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Isolation of a Novel Mutated Strain of *Xanthomonas campestris* for Xanthan Production Using Whey as the Sole Substrate

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Abstract: This study was conducted to isolate novel lactose utilizing *Xanthomonas campestris* mutants. Such a mutant will assist the utilization of whey as the sole carbon source for xanthan gum production, lower costs of fermentation process and set a precise application for whey as a waste. In this study, a mutant strain (NA1) was isolated from *Xanthomonas campestris* cells exposed to nitrous acid mutagenesis Environmental conditions were optimized and maximum activity of the β-galactosidase enzyme was obtained at pH 5.5 and 38°C following which the β-galactosidase activity in NA1 culture was increased 9.5 folds, compared to that of the wild type culture (336.1 U vs. 35.4 U). Xanthan gum production by NA1 using whey as carbon source was also studied. Using the experimental design of Plackett-Burman and statistical analysis, whey, as the main substrate and pH were the first factors affecting gum production among the seven parameters tested. Gum production using significant factors (such as substrate concentration and pH) was carried out in a lab-scale fermentor and 10 g L^{-1} xanthan was obtained.

Key words: Plackett-Burman, *Xanthomonas*, β-galactosidase, nitrous acid, whey

INTRODUCTION

Xanthan gum is a microbial polysaccharide of great commercial importance, produced by the yellow-pigmented gram-negative bacterium *Xanthomonas campestris*. Xanthan gum has unusual rheological properties and is extensively applied in various industries as a stabilizing, emulsifying, suspending and thickening agent. Because of low levels of β-galactosidase activity in *Xanthomonas campestris*, this bacterium is not able to use lactose as the sole carbon source. Consequently, very little biomass and xanthan are produced by growing this bacterium in lactose-based media. According to the literature it is evident that *Xanthomonas campestris* have indigenous β-galactosidase gene. In 1979 β-galactosidase enzyme was purified from *Xanthomonas campestris* B-1459 for the first time (Yang *et al*., 2003). In 1995, β-galactosidase gene from *Xanthomonas axonopodis* pv. *manihotis* has been cloned (Taron *et al*., 1995). Recently three β-galactosidase genes in *Xanthomonas campestris* pv. *campestris* 17L has been cloned and sequenced. The presence of a fourth β-galactosidase gene was also proposed (Yang *et al*., 2003). Whey, a by product of the cheese industry, contains 4-5% lactose, 0.8-1% proteins, small amounts of organic acids (lactic acid), mineral salts and vitamins. Whey poses a major waste disposal problem due to its high Biochemical Oxygen Demand (BOD). Its disposal has been a costly and time-consuming problem. Due to this reason and also its high lactose content, whey is considered as a suitable substrate for production of value-added products such as xanthan (Soudi *et al*., 2006).

Many efforts have been made to construct strains that can produce xanthan from lactose. A β-galactosidase expression plasmid, an *E. coli* Lac zy-containing plasmid (pKMϕLT), was constructed and transferred by conjugation to *Xanthomonas campestris* 17. This strain, Xc 17 (pKMϕLT), was able to use whey for xanthan production. Later, pKMϕ was fused with the integration vector, pS19, to form pSFϕ14 that was integrated into the chromosome of Xc17. The new strain, Xc17:: pSFϕ14, grew as well and produced as much xanthan in lactose medium as the wild type strain did in glucose medium. A lactose-utilizing strain of Xc17 was isolated by nitrous acid mutagenesis. The amounts of xanthan produced by the mutant strain (Xc17L) in lactose-based medium were comparable to those in glucose-based medium (Yang *et al*., 2002). In this study, in order to obtain improved strains, the possibility of isolating lactose

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utilizing mutant strains of *Xanthomonas campestris* b82 for xanthan production through nitrous acid mutagenesis was investigated.

MATERIALS AND METHODS

Present study has been conducted in Applied Microbiology Laboratory at Alzahra University since September 2006.

Microorganism and media: A native strain of *Xanthomonas campestris* previously isolated from soil (Soudi *et al*., 2006) was used for mutagenesis.

LB medium was used as the general-purpose medium. For propagation of the native strain, YMA slants were used (Galindo *et al*., 1994) while for the mutant strain, XOL agar medium plus 2% lactose was used. For measurements of the growth rate and β-galactosidase activity, XOLN medium plus 0.4% lactose was used and lactose was added after autoclaving (Yang *et al*., 2002). The dyes medium C was used for the estimation of lactose utilization (Lee *et al*., 2004). The same components of the synthetic medium introducing by Roseiro *et al*. (1992) was used for gum production in Plackett-Burman study.

Mutagenesis and isolation of a mutant strain: Nitrous acid was used for mutagenesis while the conditions were the same as procedures used by Yang *et al*. (2002). The isolates were cultured on dyes medium C containing lactose as the sole carbon source. The purple dye of the medium changed to yellow following lactose utilization and acid production.

Furthermore, growth rate was measured using UV-VIS spectrophotometer at OD_{620} at intervals of 3 h in XOLN plus 0.4% lactose.

Enzyme assay: The β-galactosidase activity was assessed according to the method described by Yang *et al*. (2002) which is basically as same as the Miller method with some modifications. Cells from mid log phase in XOLN medium plus 0.4% lactose was used for enzyme assay. Optimal external conditions for enzyme activity (pH and temperature) were measured. Moreover, the enzyme activity of the wild type and mutant was also compared.

One unit of β-galactosidase activity was defined as the amount of enzyme that produced 1 nmol of ONP (ortho-nitrophenol) per min.

Evaluation of significant factors involved in xanthan production: Amounts of xanthan and biomass were measured as production parameters. A certain volume of broth (containing xanthan and biomass) was precipitated using organic solvent and $CaCl_2$ and then dried and weighed as raw product. Processes to estimate the amounts of xanthan and biomass were carried out by heat-treatment of xanthan solution obtained from reconstituted raw product under 60°C, which was followed by the enzymatic treatment (1000 U g^{-1} xanthan) of the broth and thereafter separating solid xanthan gum by extraction from the treated broth using a specific organic solvent, isopropanol and $CaCl_2$. Subsequently, the precipitate was dried and weighed (Murofushi *et al*., 1999). Twelve-trial Plackett-Burman experimental design was used to evaluate significance of seven factors, including initial pH and various nutrients (whey, lactose, sucrose, salts, sodium citrate and phosphate), in xanthan gum production. Experiments were carried out in 500 mL Erlenmeyer flasks containing 80 mL of culture medium in duplicates. p-value was calculated to assess significance of each process factor (Strobel and Sullivan, 1999). Minitab software was used for data processing.

Gum production in fermentor: Preculture preparation was carried out by inoculation of 7 mL YMB from stock culture on XOL-agar plus 2% lactose and incubation in a shaking incubator at 28°C and 150 rpm overnight. The whey medium was then inoculated with this culture (10% v/v) and incubated in the same conditions for 14 h. This culture was used for inoculation of 2 L bench-top Fermentor (Biostat B, B-Braun). Fermentor medium was whey and the pH was adjusted to 6. Fermentation was carried out at 28°C for 72 h. Air flow rate was adjusted at 2 vvm and by increasing of viscosity driving speed was increased from 150 to 250 rpm.

RESULTS

Nitrous acid mutagenesis was performed as described by Yang *et al*. (2002). Following nitrous acid mutagenesis, large lactose utilizing colonies were isolated on XOL plates containing 2% lactose. Growth capability was checked in repeated passages to monitor lactose utilization ability as the sole carbon source. One of lactose utilizing isolates was selected for further assays (NA1). In contrast to the wild type, this isolate was able to grow in Dyes medium C containing lactose as the sole carbon source and changed the purple dye of medium to yellow (Fig. 1). Growth curve of the wild strain and NA1 in XOLN plus 0.4% lactose is shown in Fig. 2. Wild strain reached the late exponential phase and OD of 0.78 after 24 h, but mutant strain reached maximum OD of 3 after 30 h. This

Table 1: Effect of temperature changes on activity of β-galactosidase enzyme

Temperature (°C)	26	28	30	32	34	36	38	40	42	44
U mL^{-1}	208.06	241.06	274.51	314.38	341.88	383.12	434.45	430.78	414.29	371.21

Table 2: Twelve-trial Plackett-Burman design used to study seven factors in xanthan production. Low (-1) and high (+1) levels of factors are, g L^{-1}, whey (0, 30) lactose (0, 20), sucrose (0, 20), salts (0, 3.2), citric acid (0, 2.7), phosphate (0, 5) and pH (6,7)

	Coded setting for factor production (g L^{-1})								
Trials	Whey	Lactose	Sucrose	Salts	Citric acid	Phosphate	pH	Xanthan	Biomass
1	-1	-1	-1	+1	-1	-1	+1	0.61	0.17
2	-1	-1	+1	-1	-1	+1	-1	0.95	0.29
3	-1	-1	+1	-1	+1	+1	+1	1.23	0.43
4	-1	+1	-1	-1	+1	-1	+1	0.82	0.33
5	-1	+1	-1	+1	+1	+1	-1	7.23	1.94
6	-1	+1	+1	+1	-1	-1	-1	2.66	1.37
7	+1	-1	-1	-1	+1	-1	-1	8.84	2.28
8	+1	-1	-1	+1	-1	+1	+1	1.85	1.01
9	+1	-1	+1	+1	+1	-1	-1	8.50	3.22
10	+1	+1	-1	-1	-1	+1	-1	6.63	3.16
11	+1	+1	+1	-1	-1	-1	+1	8.58	3.56
12	+1	+1	+1	+1	+1	+1	+1	2.56	3.43

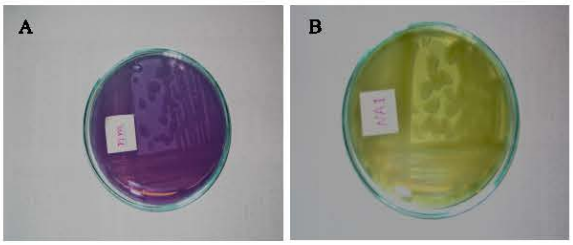

Fig. 1: Growth of mutant and wild type strains of *Xanthomonas campestris* on dye's medium C. (A) wild type strain and (B) NA1 strain

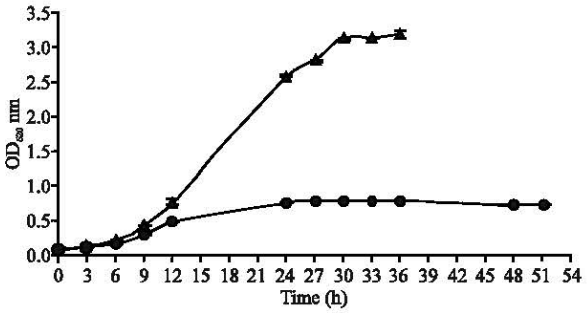

Fig. 2: Growth curve of NA1 (●) and wild type (▲) in XOLN 0.4% lactose < 0.05

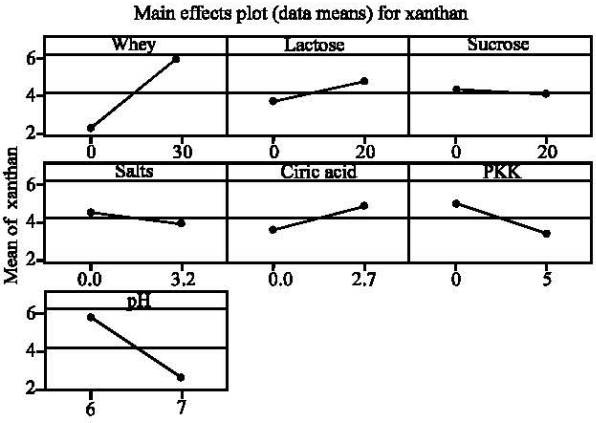

Fig. 3: Main effects plot of factors affecting xanthan production from whey

result indicates that the mutant strain has better growth capabilities in lactose based medium in comparison to the wild type.

Optimal condition for β-galactosidase activity was assessed. Optimal pH and temperature were found to be 5.5 and 38°C, respectively (Table 1).

The β-galactosidase activity of the wild type and NA1 was measured and compared under optimal conditions. The β-galactosidase activity of NA1 was about 9.5 folds greater than that of the wild type (336.1 U vs. 35.4 U). This increase in enzyme activity is sufficient to support growth.

Plackett-Burman experimental design was used to identify significant factors in xanthan production by NA1 strain in whey medium. Design of experiments and results for xanthan production in each trial are shown in Table 2. Once data was obtained for each trial, Minitab software and statistical analysis were used to evaluate the rank factors by their degree of impact on the fermentation process. p-value of < 0.05 was assumed as the cut-off point for significance. Therefore, factors with p-value ≥ 0.05 are defined as non-significant factors in gum production. p-values in Plackett-Burman experimental design were obtained for different studied factors: whey (0.000), lactose (0.243), sucrose (0.781), salts (0.505), citric acid (0.159), phosphate (0.093) and pH (0.002). The main effects plot for factors affecting xanthan production is shown in Fig. 3. Increase in concentration of sucrose,

mineral salts and phosphate showed adverse effects on gum production, but addition of citrate and lactose revealed relative increase in xanthan production but as far as the p-value is concerned, this effect was not noticeable. Addition of whey and adjustment of pH to 6 resulted in a remarkable increase in xanthan production. Regarding the p-value, these two factors are the most significant in gum production.

Furthermore, using defined significant factors (high level of whey and pH of 6), gum production was carried out in a lab-scale fermentor. Xanthan gum and biomass were obtained measuring 10 and 2.376 g L^{-1}, respectively.

DISCUSSION

Thus far, several efforts have been directed at obtaining improved strains of *Xanthomonas campestris* by mutagenesis. Effects of mutation on structure and production of xanthan can be very extensive. One example is the isolation of a mutant strain of *Xanthomonas campestris* which has trisaccharide structure composed of glucose and mannose instead of pentamer repeating unit. This polymer was identified as a type of substituted cellulose (Vojnov *et al*., 2002). Isolation of mutants with increased viscosity and gum production has been reported previously. Often it is too difficult to use direct criteria, but some investigators have used indirect measures such as increase in halo size on starch agar plates used for the selection of strain M11 (Kamal *et al*., 2003). Antibiotic resistance was selected as another indirect criterion (Rodriguez *et al*., 1997). Enhanced xanthan production using ethyl methanesulfonate as mutagenic agent (Kamal *et al*., 2003) and elevated β-galactosidase activity by nitrous acid (Yang *et al*., 2002) are a few of the well-known examples of direct selection. Isolation of lactose utilizing mutant strains is possible with direct selection criteria, as evident from this study, in the course of which β-galactosidase activity of mutant strain NA1 (that was obtained from a native wild strain of *Xanthomonas campestris*) was increased after using nitrous acid mutagenesis. This result confirms the idea fromYang and co-workers that the gene coding for β-galactosidase is present in the wild strains of *Xanthomonas campestris* and has degenerated as a result of the absence of selective pressure in soil and plant environment where lactose is not readily available. Genetically-constructed lactose utilizing strains produce much more β-galactosidase (40-200 folds) than the wild type strains (Yang *et al*., 2002), but mutated strains e.g., strain NA1 produced β-galactosidase enzyme only 9.5 folds greater than the wild type. Although genetically constructed strains seem to be superior, but mutated strains are preferred since first, a moderate increase of 9.5 in β-galactosidase activity of NA1 is already enough to support growth. Keeping the enzyme level low but sufficient for growth should be advantageous so as to conserve energy for xanthan production. Second, no incorporation of foreign DNA or antibiotic resistance genes is involved for the strain improvement and therefore the concern of a genetically modified organism and the fear of the spread of an antibiotic resistance gene are circumvented. Comparable results were obtained from β-galactosidase assay of NA1, the mutant isolated in our laboratory, with those from Xc17L mutant strain (Yang *et al*., 2002) and Xc-B1459 wild strain (Yang *et al*., 2003). Optimum temperature in NA1 (38°C) was different from that of Xc17L strain (28°C), but optimum pH was similar in both (pH; 5.5) (Yang *et al*., 2002). For B-1459, optimum pH of 5.5-5.8 for β-galactosidase activity and optimum temperature of 32°C-37°C were reported (Yang *et al*., 2003). Xanthan production in majority of the strains take place at 24-30°C but growth can continue up to 37°C. Thus, using two-stage temperature conduction is suggested in case of our strain. Using high temperatures in the first hours of fermentation helps improve carbon utilization for biomass production and cleavage of lactose to simple sugars which can then be used for gum production in the next step i.e., at low temperatures. β-galactosidase activity of Xc17L was 3.5 folds greater than that of the wild type, Xc17, but β-galactosidase activity of NA1 is 9.5 folds greater than that of the wild type. Since NA1 strain has shown potential in lactose utilization to produce xanthan, the use of whey as a cheap and rich source of lactose and a pollutant of the environment holds promise as a substrate for this microorganism. Our previous study showed that peptonized whey can be used to propagate *Xanthomonas campestris* cells and whey could successfully replace YM broth for preculture preparation (Soudi *et al*., 2006).

For medium optimization, a practical method (such as Plackett-Burman design) is recommended when more than five independent variables are to be investigated. Interactions between the factors can not be determined by using this design (Hagen, 2006). Thus to assess important factors affecting xanthan gum production by the strain NA1 in whey as lactose based medium, Plackett-Burman design was used.

Data analysis indicate that amount of whey and pH of the medium show the lowest p-values (lower than 0.05) and thus, are the most effective factors. The effectiveness of amount of whey can be described by the fact that *X. campestris* yields more when greater amounts of fermentable carbon source are supplied. Alternatively, the

accompanied sources of nitrogen i.e., whey proteins impose unfavorable effects and act as limiting factor, since they adversely effect the C/N ratio; however, controlling of other conditions can assist in keeping the C/N ratio constant.

The positive effect of acidic pH can be explained by optimum pH range for β-galactosidase enzyme, which remains between 5 and 6.

Further optimization of these two factors plus other influencing agents such as phosphate and citrate, will result in low cost production of food-grade xanthan gum. *Xanthomonas campestris* produces about 10-30 g L^{-1} xanthan and 1-10 g L^{-1} biomass in a glucose containing medium (Garcia-Ochoa *et al*., 2000). Yang *et al*. (2002, 2003) showed that Xc17L in a lactose containing medium produced 1.852 g L^{-1} xanthan. However, our mutant strain produced 10 g L^{-1} xanthan using whey as the sole carbon source. These results confirm the importance of NA1 as a xanthan producing strain of *Xanthomonas campestris* with capabilities of using whey as a cheap and readily available source of lactose.

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