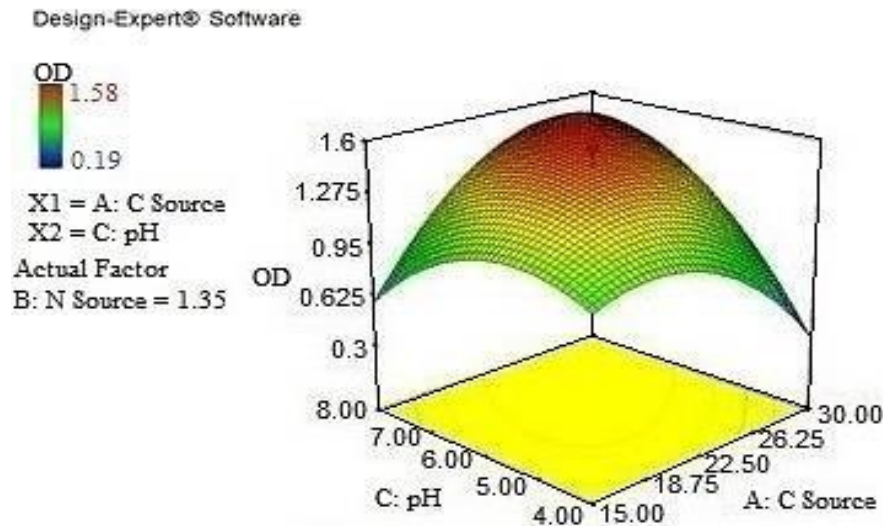

Figure 3. Effects of nitrogen concentration and pH on growth rate.

increases with increasing the yeast extract concentration from 0.7 gl^{-1} to 2 gl^{-1}. However, at pH of 4, the growth level initially increases, and then reduces with increasing the yeast extract concentration from 0.45 to 1.35 gl^{-1}. At high yeast extract concentration (2 gl^{-1}), the growth rate significantly increases with increasing the pH, while at low concentration of the yeast extract, increasing the pH value has no significant effect on the growth rate (Figure 3). The second order polynomial equation provided optimal values of independent variables and proposed maximum optical absorption. Optimal conditions for the above bacterium are provided by the following values:

pH=7.26, glucose=25.96 gl^{-1}, and yeast extract=1.82 gl^{-1}

To examine the predicted results after confirmatory test and OD measurement, optical absorption in these conditions was found 1.85, which is close to the experimented result that was 1.70.

4. Conclusion

Given the increasing spread of industrial dairy products instead of traditional ones, it is possible that many probiotic bacteria are lost. It is, therefore, necessary to isolate and identify these bacteria from traditional food sources, and use them in dairy products. Thus, optimization of production in a fermentor is the first step in producing a wide range of probiotic products based on these bacteria.

In this study, pH, glucose and yeast extract concentrations were considered as influential factors in growth optimization using CCD method. The parameters were benchmarked according to the initial experiments of influential factors and OD results.

On the basis of medium optimization, the second degree polynomial model predicted the maximum OD_{600} by *L. plantarum* as 1.85 (equal to 1.25×10^{10} CFU ml^{-1}) in the medium containing 25.96 and 1.82 gl^{-1} of glucose and yeast extract, respectively, in pH 7.26, temperature 37-40°C, and stirring at 40 rpm. Experimental studies showed a good correlation between the predicted and experimental OD values.

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6. Conflict of interests

None of the authors had any personal or financial conflict of interest.

References

1. Esteban-Torres M, Mancheño JM, de las Rivas B, Munoz R. Characterization of a halotolerant lipase from the lactic acid bacteria Lactobacillus plantarum useful in food fermentations. LWT-Food Sci Technol. 2015; 60(1): 246-252. doi:10.1016/j. lwt.2014.05.063
2. Granato D, Branco GF, Nazzaro F, Cruz AG, Faria JA. Functional foods and nondairy probiotic food development: trends, concepts, and products. Compr Rev Food Sci Food Safety. 2010; 9(3): 292-302. doi: 10.1111/j.1541-4337.2010.00110.x
3. Mattila-Sandholm T, Myllärinen P,Crittenden R, Mogensen G, Fondén R, Saarela M. Technological challenges for future probiotic foods. Int Dairy J. 2002; 12(2): 173-182. doi:10.1016/S0958-6946(01-)00099-1
4. Pineiro M, Stanton C. Probiotic bacteria: Legislative framework-requirements to evidence basis. J Nutr. 2007; 137(3): 850S-853S.
5. Tannock G.W.Probiotic properties of lactic-acid bacteria: Plenty of scope for fundamental R & D. Trend Biotechnol. 1997; 15(7): 270-274.
6. Dunne C, O'Mahony L, Murphy L, Thornton G, Morrissey D, O'Halloran S, Feeney M, Flynn S, Fitzgerald G, Daly Ch, Kiely B , O'Sullivan GC, Shanahan F, Collins JK. In vitro selection criteria for

probiotic bacteria of human origin: Correlation with in vivo findings. Am J Clin Nutr. 2001;73(2): 386s-392s.

7. Collins J, Thornton G, Sullivan G. Selection of probiotic strains for human applications. Int Dairy J. 1998; 8(5): 487-490. doi: 10.1016/S0958-6946(9-8)00073-9
8. Wood BJB, Warner PJ. Genetics of lactic acid bacteria. Vol. 3. New York: Kluwer Academic/Plenum Publishers, 2012.
9. Senthilkumar P. Antibacterial potential of lactic acid bacteria and its metabolites against food borne pathogens. Int J Pharm Biol Sci Arch 2012; 3(2):
10. Lee YK, Salminen S. Handbook of probiotics and prebiotics. John Wiley & Sons. 2nd edition. New Jersey, USA, 2009.
11. Jung-Woo L, Jung-Gul Sh, Eun Hee K, Hae Eun K, In Been Y, Ji Yeon K, Hong-Gu J, Hee Jong W. Immunomodulatory and antitumor effects in vivo by the cytoplasmic fraction of Lactobacillus casei and Bifidobacterium longum. J Vet Sci 2004; 5(1): 41-48.
12. Matsuzaki T, Chin J. Modulating immune responses with probiotic bacteria. Immunol.Cell Biol. 2000; 78(1): 67-73. doi: 10.1046/j.1440-1711.2000.00887.x
13. Macfarlane GT, Cummings JH. Probiotics, infection and immunity. Current Opin Inf Dis. 2002; 15(5): 501-506.
14. O'Shea EF, Cotter PD, Ross RP, Hill C. Strategies to improve the bacteriocin protection provided by lactic acid bacteria. Curr Opin Biotechnol, 2013; 24(2): 130-134. doi:10.1016/j.copbio.2012.12.003
15. Katharina E. Scholz A, Berit A, Florence R, Denis V B, Michael de V, Yahya Al, Jürgen S. Effects of probiotics, prebiotics, and synbiotics on mineral metabolism in ovariectomized rats -impact of bacterial mass, intestinal absorptive area and reduction of bone turn-over. NFS J. 2016; 3: 41-50. doi:10.-1016/j.nfs.2016.03.001
16. Jiang Q, Stamatova I, Kari K, Meurman JH. Inhibitory activity in vitro of probiotic lactobacilli against oral Candida under different fermentation conditions. Beneficial Microbes. 2014; 6(3): 361-368. doi: 10.39-20/BM2014.0054
17. Khuri AI, Mukhopadhyay S. Response surface methodology. WIREs CompStat. 2010; 2(2): 128-149. doi: 10.1002/wics.73
18. Lew LC, Liong MT, Gan CY. Growth optimization of Lactobacillus *rhamnosus* FTDC 8313 and the production of putative dermal bioactives in the presence of manganese and magnesium ions. J Appl Microbiol. 2013; 114(2): 526-535. doi:10.111-1/jam.12044
19. Burket M, Antonio Davila , Kandarp Mehta, Daniel Oyon.Relating alternative forms of contingency fit to the appropriate methods to test them. Manag Accoun Res. 2014; 25(1): 6-29. doi:10.1016/j.mar.201-3.07.008
20. Falah B, Akour M, Bouriat S. RSM: Reducing mutation testing cost using random selective mutation technique. Malays J Comput Sci 2015; 28(4): 338-347.
21. Tafvizi F, Tajabadi EM, KHojareh L. Molecular identification of probiotic lactobacill bacteria from tarkhineh and tarkhineh dough by 16s RDNA sequencing. J FoodTech Nutr. 2013; 10; (38) 61-68.
22. Tafvizi F, Tajabadi EM. Application of repetitive extragenic palindromic elements based on pcr in detection of genetic relationship of lactic acid bacteria species isolated from traditional fermented food products. J Agric Sci Technol. 2015; 17(1): 87-98.
23. Chen Q, He G, Ali MA. Optimization of medium composition for the production of elastase by Bacillus sp. EL31410 with response surface methodology. Enz Microb Technol 2002; 30(5): 667-672. doi: 10.1016/S0141-0229(02)00028-5
24. Heller KJ. Probiotic bacteria in fermented foods: product characteristics and starter organisms. Am J Clin Nutr. 2001; 73(2): 374s-379s.
25. Beukes EM, Bester BH, Mostert JF. The microbiology of South African traditional fermented milks. Int J Food Microbiol. 2001; 63(3): 189-197. doi:10.-1016/S0168-1605(00)00417-7
26. Sathyanarayanan J, Kunthala J, Gurumurthy K. Optimization of MRS media components using response surface methodology for the riboflavin production by *Lactobacillus fermentum* isolated from yoghurt sample. Int Food Res J. 2011; 18(1): 149-158.
27. Ghasemi S, Ahmadzadeh M. Optimisation of a cost-effective culture medium for the large-scale production of Bacillus subtilis UTB96. Arch Phytopathology Plant Prot. 2013; 46(13): 1552-1563. doi: 10.1080/03235408.2013.771469