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ORIGINAL ARTICLE

Optimization of the Yield and Quality of Agar from *Gracilariopsis persica*

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ABSTRACT

In this study, different conditions of pretreatment on physicochemical characteristics (quality) of the agar obtained from Gracilariopsis persica were optimized using response surface methodology (RMS). Pretreatment conditions were determined in five levels affected by three independent variables of alkali concentration including 2, 2.8, 4, 5.2 and 6% in periods (30, 60, 105, 150, 180 minutes) and thermal ranges of 60, 68, 77.5, 88 and 95 ^{0}C. Extraction yield, gel strength, sulfates and 3, 6 anhydro-L-galactose content were evaluated as dependent variables for a combination of independent variables. Variance analysis of overall effect of the process variables in regression models showed that, all input variables had a significant impact on gel strength and 3, 6 anhydro-L-galactose content ($p<0.05$) while temperature and time of pre-treatment were significant parameters on the extraction yield. Alkali concentration and pre-treatment time were significant parameters for the sulfate content. Polynomial models and optimal conditions were determined to extract agar with the maximum yield, gel strength, 3, 6 anhydro-L-galactose and minimum sulfate. Pre-treatment with concentration by 5.2% at temperature of 80^{0}C for one hour was suggested as optimal point. Actual values with regard to consistency with the model suggested values were satisfying which represent the model utility for optimizing agar pre-treatment.

***Key words**: Agar, Gracilariopsis persica, Alkali pretreatment, Optimization, Response Surface Methodology (RSM)*

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INTRODUCTION

Agar is a high molecular weight polysaccharide which has been derived from cell walls of some red algae. This biopolymer is the first used Phycocolloid as food additives in the Far East more than 300 years ago [2]. This hydrocolloid contains two polysaccharides namely Agarose and Agaropectin. Its gelatin property provides adhesion due to agarose and agaropectin compound. Repeating units of agarose include alternating groups of 3.6-anhydro-α-l-galactopyranos and β-D-galactopyranose. Agaropectin a non-uniform mixture of smaller molecules which is a structure similar to agarose but a little branched and sulfated. Actually, agaropectin in terms of structure is a little complicated and contains sulfate, pyruvate and gluconate groups [2, 1]. Agar has properties such as gelling, thickening, stabilizing, emulsifying, absorbing and being facilitators (lubricant). Therefore, agar has a wide application in food, medicine and biotechnological industries due to its colloidal and gel state [1]. Among agar resources (Pterocladia, Gelidium ،Gelidiella، Gracilaria), extracted agar from Gelidium algae had more appropriate quality but large population and growing of Gracilaria algae have caused these algae to be the main source of agar extraction throughout the world [8]. Therefore, alkaline pre-treatment is accomplished in order to improve qualitative traits of Gracilaria agar. This process leads to better extracting of polysaccharide from cell wall by alkaline hydrolysis of sulfate groups [3].

Collection of conducted researches show that, produced agar depends on some conditions such as considered species, season, environmental conditions, various stages of algae life cycle and extraction method. So optimizing the process of agar production and determination of extracted agar are much important [4], on the other hand, analysis of all factors affecting the extraction is costly and time consuming. There are various methods to reduce the number of effective treatments among which statistical method of response surface methodology is more efficient in the assessment of materials production processes [5, 6].

Nowadays, need for greater amounts of agar along with improvement of qualitative traits have cause to introduce various species and different methods of extraction. Therefore, various aspects of agarophyte must be investigated before introducing it and its extraction method. Although, many studies have been conducted about alkali pre-treatment and agar extraction from diverse agarophytes [7, 14, 10,25], but doing more researches about agar and introducing other agarophytes are required due to the wide application of agar. Therefore, the objective of this research is to determine the optimal conditions of alkali pretreatment using response surface methodology, considering that the studies on pretreatment optimization of agar extraction from *Grasilariopsis persica* and assessment of its physicochemical traits have not been yet, and also, Iran has these potential algal sources in the southern coasts (Persian Gulf).

MATERIALS AND METHODS

Sample preparation

Gracilariopsis persica were collected after a two-month growth period in May 2012 from site located in Minab (Hormozgan province, South of Iran).Washing operations were conducted immediately after collection, and the alga were immersed in 10% formalin for one hour to prevent enzymatic hydrolysis and microorganisms growth. Then, they were washed and dried in a proper place under sunlight and after that, they were kept in 60^{0}C oven for 8 hours. Then the samples were grinded (the size by 300-400 micro meters) and were packed in polyethylene bags to accomplish agar extraction process with assistance of pre-treatment stage [10].

Alkali pretreatment and agar extraction

For pretreatment operations, 10 grams of alga powder was heated in 200 ml of alkali solution in five points of sodium hydroxide concentrations of 2, 2.8, 4, 5.2 and 6% in periods of 30, 60, 105, 150 and 180 minutes and thermal ranges of 60, 68, 77.5, 88 and 95 ^{0}C. After conducting pre-treatment, alkali-alga mixture was chilled in the environment temperature, and alkali solution was separated from alga and washed by urban water. The algae were wetted by a 0.02 solution of sulfuric acid for 10 minutes and washed by distilled water. Then, the algae remained in distilled water for a night and washing water was isolated from algae in the next day. Agar extraction process was conducted at temperature by 121^{0}C for 30 minutes in 300 ml of 0.1 M phosphate buffer with a pH by 6.2. Distillate of extracted agar was filtered by a vacuumed filter and it was frozen for 12 hours. Liquid ejected from the sample was discarded, then, the achieved gel was washed by 70% ethanol for 15 minutes. After that, it was washed two times by thick ethanol for 30 minutes. Finally, the sample was died at temperature by 60 ^{0}C for 72 hours [9].

Evaluation of physical properties of agar

Extraction yield of agar was determined based on [10].

Agar Yield % = (Dry weight of agar) / (Dry weight of seaweed) × 100.

The gel strength was measured as described by [4] with minor modifications by using a CT3 texture analyzer (Brookfield Engineering, Middleboro, MA, USA). In this regard, a 1.5% (w/v) agar solution was prepared with distilled boiling water. When the hydrocolloid had dissolved completely, it was immediately disembogued into a cylindrical container (height by 45 mm and diameter by 30 mm) and then the lid was closed, the gel became ripe within a day at the environment temperature (25±1^{0}C). Finally, the gel strength (gr/cm^2) was recorded using a cylindrical plastic probe with a diameter of 10 mm, speed and depth of penetration of 1 mm/s and 20 mm respectively.

Determination of chemical properties of agar

The agar sulfate content was determined according to [10] and [11] methods. 0.5 gr samples of dried agar were thrown into the standard digestion tubes and 10 ml of thick HNO_3 was added to it. Digestion tubes were transported to a digester at temperature of 123 ^{0}C for 30 minutes to reach ultimate volume by 2-3 ml. After cooling the samples, 2-3 drops of 40% HCHO solution were added to it to eliminate extra HNO_3. The solution was filtered into a 250 ml flask and 0.5 mm of thick HCl was added to it and was reached to volume of 250 ml by distilled water. The solution was heated until becoming boiled and then, 10 ml of $BaCl_2$ was added to the solution as drop by drop and by stirrer during five minutes. After that, the solution was held in a warm place for 5 hours. $BaSO_4$ deposits were filtered by filter paper (Whatman No. 5). Obtained deposit became ash after transferring to a porcelain crucible for 1 hour at temperature of 700

^{0}C. Then, porcelain crucible was cooled in a desiccator and was weighted to achieve the amount of $BaSO_4$. Finally, sulfate percentage was computed through the following equation:
Sulphate (% db) = [(41.16 × Weight of BaSO4) / Sample Weight] × 100
The amount of 3, 6 Anhydro-L-galactose was calculated by colorimetric method of Yaphe and Arsenault [12], with some modifications. In this method, resorcinol–acetal and fructose were used as reagent and standard respectively.

Experimental design and statistical analysis

According to the conducted pre-tests, three independent variables of alkali concentration (2, 2.8, 4, 5.2 and 6%, X1), pretreatment time (30, 60, 105, 150 and 180 minutes, X2) and pretreatment temperature (60, 68, 77.5, 88, and 95 ^{0}C, X3) were determined in five levels.
The experiments were analyzed under central composite rotatable design (CCRD) by Design Expert software (version 7). In this layout, totally 20 experiments were planned with 6 replications in the central points. In this method, modeling of each dependent variable (Y) was presented in the framework of the following polynomial regression model as a function of independent variables (X).

$$Y= \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \sum_{j=i+1}^{k} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2$$

Y represents the amount of predicted response (extraction efficiency, gel strength, etc), K represents the number of input variables, xi is independent variables (i=1, 2,), βi, βii, βij are linear coefficient, interaction and quadratic respectively [22]. Table 1 shows the matrix of experiments and obtained response for extraction yield (Y_1), gel strength (Y_2), sulfate content (Y_3) and 3,6- Anhydrogalactose content (Y_4).

RESULTS

Codded values and obtained responses for each variable have been evaluated. Possible factors were assessed to use in the modeling process according to p-value and fitting. P-values less than 0.0001 (with significance values less than 0.05) were obtained for regression model of all responses which represented the model significance. On the other hand, the values of the lack of fit were obtained by 0.0671, 0.4658, 0.7131, and 0.8352 for extraction yield, gel strength, sulfate and 3, 6- Anhydrogalactose content respectively, and their lack of significance represents the model appropriateness (Table 1). The results of variance analysis showed that which component had significant impact on the considered response among components of linear, quadratic and interaction. Only some components which had no significant effect were eliminated from the next analyses to improve the model. High values of R^2 and also, closeness of adjusted R^2 and predicted R^2 shows the models satisfaction. After investigating the traits of selected statistical model, multiple regression analysis was conducted and polynomial mathematical model for each response was obtained (Table 2).
Impact of different pretreatment condition on each response has been shown in three-dimension graphs and contours. It seems that, alkali concentration and pretreatment temperature have found their convergence in alkali concentrations of 4% and temperature of 78 ^{0}C. Although, the proper point for these two factors is in this range, but, there is ascending trend of efficiency by increasing alkali concentration and pretreatment temperature from the beginning of determined range up to the middle limit while the effect of pretreatment is greater in this trend.

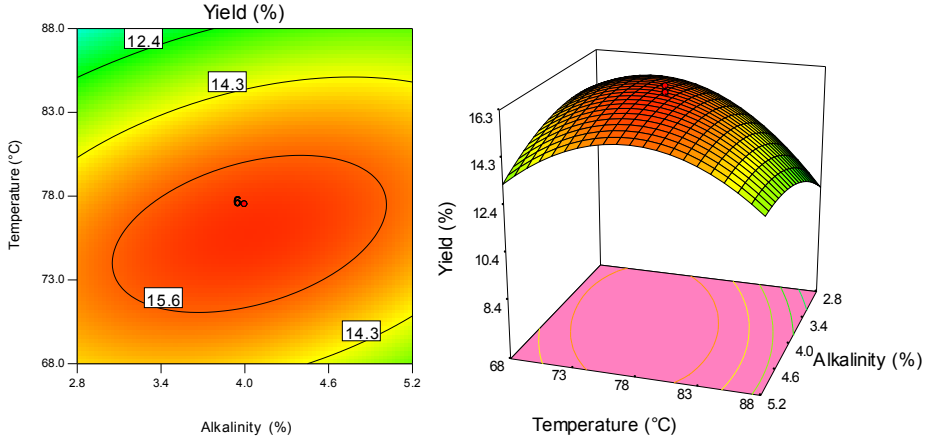

Fig. 1. Simultaneous effects of alkali concentration and pretreatment temperature on extraction yield

Negative interaction between pretreatment temperature and time, and extraction yield is appeared in Fig. 2. So that, extraction yield simultaneously decreases by increase of pretreatment time and temperature.

Desirable efficiency has been achieved at the minimum temperature and time of pre-treatment. According to F-value, pretreatment has a more significant effect in this interaction.

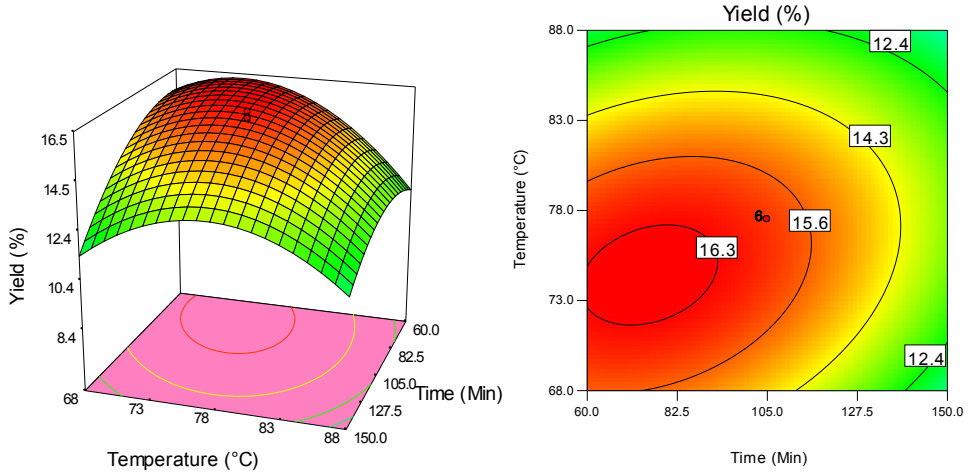

Fig. 2. Simultaneous effects of pre-treatment temperature and time on extraction yield

For gel strength, the simultaneous effect of alkali concentration and pretreatment time has been indicated in Fig. 3. Stronger gels can be extracted in the maximum alkali and minimum time of pre-treatment. According to the graph, it can be found that, the effect of alkali concentration on gel strength has been much greater than pretreatment time.

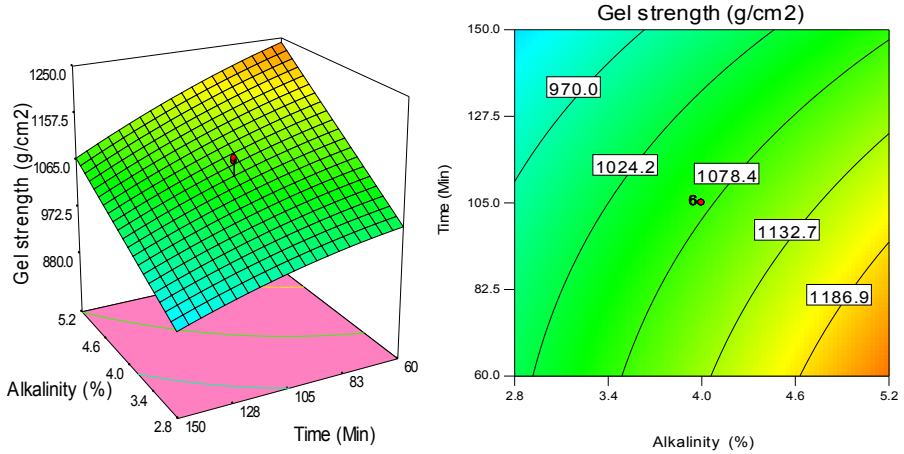

Fig. 3. Simultaneous effects of alkali concentration and pre-treatment time on agar gel strength

Simultaneous influence of pre-treatment time and temperature on the gel strength has been shown in Graph 4. According to the graph, strong gels are obtained at high temperatures and low time. Therefore, at high temperatures and low time of pre-treatment, an agar with high gel strength can be produced.

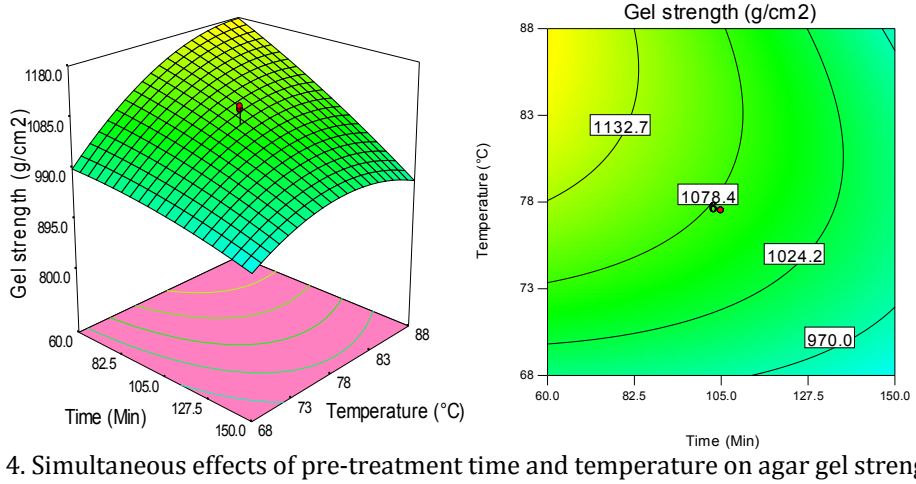

Fig. 4. Simultaneous effects of pre-treatment time and temperature on agar gel strength

Graph 5 shows the interaction of alkali concentration and pretreatment temperature on the sulfate content. Maximum amount of sulfate can be found in the lowest pretreatment conditions. Actually, by increasing alkali concentration and pretreatment temperature, the amount of sulfate existing in biopolymer can be reduced. This trend becomes very slow or stopped from the concentration of 4.6 and temperature of 78 ^{0}C. It is required to be noted that, alkali concentration has a higher impact on the trend of sulfate variations.

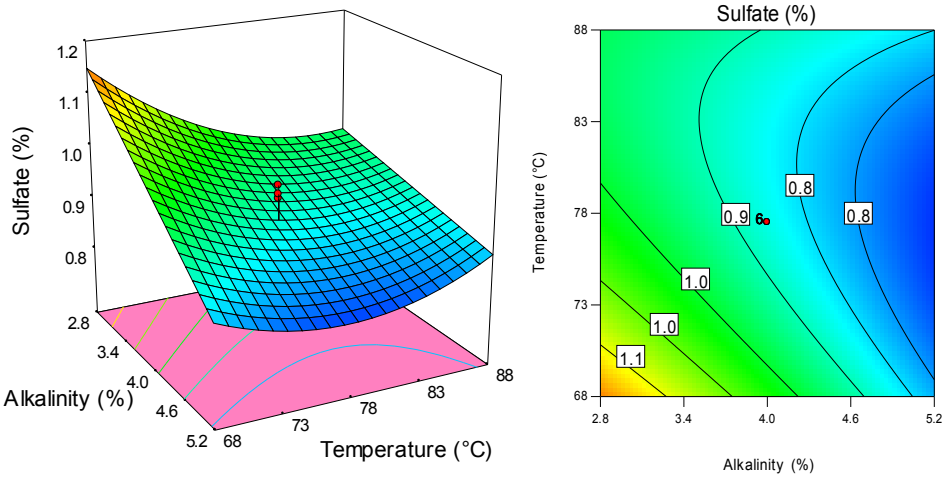

Fig. 5. Simultaneous effects of alkali concentration and pretreatment temperature on sulfate content

Graph 6 shows that, by increasing pre-treatment temperature and alkali concentration, the amount of 3, 6 AG increases simultaneously, and the agar containing high 3, 6 AG has been extracted from pretreated agarophyte with high concentrations of alkali and higher temperatures. This ascending trend reaches its peak at temperatures of 73-83 in the highest alkali concentrations.

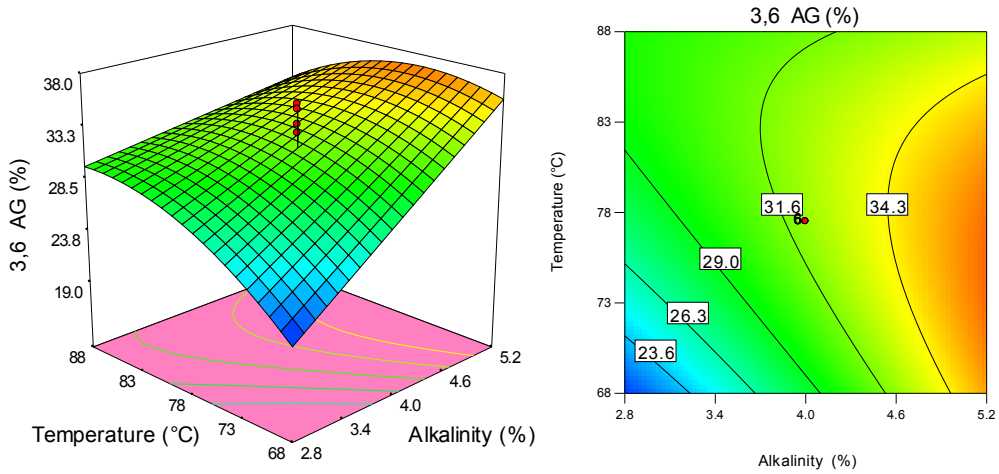

Figure 6. Simultaneous effects of alkali concentration and pre-treatment temperature on 3, 6 AG content

Interaction of pre-treatment temperature and time on 3, 6 AG content has been shown in Graph 7. According to the graph, by increasing pretreatment time and temperature, 3, 6 AG content increases if pretreatment time is low. Therefore, negative impact of the interaction of these two variables is assumed, and it can be predicted that, by increasing pretreatment temperature and decreasing pretreatment time, 3, 6 AG content increases.

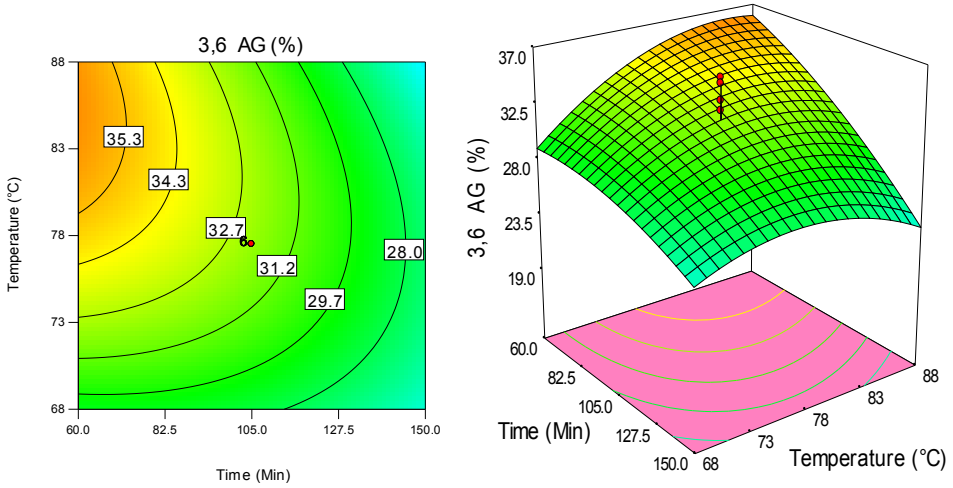

Fig 7. Simultaneous effects of pretreatment time and temperature on 3, 6 AG content

Table 1. Results of responses variance analysis test

Response	source	Sum of square	df	Mean of square	F-value	p-value
	Regression model	137.03	8	17.13	42.61	<0.0001
	remnant	4.42	11	0.40		
Yield	lack of fit	3.70	6	0.62	4.24	0.0671
	net error	0.73	5	0.15		
	total	141.45	19			
	Regression model	2.867E+005	7	40955	49.19	<0.0001
	remnant	9990.91	12	58.832		
Gel Strength	lack of fit	6104.37	7	872.05	1.12	0.4658
	net error	3886.54	5	777.31		
	total	2.967E+005	19			
	Regression model	0.49	6	0.082	16.53	<0.0001
	remnant	0.064	13	4.961E-003		
Sulfate content	lack of fit	0.033	8	4.144E-003	0.66	0.7131
	net error	0.031	5	6.267E-003		
	total	0.56	19			
	Regression model	730	7	104.29	15.22	<0.0001
	remnant	82.2	12	6.85		
3.6 AG	lack of fit	31.86	7	4.55	0.45	0.8352
3,6-Anhydrogalactose content	net error	50.34	5	10.07		
	total	812.2	19			

Table 2. Regression model and responses coefficient

Response	R^2	Adj- R^2	Pred- R^2	CV	Adeq precision	PRESS	Regression Model
Y	0.9687	0.9460	0.8447	4.86	18.8	21.97	$15.9+0.2X_1-1.24X_2-0.82X_3+0.96X_1X_3+0.86X_2X_3-0.7X_1^2-1.13X_2^2-2.34X_3^2$
G.S	0.9663	0.9467	0.8963	2.8	25.328	30760.32	$1072.26+95.22X_1-65.95X_2+59.89X_3-17.56X_1X_2-41.59X_2X_3-11.48X_2^2-5.73X_3^2$
S	0.8841	0.8306	0.7553	7.44	15.343	0.14	$0.87-0.11X_1+0.096X_2-0.062X_3+0.65X_1X_3+0.30X_2^2+0.082X_3^2$
3,6 AG	0.8988	0.8398	0.7469	8.83	13.501	205.57	$32.15+4.6X_1-3.66X_2+1.80X_3-2.97X_1X_3-2.10X_2X_3-1.10X_2^2-2.58X_3^2$

Y: Yield (%), G.S: gel strength (g/cm²), S: sulfate content (%), 3, 6 AG: 3, 6 Anhydrogalactose (%).

DISCUSSION

Statistical methods based on experiments layout such as response surface methodology act in more appropriate understanding and evaluation of the impact of simultaneous variables on response. The used

models in this study have analyzed properly the simultaneous effect of alkalinity concentration, pretreatment time and temperature on physicochemical properties of the extracted agar. P-values for polynomial regression model ($p<0.05$) and lack of fit ($p>0.05$) represent model appropriateness and significance. High values of R demonstrate appropriate correlation between real and predicted values [26]. Low amounts of variations coefficient represent trust to the model. In this study, all responses of native agar had considerable variations compared to the extracted agar which represents the experiments conditions and effects of pretreatment variables. Although, the native sample yield was higher than pretreated agar but *Gracilariopsis persica* extracted agar was higher than required amount for industry (>8%) [3]. Researchers reported that, relative decrease of pretreated extraction yield compared to the native sample is due to Polysaccharide degradation and digestion during pretreatment process and agar reduction [4]. Alkali Pretreatment plays an important role in preventing of agar diffusion from algae [13].

According to Arvizu-Higuera, pretreatment time also has a negative significant effect on the agar of *Gracilaria vermiculophylla* species and by increasing pretreatment time, the extraction yield reduced [14] while during washing process, agar diffusion was observed as a viscose liquid which represents agar exit at the time of pretreated alga washing [13, 4].

Sousa *et al.* [25] stated that complete degradation of polysaccharides occurs at temperature of 120 ^{0}C due to excess heat. On the other hand, low temperatures (less than 60 ^{0}C) have an impact in weak polymer production. According to the conducted studies, extraction yield depends on numerous factors such as agarophyte type, collection time and region, and environmental and physical factors of the species. On the other hand, difference in extraction yield can be due to independent variables used in this study [16, 10, and 14]. In the studies conducted by Arvizu-Higuera *et al.* [14], low pretreatment time had significant effect on improvement of gel characteristics. Conversion of L-galactose-6-sulphate to 3.6 anhydro-L-galactose leads to increase of gel strength [4]. In synchronization of pretreatment time and temperature, greater number of sulfate groups has been converted into gel amplifier units.

Abundant hydrolysis of sulfate groups in the presence of alkali can be considered as the cause of existing trend in interaction of alkali concentration and pretreatment temperature on sulfate content [14]. Actually, by conducting pretreatment operations, sulfate precursor sequences occur which ultimately cause to reduce sulfate precursor sequences [5].

Conducted studies also show that, environmental conditions of alga growth affect the amount of extracted agar sulfate so that, agar resulted from grown agarophyte at high temperatures contains more sulfate compared to the agar produced from grown agarophyte at lower temperatures [18].

Alkali pretreatment has a significant impact in the increase of anhydrogalactose-3.6 and has an important role in agar quality [20]. Alkali causes to reduce sulfate groups and increase anhydrogalactose-3.6 through converting L-galactose-6 of sulfate to anhydro-L-galactose 6.3 [5, 19].

DEGREE OF DESIRABILITY

In order to choose optimal conditions for agar extraction, numerical optimization technique was used. In this regard, high, low and desirable limits of each dependent and independent variables and their importance were determined. The applied settings included maximum extraction yield, (with importance degree by +5) gel strength (+3), 3, 6 AG (+5) and minimum sulfate content (+5). To investigate accuracy of the predicted model, the proposed treatment was produced in three replications at the same conditions, and the obtained results were compared to the results predicted by model. Lack of significance difference between the model and experimental observations proves the models efficiency properly ($p>0.05$).

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