Xanthan production by a novel mutant strain of *Xanthomonas campestris*: Application of statistical design for optimization of process parameters

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Abstract: Xanthan gum is a microbial polysaccharide of great commercial importance as it has unusual rheological properties in solution and consequent range of applications. In this study, a series of mutants were isolated from *Xanthomonas campestris* by acridine orange mutagenesis. The gum yield of XC5 mutant was 15.6 % better than that of the parent strain. Sequential methodology based on the application of two types of experimental designs was used to optimize the fermentation conditions for xanthan production from *X. campestris* strain XC5 in shaking flask cultures using beet molasses as the sole substrate. Using Plackett–Burman design, beet molasses and KH_2PO_4 were identified as significant variables which highly influenced xanthan gum production and these variables were subsequently optimized using a steepest ascent design. The steepest ascent method was demonstrated effectively and efficiently to approach the neighborhood of the optimum. The optimum medium composition was found to be: Beet molasses, 100; KH_2PO_4, 10; $MgCl_2.6H_2O$, 0.3; citric acid, 0.5; $CaCl_2.2H_2O$, 0.006; NH_4Cl, 0.2. Xanthan production increased markedly from 11.5 to 28g/l, when XC5 strain was cultivated in the optimal medium, compared to the basal.

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Introduction

Xanthan is a water-soluble hetero-polysaccharide produced by the Gram-negative bacterium *Xanthomonas campestris* (Borges et al., 2009). This polymer is one of the major microbial polysaccharides actually employed in many industrial processes because of its unique rheological behavior. Solutions of xanthan are highly pseudoplastic, have high viscosity and solubility, enhanced stability over a wide range of pH values and temperatures, as well as compatibility with many salts, food ingredients and other polysaccharides used as thickening agents and show very good suspending properties (Rosalam and England, 2006; Melo et al., 2011).These properties account for its widely applications in food ,cosmetics, and pharmaceutical industries ,agricultural products and other industries as stabilizing , suspension - thickening as well as an emulsifier (Zabot et al., 2012). Nowadays, the global xanthan market has progressively increased, at an annual rate of 5–10% (Ben Salah et al., 2010).

Many variables, such as composition of the culture medium, temperature, pH, and oxygen transfer rate affect the production of xanthan. Commercially available xanthan is relatively expensive, since it is manufactured from glucose or sucrose (Yoo and Harcum, 1999). However, prices would be reduced if cheaper suitable substrates could be found. In recent years, considerable research has been carried out using agro-industrial wastes, which are renewable and abundantly available to produce value-added products. For example olive mill waste waters (Lopez et al., 2004), sugar beet molasses (Kalogiannis et al., 2003), cheese whey (Silva et al., 2009), sugarcane broth (Faria et al., 2010), raw cassava starch (Kerdsup et al., 2011), date palm juice by-products (Ben Salah et al., 2010), and whey permeate (Savvides et al., 2012) are used as substrates for xanthan production.

In order to reduce production costs and to improve the competitive position of xanthan, its productivity should be increased (Carignatto et al., 2011). Various approaches have been suggested for this purpose, including (I) development of more efficient fermentation processes (Sabra and Hassan, 2008; Kassim, 2011); (II) optimization of culture media used for the gum production (Faria et al., 2010; Khosravi-Darani et al., 2011), and (III) isolation of *X. campestris* mutants with enhanced xanthan production (Ashraf et al., 2008; Kim et al., 2009; Kerdsup et al., 2011).

Statistical experimental design methods provide a systematic and efficient means of reaching particular goals and simultaneously studying several control factors. Hence, these methods can be used to examine and optimize the operational variables. Experimental designs for first-order models, such as the factorial

design or Plackett–Burman design (Plackett and Burman, 1946), can be used. The advantages of the Plackett –Burman are that numerous factors can be simultaneously screened and much quantitative information can be extracted from only a few experimental trials (Yong et al., 2011). Therefore, this method has been widely applied in industry. The steepest ascent method (Wang and Wan, 2009) is an effective experimental procedure for moving sequentially along the direction of the maximum increase in the response, and thus, can approach the optimum neighborhood rapidly and efficiently.

This work is concerned with the economic achievement of highest yield of xanthan gum via two approaches; the first is the utilization of agro-industrial wastes abundantly produced in Egypt as a lower-cost alternative to the high cost substrates. The second approach is via mutagenesis aiming to obtain a hyperproducing strain.

Materials and methods

Bacterial Strain and Inoculum Preparation

The bacterial strain used throughout this work was *Xanthomonas campestris*BRCS000093B, kindly provided in a lyophilized form from the Institute of Agriculture Research, Cairo University, Egypt. Inoculum preparation was performed by transferring the microorganism from stock culture to YM agar plate containing(g/L) yeast extract, 3; malt extract, 3; peptone, 5; glucose , 10 and agar 2, pH7.0 , with subsequent incubation for 72h at 30°C. A loopful of cells from the YM plates was then transferred to a 250mL Erlenmeyer flask containing sterile 50mL of YM broth and incubated on a rotary shaker for 24h at 30°C , 180 rpm to obtain an initial cell concentration with optical density (600nm) =1.2 for ultimate use as inoculum with concentration of 2 %(v/v) .

Fermentation Conditions

For primary evaluation for medium optimization process, three different types of broth medium known to support high xanthan production were used. The composition (gL^{-1}) was as follows: Medium A : Glucose, 55; Citric acid, 2.3; KH_2PO_4, 5; Na_2SO_4, 0.114; $MgCl_2.6H_2O$, 0.163; $FeCl_3$, 0.0014; $ZnCl_2$, 0.0067; $CaCl_2.2H_2O$, 0.012; H_3BO_3, 0.006; NH_4Cl, 0.62 (Flores-Candia and Deckwer, 1999). Medium B : Glucose, 20; KH_2PO_4 ,5; $MgSO_4.7H_2O$ 0.2; $(NH_4)_2SO_4$, 2; Citric acid, 2; H_3BO_3, 0.006;ZnO, 0.006; $FeCl_3.6H_2O$, 0.0024; $CaCO_3$, 0.02;HCl, 0.13ml (Souw and Demain, 1979). Medium C: Sucrose, 40; Citric acid, 2.1; NH_4NO_3, 1.144; KH_2PO_4, 2.866; $MgCl_2$, 0.507; Na_2SO_4, 0.089; H_3BO_3, 0.006; ZnO, 0.006; $FeCl_3$.$6H_2O$, 0.02; $CaCO_3$, 0.02; HCl, 0.13ml (Garcia-Ochoa et al., 1992). For all media used, the pH was adjusted to 7.0 with 1M of HCl or NaOH before sterilization. The carbon source was autoclaved separately and added to the fermentation medium before inoculation. All fermentations were carried out using 100-mL Erlenmeyer flasks with 30ml sterile production medium. For optimization studies, the composition of the medium was varied according to the experimental design. The medium in the flasks was then sterilized at 121°C for 15min. After inoculation with 2 %(v/v) of seed culture, the flasks were incubated at 28ºC on a rotary shaker agitated at 180 rpm/min for 72 h then cultures were examined for growth and xanthan gum production (Rosalam and England, 2006).

Mutagenesis and isolation of mutant

The technique for mutagenesis is based on the method described by Wu et al., (2006) with some modifications. Wild type of *X. campestris* was grown at 28 ºC in 2ml YM broth to optical density (OD) of 1.00±0.10 at 600nm.The cultures were centrifuged at 10,000 rpm for 15 min, cells were collected, washed twice in 0.9%NaCl solution and then resuspended in 2ml of the same solution. Different concentrations (1, 2, 3, 4, 5, 18µl) of freshly prepared sterile acridine orange solution (1%, w/v)were then added to each tube as a chemical mutagen, followed by incubation at 28 ºC with mild shaking for 24h. The treated cells were collected, washed twice in 0.9% NaCl solution and resuspended in 2ml of same solution. Each was diluted to appropriate concentration and 200µl of bacterial suspension were spread on YM agar. After incubation at 28°C for 48h, the surviving cell colonies were determined.

Xanthan gum production from agro-industrial wastes

Different wastes (potato peels, oil cakes of olive and cotton seeds and soybean meal as well as beet and sugarcane molasses) were tested for xanthan production. Glucose in medium A was replaced by each of the above mentioned wastes while Na_2SO_4, $FeCl_3$, $ZnCl_2$, H_3BO_3, were omitted from the media.

Analytical methods

Growth determination

Bacterial growth in synthetic medium was assessed by measuring the optical density (OD) at 600nm with a Pharmacia Biotech spectrophotometer. Biomass of medium containing waste was determined by centrifuging the fermented broth at 10,000 rpm for 30min; the biomass was then washed twice with distilled water before following another centrifugation. Finally, biomass was dried in an oven at 90°C to constant weight. The results were expressed in gram dry biomass per liter (Borges et al., 2009).

Xanthan gum concentration

Xanthan gum was determined in culture supernatants as the method described by Rottava et al., (2009). To 10 ml cell-free supernatant, three volumes of ice ethanol (1:3v/v), were added and the mixture was maintained at 4 °C for 12h to precipitate xanthan gum. Afterwards, the precipitate was recovered by centrifugation at 10,000 rpm, for 30 min at 4°C. The precipitated xanthan was washed three times with ethanol, and dried to constant weight at 50°C. Xanthan concentration was evaluated by measuring the weight of dry product in grams per liter of fermented broth. The dried gum was ground with mortar, pistil and stored in a sealed flask. All points on graphs are the mean of three independent experiments.

$$\text{Crude xanthan production } (g/l) = \frac{\text{Dried weight of precipitant } (g)}{\text{Culture medium } (l)}$$

Optimization procedure

Plackett–Burman design

Plackett–Burman design is a very useful tool for screening 'n'variables in just'n +1'tests (Yong et al., 2011). This design is very practical, especially when the investigator is faced with a large number of factors and is unsure which settings are likely to be close to optimum responses. In this part, it was used to identify the major fermentation parameters that affect xanthan production. Each variable is represented at two levels, high and low, which are denoted by (+) and (−), respectively. The chosen levels of the culture components are given in Table1.The effect of each variable on xanthan production was determined by the standard equation:

Main effect = [∑R (H) - ∑R (L)]/N

where R(H)=total responses when component was at high levels, R(L)=total responses when component was in low levels, N= number of trials divided by 2.

The experimental design was applied with 7 different variables and hence 8 different growth conditions are presented in Table 2 and the design matrix is developed by using Statistica"software (trial version 6.0, StatSoft, USA). All experiments were carried out in triplicate and the averages of the results were taken as response value.

Steepest ascent method

The next step in this study involved the determination of the optimum levels of the significant variables from the Plackett –Burman design. Steepest ascent method was used to optimize the levels of these variables. The zero level of the chosen variables in Plackett–Burman design was identified as starting point of steepest ascent path. Based on the results of starting experiments, a series of exploratory runs were performed. The path of steepest ascent through the experimental region followed the direction of maximum increase in the predicted response (Montgomery, 1997). All experiments were performed in triplicate and response values were the averages of the corresponding results. The experimental design of steepest ascent method and corresponding responses are shown in Table 5.

Rheological properties of the polymer

Xanthan gum, obtained from *X. campestris* XC5 was dissolved in deionised water (0.1% w/v) and centrifuged (20min at 12,000 rpm). Supernatant was dialyzed against deionised water for 24 h at pH 7 ,4 °C and solutions were precipitated by adding ethyl alcohol (1:3, v/v), and then dispersed in deionised water. The solutions were gently stirred at 4 °C using a magnetic stirrer for 8–12 h to remove bubbles and foam, then precipitated again with ethanol. After centrifugation (20min at 12,000rpm), the precipitate was finally dried at 50°C and ground to obtain creamy powder stored in a sealed flask. Effects of different parameters (polymer concentration, pH and temperature) on polymer viscosity were measured using a conventional Ostwald viscometer (Berekaa et al., 2009). The apparent relative viscosity (app) of xanthan suspension was determined as follows:

$$\eta_{(app)} = t_s / t_o$$

where t_s is the falling time of the sample at 30° C and t_o is that of water under the same conditions.

Results and discussion

Xanthan production using different media

Results obtained in this study support the previously reported data (Lo et al., 1997) that medium formulation affects xanthan biosynthesis. As shown in Figure 1 maximal growth was obtained in medium B (OD_{600}=0.802). On the other hand, maximal xanthan production (9.7 g/L) was obtained in medium A followed by medium B (8.2 g/L). Media A and B containing glucose as sole carbon source were most efficient in producing high yield of xanthan compared to medium C (6.7 g/L) with sucrose as a carbon source. Glucose and sucrose were always the preferred carbon sources for xanthan production (El Enshasy et al., 2011), due to their ease of assimilation and their direct integration in xanthan biosynthesis pathway (Rosalam and England, 2006). Therefore, medium A was used for further medium optimization.

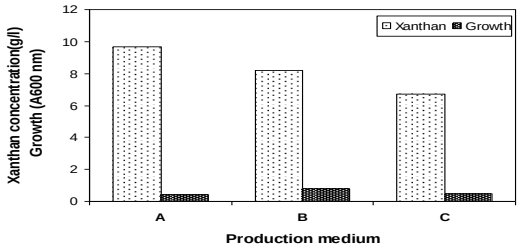

Fig.1. Influence of different production media on growth and xanthan concentration by *X. campestris* incubated at 28°C on a rotary shaker for 72h.

Mutation of *X. campestris* by Acridine orange

Mutation and screening industrially useful microorganisms are important for the successful development of the various strains required in the fermentation industry. Several publications related to mutagenesis of *X. campestris* and selections of strains with enhanced xanthan production have been reported in the literature (Wu et al., 2006; Kerdsup et al., 2011). In the present work, exposing the wild type of *X. campestris* to acridine orange generated a collection of mutants, 10 colonies were selected according to viscous formation. These isolates were then checked for xanthan producing ability in medium A. The yield of xanthan ranged from 6.55 to 11.15 g/l compared to 9.7g/l obtained with the parent strain (data not shown). The mutant strain giving the highest xanthan yield (1.5 fold increase than parent strain) was designated XC-5 and used for further studies.

Utilization of agro-industrial by- products

A number of byproducts readily available in Egypt were screened as substrates for xanthan gum production. Six different substrates; potato peels, oil cakes of olive and cotton seeds and soybean meal in addition to beet and sugarcane molasses were used instead of glucose. Both strains *X. campestris* wild type and XC5 mutant were examined for comparison.

Data presented in Figure. 2 depict that beet molasses was the best substrate supporting the production of 11.7 and 14.8 g/l xanthan for wild type and mutant, respectively. These values were 1.21 and 1.33 fold increase compared to medium containing glucose as carbon source. Bioconversion of sugar beet or sugar cane molasses to xanthan by different strains of *X. campestris* was investigated (Faria et al., 2010). Moreover, *X. campestris* XC5 produced higher amounts of xanthan compared to wild type when grown on soybean oil cake, sugarcane molasses and potato peels. Neither the wild type nor its mutant was able to grow on olive oil cake or cotton oil cake.

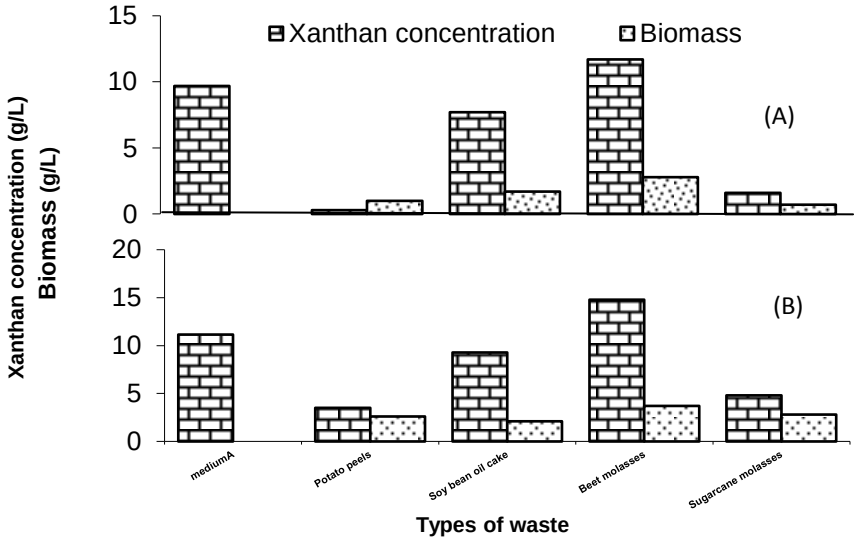

Fig. 2 Effect of different substrates on growth and xanthan production by *X. campestris* (A) and mutant strain XC5 (B) .Cells were grown in medium A and incubated at 28°C on a rotary shaker for 72h.

Two-level fractional factorial design

Plackett–Burman design was chosen as the first approach to optimize xanthan production by the promising candidate strain XC5. The influence of 7 quantitative factors (beet molasses, KH_2PO_4, citric acid, calcium chloride, ammonium chloride, magnesium chloride and culture volume) were evaluated in a fractional factorial design at 2 levels (coded -1 and +1) together with a center point replication (coded 0) (Table1).Table 2 represents the Plackett–Burman experimental design for 8 trials at two levels of concentration for each variable along with the responses xanthan yield (g/l).

The responses in Table 2 show a wide variation, which reflects the importance of parameter optimization to reach higher productivity. Highest xanthan gum concentration (21.9g/L) was demonstrated in trial 2. The lowest amount (8.1g/L) was obtained in trial 8. The main effect of each variable upon response was estimated as the difference between both averages of measurements made at the high level and at the low level of that factor. Data represented graphically in Figure. 3, indicate that the main variables which positively affected xanthan production by mutant XC5 were beet molasses, KH_2PO_4 and $MgCl_2.2H_2O$. Yet, citric acid, $CaCl_2$ and NH_4Cl showed a negative effect, i.e., low amounts of these compounds in the culture medium increased xanthan yield. The deleterious effect of citric acid might be attributed to significant amounts of metabolizable organic acids in sugar beet molasses as observed earlier by Souw and Demain, (1979). The significant variables were identified by statistical analysis of the Plackett - Burman results using the t - test supported by Excel Microsoft Office to determine the statistical significance of the measured response (Table 3). In the present study, the variables with confidence level greater than 80% were considered as significant factors. The *t* test for each effect allows an evaluation of a probability, *P*, which showed that $KH_2PO_4$and beet molasses were the most statistically significant variables at 97% and 81% respectively (Table 3). Kalogiannis et al., (2003) reported that K_2HPO_4 and beet molasses had a significant positive effect on xanthan gum production and the confidence level of the calculation was higher than 95%. Silva et al., (2009) also reported that K_2HPO_4 had a significant positive effect on xanthan gum production. Ammonium chloride had a negative effect on xanthan production, probably because organic nitrogen content in molasses is sufficient to support growth of *X. campestris* XC5 while further increase by the addition of ammonium chloride had a detrimental effect, since they affect the C/N ratio. This is also supported by earlier findings referring to the inhibitory effect of high nitrogen concentrations on both growth and xanthan gum production (Kalogiannis et al., 2003). Beet molasses and KH_2PO_4 were selected for further optimization in the next stage considering their importance in xanthan production.

Table 1. Factors examined as independent variables affecting xanthan production and their level in the Plackett-Burman experiment

Factors	Level		
	+1	0	-1
Beet molasses (g/l)	100	58	40
Citric acid (g/l)	5	2.3	0.5
KH_2PO_4 (g/l)	10	5	0.5
$MgCl_2.6H_2O$(g/l)	0.3	0.163	0.06
$CaCl_2$. H_2O (g/l)	0.1	0.012	0.006
NH_4Cl (g/l)	2	0.62	0.2
Culture volume	50ml	30ml	20ml

Table 2. Plackett –Burman design matrix for 7 cultural variables and the results for evaluation of the relative importance of selected factors for xanthan production by *X. campestris* XC5

Trial No.	Variables							Response	
	KH_2PO_4	$MgCl_2$	Citric acid	NH_4Cl	Beet molasses	$CaCl_2$	Culture Volume	Biomass (g/l)	Xanthan (g/l)
1	+1	-1	-1	+1	-1	+1	+1	4.8	11.39
2	+1	+1	-1	-1	+1	-1	+1	6.35	21.9
3	+1	+1	+1	-1	-1	+1	-1	4.88	13
4	-1	+1	+1	+1	-1	-1	+1	1.45	6.3
5	+1	-1	+1	+1	+1	-1	-1	5.98	19.5
6	-1	+1	-1	+1	+1	+1	-1	1.92	8.96
7	-1	-1	+1	-1	+1	+1	+1	3.6	9.96
8	-1	-1	-1	-1	-1	-1	-1	1.62	8.1

Table 3. Statistical analysis of the Plackett-Burman experimental results

Variables	Xanthan production		
	Effect	t value	Confidence%
Beet molasses	5.4	1.48	81
Citric acid	-0.397	-0.093	
KH_2PO_4	8.1	3.07	97
$MgCl_2.6H_2O$	0.3	0.07	
$CaCl_2.H_2O$	-3.12	-0.77	
NH_4Cl	-1.7	-0.407	
Culture volume	-0.0025	-0.00059	

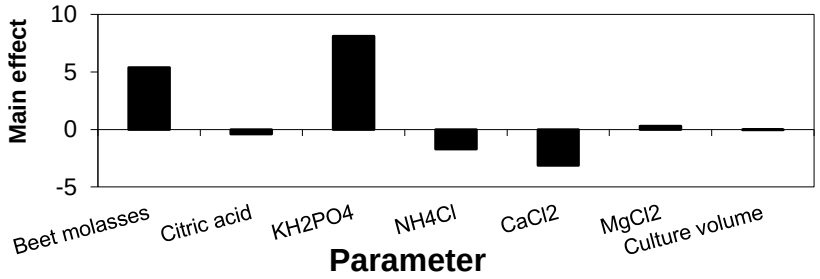

Fig.3 Elucidation of different components of medium affecting xanthan production by the mutant strain XC5.

Steepest ascent path

Based on the Plackett–Burman design, beet molasses and KH_2PO_4 showed positive effect on gum yield. In order to approach the optimum medium formula for xanthan production by strain XC5, a statistical design known as the steepest ascent method (Wang and Wan, 2009) was applied to improve performance. The path of steepest ascent is the direction in which beet molasses and KH_2PO_4 increased most quickly, while; the other factors were omitted as variables from the model. Since the two factors had identical figures of the effect total parameter in complete factorial design, the relative concentration change unit of beet molasses was 1 and the change unit of KH_2PO_4 was 1.4 (Table 4). Trial 4 gave the highest value, and all the other runs exhibited decreased value (Table 5). Assessed beet molasses and potassium dihydrogen phosphate concentrations up to 100g/l, and 10 g/l respectively resulted in increased xanthan production.It can thus be concluded that the level of molasses and KH_2PO_4 in trial 4 are the closest to the optimum point.

Table 4. Statistical analysis of the second phase factorial experiment (steepest ascent method)

Factor	Mutant XC5	
	Molasses	KH_2PO_4
Effect total	5.4	8.1
Slope	0.7	1.01
Change unit	0.35	0.51
Relative change unit	1	1.4

The statistical optimization experiments enabled us, to formulate a suitable medium that can increase xanthan production by *X. campestris* XC5. Consequently, the following optimized medium composition was postulated to maximize xanthan production which contained in g/l: Beet molasses, 100; KH_2PO_4, 10; $MgCl_2.6H_2O$, 0.3; citric acid, 0.5; $CaCl_2.2H_2O$, 0.006; NH_4Cl, 0.2. Under these conditions, the maximum xanthan yield was 27.9 g /l. with an increase of 1.88 fold, compared with the pre optimized fermentation conditions.

Table 5. Gradual increases in beet molasses and potassium dihydrogen phosphate concentrations and the experimental results according to the steepest ascent method

Trials	Factors		Xanthan (g/l)
	Molasses (g/l)	KH_2PO_4 (g/l)	
1	**97**	**5.8**	**22.4**
2	**98**	**7.2**	**24.4**
3	**99**	**8.6**	**26.3**
4	**100**	**10**	**27.9**
5	**101**	**11.4**	**24.9**
6	**102**	**12.8**	**19.3**
7	**103**	**14.2**	**19.1**
8	**104**	**15.6**	**17.2**
9	**105**	**17**	**16.4**
10	**106**	**18.4**	**12.1**

Properties of xanthan gum

The concentration of polysaccharide in solution is known to affect directly the viscosity (Sutherland, 1994). As shown in Figure 4, the viscosity exponentially increased with xanthan concentration. Other researchers have found similar relationships between concentration and viscosity of xanthan solutions (Lopez et al., 2004). Gum solutions

subjected to increase in temperature showed reduced viscosity (Figure 5). Temperature is known to affect mainly the conformational structure of polysaccharide in solution (Milas and Rinaudo, 1986). Viscosity increased as the pH increased till reach maximum at neutrality then decreased as the pH directed to the alkaline range (Figure 6). Lopez et al., (2004) reported similar response.

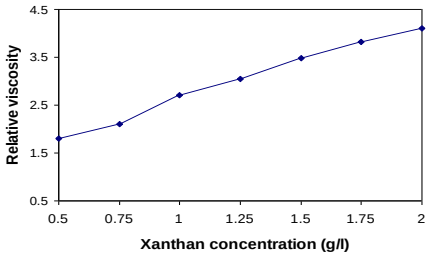

Fig.4 Effect of xanthan gum concentration on viscosity

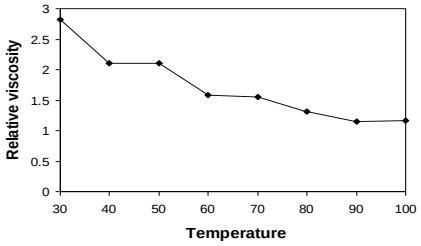

Fig.5 Effect of temperature on viscosity of xanthan gum

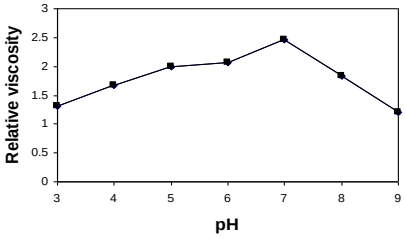

Fig.6 Effect of pH on viscosity of xanthan gum

Conclusions

The present study reveals that increased xanthan production by *X. campestris* can be achieved via chemical-induced mutagenesis. We can increase the xanthan production from 9.7g/L using wild type to 11.15g/L with mutant strain. Sequential methodology based on the application of Plackett–Burman design and steepest ascent method to optimize the fermentation conditions of xanthan production by mutant strain XC5 would be viable and effective. We conclude that beet molasses and KH_2PO_4 are key factors affecting xanthan production. After optimization a maximum yield of 27.9 g/L xanthan was reached.

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