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Mutagenic effect of microwave radiation on exopolysaccharide production in *Xanthomonas campestris*

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Abstract

The exopolysaccharide produced by the gram-negative bacterium *Xanthomonas campestris* (known as xanthan gum) is a product of commercial importance. In the present study *X. campestris* was subjected to two different microwave (MW) powers (90 W and 450 W) for different time durations, and its effect on growth and exopolysaccharide (EPS) production was studied for multiple generations of the xanthan overproducing mutants obtained after MW treatment. As the MW treatment was performed under temperature-controlled condition (by placing the inoculum vial in an ice containing beaker, while exposing to MW), where the temperature did not cross 25°C, whatever alterations were observed in growth and xanthan production seem largely to have resulted from non-thermal effects of MW radiation. As the trait of xanthan overproduction was transferred from one generation of MW treated organism to the next, the MW effect can be said to be mutagenic. However this MW induced mutation was not found to be stable over multiple generations. Both the xanthan overproducing mutants obtained by two different MW treatments reverted back to the parent phenotype, suggesting the reversible nature of the MW induced mutations. Identifying mutagenic frequencies of MW radiation can pave way for large scale screening programmes employing MW mutagenesis as a tool for strain improvement, and yield genetically stable overproducing mutants.

Keywords: Microwave mutagenesis; Xanthan gum; Exopolysaccharide; Microwave specific effects; Non-thermal effects.

Introduction

Xanthomonas campestris is a gram-negative bacterium belonging to γ subdivision of the *Proteobacteria*. It is well known for its pathogenicity to cruciferous plants, and also as a producer of the acid exopolysaccharide xanthan (xanthan gum). Xanthan is a heteropolysaccharide with a cellulose-like backbone and trisaccharide side-chains of one glucuronate and two mannose residues that are attached to every second glucose moiety of the β (1-4)- linked glucan main chain (1). Xanthan is of commercial importance owing to its applications as a thickening agent and emulsifier in the pharmaceutical, nutritional, and oil drilling industries. It is produced by fermentation, and production exceeds 86,000 tons per annum (2). Xanthan suspensions are particularly attractive for the frozen-food industry owing to their high freeze-thaw stability. It has also found application as an additive to fruit drinks to reduce tooth decay, and as a hydrophilic matrix carrier for pharmaceuticals. Xanthan has also been suggested as a good base for shampoos, and for suspending adhesive agents for wallpapers. Suspension-stabilizing property of xanthan makes it suitable for production of sharp prints from dyes (1).

Strain improvement through various mutagenesis strategies has been an integral part

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of any successful microbial fermentation process. Mutation has been a major factor involved in the several- fold increases obtained in the production of microbial metabolites. The ability to genetically modify a microbial culture to higher productivity has been the most notable factor in keeping the fermentation industry in its viable and healthy state (3). Strain improvement strategies based on use of mutagenic chemicals, viruses or transposons are being used at a decreasing frequency, due to complexity of these processes. Radiation mutagenesis is an attractive alternative owing to its convenience, safety, and improved mutagenicity results. However, industrial bacterial strains can exhibit intolerance to ultraviolet, X-ray and γ-ray radiation (4). Microwave (MW) radiation breeding can emerge as a clean, effective, and easily applicable tool for strain improvement. It is claimed to deliver a high rate of positive mutation, and the mutant strains thus generated can be sufficiently stable (5). MW mutagenesis can also avoid the problem of photo-reactivation, which often is observed with ultraviolet radiation based mutagenesis.

MWs are electromagnetic waves with frequency between 0.3-300 GHz, equivalent to wavelength range of 1m - 1mm. Several biological applications of MW have been developed (6) e.g., MW sterilization (7-9) microwave assisted extraction (MAE) (10-11), etc. MWs have been used for mutagenesis in plants (12) as well as microorganisms (4, 13). Thermal and non thermal effects of MW on microorganisms have been reported by many researchers (14-18). In the present study *X. campestris* was subjected to two different MW powers (90 W and 450 W) for different time durations, and its effect on growth and exopolysaccharide (EPS) production was studied for multiple generations of the MW treated parent strain.

Materials and Methods

Test organism: *Xanthomonas campestris* (MTCC 2286) was procured from Microbial Type Culture Collection, Chandigarh.

MW treatment: Bacterial suspension was prepared in sterile normal saline, from an active culture growing on nutrient agar, whose turbidity was adjusted to that of 0.5 McFarland standard. Test culture (5 mL) in sterile screw capped glass vials (15 mL, Merck) was exposed to MW radiation (90 W or 450 W; 2450 MHz) in a domestic MW oven (Electrolux® EM30EC90SS). MW treatment at 90 W was given for three different time durations viz. 2, 4, and 6 min, whereas MW treatment at 450 W was given for 3 min. Vials inside the MW oven were placed in an ice containing beaker, so as to avoid any thermal heating. Temperature of the microbial suspension after MW treatment at 90 W did not go beyond 15°C, and while using MW at 450 W, it did not go beyond 25°C. The whole MW treatment was performed in an air-conditioned room. Untreated inoculum was used as control. Before MW treatment all the inoculum vials were put in ice for 5 min to nullify any variations in initial temperature. Test organism was immediately (in less than 5 min) inoculated into TY broth (HiMedia, Mumbai) supplemented with calcium chloride (0.7 g/L) (19) following MW treatment. Incubation was made at room temperature under shaking condition (~180 rpm) for 72 h.

EPS quantification: Once the incubation was over, following estimation of growth by measuring OD at 625 nm, culture broth was subjected to centrifugation at 7500 rpm for 10 min, and the cell-free supernatant (CFS) was used for EPS quantification using the method described in Li et al. (2012) with some modification. Briefly, 40 mL of chilled acetone was added to 20 mL of CFS, and allowed to stand for 30 min. The EPS precipitated thus was separated by filtration through pre-weighed Whatman # 1 filter paper (Whatman International Ltd., England). Filter paper was dried at 60°C for 24 h, and weight of EPS on paper was calculated.

Screening for mutants: Following the MW treatment, the treated inoculum was streaked on nutrient agar plate, and incubated at 28°C till appearance of visible growth (which usually took

72 h). After the incubation, 3-4 colonies from each plate corresponding to different MW treatments were picked randomly, and each colony (a separate code was given to each picked colony) was streaked on to a separate nutrient agar plate. Daughter populations thus generated from a single parent colony were then inoculated into the TY broth described above for EPS quantification, and simultaneously streaked on to new nutrient agar plates.

Initially EPS estimation was made for all the MW treated inoculums. Then randomly selected colonies, from the plate corresponding to the MW treatment yielding highest EPS production, were selected for further experiments. Among these randomly picked 3-4 colonies, the highest (compared to control) EPS producer was identified as the best overproducer. Those producing EPS equivalent to control were discarded. EPS production in thus selected overproducer was studied over 3 or 4 generations until the trait of EPS overproduction was maintained by the mutant.

Statistical Analysis: All the experiments were performed in triplicate, and measurements are reported as mean ± standard deviation (SD). Statistical significance of the data was evaluated by applying *t*-test using Microsoft Excel®. *P* values less than 0.05 were considered to be statistically significant.

Results and Discussion

Results of experiments performed at 90 W: Estimation of growth and EPS production was made in three different inoculums, which received MW (90 W) treatment for 2, 4, or 6 min. Comparison of their growth and EPS production with that of control (Table 1) was made. A significant increase in EPS production was observed from the *X. campestris* culture corresponding to 6 min MW treatment; however this was not accompanied by any increase in growth. Thus, growth and xanthan production in this case seemed to be affected by MW independent of each other.

After 90 W MW treatment, the culture exposed to MW for 6 min was found to produce xanthan appreciably higher than the parent control, so it was selected for further experiments. Three colonies from the plate on to which the culture exposed to 6 min MW treatment was plated, were picked randomly and designated as 6A, 6B, and 6C. These three colonies were streaked on three separate nutrient agar plates, and the resulting population was studied for xanthan production after inoculation into TY broth (Table 2). Out of these three colonies, 6A was found to produce 43.75% higher xanthan as compared to the parent strain, accompanied by a significant increase in growth too. However, the magnitude of xanthan overproduction was much higher than the increase in growth, suggesting that xanthan overproduction was entirely not due to increased cell density in the 6A culture. The magnitude of increase in xanthan production shown by 6A was almost identical to that achieved in the first experiment for 6 min treatment (Table 1).

Further generations of 6A were then also studied for xanthan production, to investigate whether the trait of xanthan overproduction is maintained stably over generations. Table 3 lists measurements of growth and xanthan production in all the generations studied of 6A, and their comparison with those of the parent strain. The first results obtained with 6A are designated as those of generation 'zero'. MW treatment at 90 W for 6 min duration was able to alter the xanthan production in *X. campestris* to a notable extent, while growth was affected to a lesser extent. The trait of xanthan overproduction in the mutant 6A was stable only till one generation, thereafter it was able to produce xanthan equivalent to the parent strain. The mutant strain reverted back to the parent phenotype from second generation onwards.

Results of experiments performed at 450 W: Effect of MW on *X. campestris* was also investigated at 450 W, where duration of MW exposure was kept 3 min (Table 4). This MW treatment was able to induce xanthan production

in *X. campestris*, but growth was lesser than control. Four colonies from the plate on to which the culture exposed to 3 min MW (450 W) treatment was plated, were picked randomly and designated as 3A, 3B, 3C, and 3D. These four colonies were streaked on four separate nutrient agar plates, and the resulting population was studied for xanthan production after inoculation into TY broth (Table 5). Out of these four colonies, 3C was found to produce 50% higher xanthan as compared to the parent strain, although its growth experienced a decrease. This suggests that increase in xanthan production was not linked to the growth of the test organism. The magnitude of increase in xanthan production shown by 3C (Table 5) was much higher than that achieved in the first experiment for 3 min MW (450 W) treatment (Table 4).

Further generations of 3C were then also studied for xanthan production, to investigate whether the trait of xanthan overproduction is maintained stably over generations. Table 6 lists measurements of growth and xanthan production in all the generations studied of 3C, and their comparison with those of the parent strain. The first results obtained with 3C are designated as those of generation 'zero'. The trait of xanthan overproduction in the mutant 3C was inherited only upto one more generation, however the magnitude of overproduction was lesser than generation 'zero'. From second generation onwards it was able to produce xanthan equivalent to the parent strain. The mutant strain 3C reverted back to the parent phenotype from second generation onwards.

MW treatments at two different powers (90 and 450 W) employed in this study were able to alter growth and xanthan production in some of the *X. campestris* isolates obtained from MW treated suspensions. As the MW treatment was performed by placing the inoculum vial in an ice containing beaker, and the temperature did not cross 25°C, whatever alterations were observed in growth and xanthan production seem largely to have resulted from non-thermal effects of MW radiation. As the trait of xanthan overproduction was transferred from one generation of MW treated organism to the next, the MW effect can be said to be mutagenic. However this MW induced mutation was not found to be stable over multiple generations. Both the xanthan overproducing mutants (6A and 3C) obtained by two different MW treatments reverted back to the parent phenotype, suggesting the reversible nature of the MW induced mutations.

Reports suggesting reversible nature of MW effects, as well as those suggesting the MW-induced mutations to be stable, both have accumulated in literature. Disappearance of low-level MW (35 GHz, the surface of irradiated object - 30 mcW/cm^2, exposure time 10 s) induced effects after few generations in *Drosophilla melanogaster* was demonstrated by Pasiuga et al. (20). It is possible that MW treatment has a profound effect on mutation repair system of a cell for initial few generations, but later the repair system restores its efficiency. Our results, and those reported by Pasiuga et al. (20) are in contrast with few other reports describing stable mutations generated by MW treatment. For example, Lin et al. (4) obtained lactic acid overproducing mutants of *Lactobacillus rhamnosus* by using MW radiation (2450 MHz; 400 W for 3 min), and they found the mutant to be stable for increased L-lactic acid production for at least nine generations. Li et al. (13) reported enhanced cellulase production in *Trichoderma viride* mutated by compound mutagenesis using MW (2450 MHz; 700 W for 15-195 s) and ultraviolet, and these mutants showing higher cellulase production were stable up to 9 generations. Li et al (21) employed MW (250 W for 36 s) mutagenesis for obtaining *Kleibsella pneumoniae* mutants with better N_2 fixing and P-solubilizing ability, and claimed them to be genetically stable. Jangid et al. (12) found microwave treatment (2450 MHz, 800 W cm^{-2} for 1, 3, 5 and 7 s) to be capable of inducing mutations and altering gene expression in *Vigna aconitifolia*.

Increased amount of EPS production observed in the mutants obtained in the present

Table 1. Effect of different durations of MW (90 W) treatment on growth and xanthan production in *X. campestris*

Duration of MW treatment (min)	Growth(OD_{625}) (Mean ± SD)	% change compared to control	Amount of xanthan produced(g/L)	% change compared to control
0 (control)	1.28 ± 0.01	0.00	0.72 ± 0.28	0.00
2	1.29 ± 0.02	1.17	0.80 ± 0.28	11.11
4	1.28 ± 0.01	0.00	0.88 ± 0.80	22.22
6	1.26 ± 0.00	-1.56	1.04 ± 0.40	44.44*

*p<0.05; minus sign indicates a decrease over control

Table 2. Growth and xanthan gum production by three different isolates selected randomly from plate corresponding to 6 min MW (90 W) treatment

Duration of MW treatment (min)		Growth (OD_{625}) (Mean ± SD)	% change compared to control (g/L)(Mean ± SD)	Amount of xanthan produced	% change compared to control
0 (parent control)		0.89 ± 0.00	0.00	0.64 ± 0.00	0.00
6	6A	1.04 ± 0.00	16.81**	0.92 ± 0.02	43.75**
	6B	0.96 ± 0.20	7.84	0.60 ± 0.02	-6.25
	6C	1.04 ± 0.04	16.59**	0.68 ± 0.08	6.25

**p<0.01; minus sign indicates a decrease over control

Table 3. Comparison of xanthan production of the mutant 6A with parent strain over multiple generations

Generation number	Growth			Amount of xanthan produced		
	OD_{625} (Mean ± SD)		% change compared to control	(g/L) (Mean ± SD)		% change compared to Control
	Control (Parent strain)	6A		Control (Parent strain)	6A	
0	0.89 ± 0.00	1.04 ± 0.00	16.81*	0.64 ± 0.00	0.92 ± 0.02	43.75*
1	0.87 ± 0.00	0.89 ± 0.00	2.06	1.05 ± 0.00	1.47 ± 0.00	40.47*
2	1.37 ± 0.00	1.41 ± 0.01	2.91*	0.80 ± 0.00	0.85 ± 0.00	6.25
3	1.03 ± 0.01	1.05 ± 0.01	1.74	1.10 ± 0.00	1.10 ± 0.00	0.00
4	1.03 ± 0.00	1.06 ± 0.01	3.10	1.65 ± 0.00	1.70 ± 0.00	3.03

*p<0.05

Table 4. Effect of MW (450 W) on growth and xanthan production in *X. campestris*

Duration of MW treatment(min)	Growth(OD_{625}) (Mean ± SD)	% change compared to control	Amount of xanthan produced(g/L) (Mean ± SD)	% change compared to control
0 (control)	1.13 ± 0.01	0.00	1.06 ± 0.02	0.00
3	0.94 ± 0.02	-16.37*	1.30 ± 0.05	22.06*

*$p<0.05$

Table 5. Growth and xanthan gum production by four different isolates selected randomly from plate corresponding to 3 min MW (450 W) treatment

Duration of MW treatment(min)		Growth(OD_{625}) (Mean ± SD)	% change compared to control	Amount of xanthan produced(g/L) (Mean ± SD)	% change compared to control
0(parent control)		1.40 ± 0.00	0.00	0.90 ± 0.00	0.00
3	3A	1.32 ± 0.08	-5.71	0.97 ± 0.03	-7.77
	3B	1.32 ± 0.04	-5.71	1.15 ± 0.07	27.77**
	3C	1.07 ± 0.00	-23.21**	1.35 ± 0.07	50.00**
	3D	1.39 ± 0.01	-0.71	0.85 ± 0.10	-5.55

**$p<0.01$; minus sign indicates a decrease over control

Table 6. Comparison of xanthan production in the mutant 3C with parent strain over multiple generations

Generation number	Growth			Amount of xanthan produced		
	OD_{625} (Mean ± SD)		% change compared to control	(g/L) (Mean ± SD)		% change compared to Control
	Control (Parent strain)	3C		Control (Parent strain)	3C	
0	1.40 ± 0.00	1.07 ± 0.00	-23.21**	0.90 ± 0.00	1.35 ± 0.07	50.00**
1	0.75 ± 0.02	0.83 ± 0.01	9.93	1.20 ± 0.00	1.45 ± 0.02	20.83**
2	1.14 ± 0.00	1.12 ± 0.02	-1.31	2.50 ± 0.05	2.20 ± 0.50	-12.00
3	1.35 ± 0.02	1.20 ± 0.00	11.43**	1.70 ± 0.03	1.70 ± 0.05	0.00

**$p<0.01$; minus sign indicates decrease over control

study may either be due to effect of MW on xanthan synthesizing machinery of the cell, or increased secretion of xanthan through cell membrane whose permeability might have been altered following MW treatment. Exopolysaccharides are usually synthesized at the cell membrane, and then exported from the cell (22). Alteration in the permeability of cell membrane following MW treatment has been reported by Bollet et al. (23). Influx of extracellular calcium was shown to be affected by MW-induced alterations in the bacterial cell permeability (24). Calcium is an important cell signalling molecule, and calcium chloride (which we used at 0.7 g/L as one of the medium ingredients) has been one of the major quantitative factors which can affect xanthan production (25). Alteration in cell membrane permeability may contribute, in part, to the non-thermal effects of MW on microbial cells.

Growth of *X. campestris*, and EPS production by it were affected by MW differently (Table 1-6). Increase in xanthan production could not be correlated with increase or decrease in growth. This indicates that the enhancement of EPS production by the mutant strains (6A and 3C) was independent of the effect of MW on *X. campestris* growth. Enhancement in EPS production in these mutants is likely to be due to the effect of MW radiation on production and/or secretion of the EPS. Though some work has been done on xanthan biosynthesis, the export and polymerization process for it is not well understood. The xanthan synthesis is encoded by the *gum* genes located in a single gene cluster of 12 kb (2). The *gum* gene cluster codes for transferase activities in *X. campestris*, three polymerases, and also includes a gene (*gum*J) which appears to control the export of the polysaccharide. Mutation in *gum*J is lethal but blocking xanthan synthesis suppresses the lethality. Mutants deficient in *gum*J cannot export xanthan, which accumulates and kills the cells. Mutation studies have revealed a number of strains that produce xanthan with alterations in the pattern of acetylation and pyruvylation, as well as mutants which show increases in yield, rates of production and composition of the repeat sub-unit (22).

Conclusion

This study has demonstrated the ability of low power MW radiation to influence EPS production in *X. campestris*. Though the enhanced EPS production induced by MW treatment was carried over to the daughter populations of the mutants, these mutants ultimately turned back to the parent phenotype. MW energy is believed to be able to penetrate deep into the biological materials (26). The MW frequency used in this study (2450 MHz) has been shown to cause single- and double- strand DNA breaks in rat brain cells (27). Ability of MW radiation to cause DNA damage including point mutations has also been reported (4). Effective use of MW radiation for mutation experiments has been reported by many investigators (4, 5, 13, 20, 21, 28). Identifying mutagenic frequencies (with simultaneous optimization of MW power and exposure time) can pave way for large scale screening programmes employing MW mutagenesis as a tool for strain improvement, and yield genetically stable overproducing mutants. Such experiments can also provide for better understanding of the MW specific non-thermal effects.

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