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Microwave mutagenesis for altered lactic acid production in *Lactobacillus plantarum,* and *Streptococcus mutans*

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Abstract

This study attempted at microwave mutagenesis of: (i) *Lactobacillus plantarum* for lactic acid overproduction, and (ii) *Streptococcus mutans* for reduced lactic acid production. Lactic acid is among the microbiological products with high market potential. Lactic acid is also an important virulence factor in formation of dental caries by *S. mutans*, as the acid produced by the bacteria leads to demineralisation of the teeth. Two of the mutants obtained (one from each organism) were able to maintain the altered lactic acid production till 10 generations. However the magnitude of alteration in lactic acid producing ability of the mutants went on decreasing over generations. The microwave effects observed in this study seem largely to be athermal in nature. Investigation of the mutants obtained at molecular level may result in identification of novel mutations responsible for altered lactic acid production. These mutations can then be introduced into a suitable organism either for better industrial production of lactic acid, or for constructing new probiotic strain(s) for possible application in maintenance of oral health.

Keywords: Microwave mutagenesis, Lactic acid, Probiotic, Microwave specific athermal effect**.**

Introduction

The part of electromagnetic spectrum corresponding to wavelength range of 300 MHz to 300 GHz is referred as microwave (MW) region. During last few decades this non-ionizing part of electromagnetic spectrum has experienced widespread uses (1-2), e.g. in telecommunications (3), domestic and medical microwave devices for diagnosis and/or therapy (4, 5), extraction (6, 7), sterilization (8-10), waste treatment (11), etc. MW have been described to exert two kinds of effects on biological systems, i.e. thermal and athermal (MW specific non-thermal effect). The former has been established and understood very well, whereas controversy has built up surrounding the latter (12). More research is required on the athermal MW effects, whether they affect biological entities, their possible mode of action, and if these effects are heritable (13).

One of the recently indicated applications of MW is their use as a mutagenic agent. Lin *et al.* (14) reported MW induced overproduction of lactic acid in *Lactobacillus rhamnosus*. Kothari *et al*. (15) has reported mutagenic effect of MW radiation on exopolysacchride production in *Xanthomonas campestris*. Li *et al*. (16) reported enhanced cellulase production in *Trichoderma viride* mutated by compound mutagenesis using MW (2450 MHz; 700 W for 15-195 s) and ultraviolet. Radiation mutagenesis on account of its convenience, safety, and better mutagenicity results is an attractive alternative to conventional strain improvement strategies based on use of mutagenic chemicals,

transposons or viruses. At times industrial strains can exhibit intolerance to ultraviolet and X-ray radiation. MW mutagenesis can emerge as a clean and effective tool for strain improvement. It can also help get rid of the problem of photo-reactivation, often observed with ultraviolet mutagenesis (17).

The present study attempted at MW mutagenesis of: (i) *Lactobacillus plantarum* for lactic acid overproduction, and (ii) *Streptococcus mutans* for reduced lactic acid production. Lactic acid is among the microbiological products with high market potential. Lactic acid has found application in foods, beverages, biodegradable polymer production, and hence bioproduction of L- lactic acid has become an issue of significance (18). The production of biodegradable plastic polylactide has led to increased interest in optically pure lactic acid, accounting for recent shift from chemical to microbial processes. Though lactic acid has been on the market for sometime, further strain improvement for the large scale microbial production processes is needed (19).

Lactic acid is an important virulence factor in formation of dental caries by *S. mutans*, as the acid produced by the bacteria leads to demineralisation of the teeth (20). The mutant and/or recombinant strains capable of producing lower or no lactic acid can find application as a probiotic in maintenance of oral health. Probiotic methods are currently under focus as an alternative means of caries management (21).

Materials and Methods

Test Organisms: *L. plantarum* (MTCC 2621), and *S. mutans* (MTCC 497) were procured from Microbial Type Culture Collection (MTCC), Chandigarh.

Microwave Treatment: Bacterial suspension was prepared in sterile normal saline, from an active culture growing on brain heart infusion agar (BHI; HiMedia, Mumbai) (22, 23), and MRS agar (HiMedia) (24, 25, 14), in case of *S. mutans* and *L. Plantarum* respectively. Inoculum 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/ 270 W/ 450 W; 2450 MHz) in a domestic MW apparatus (Electrolux® EM30EC90SS). MW treatment at 90 W was given for three different time durations viz. 2, 4, and 6 min; MW treatment at 270 W was given for 2, and 5 min; whereas MW treatment at 450 W was given for 2 min. Vials inside the MW apparatus were placed in an ice containing beaker, so as to avoid/minimize any thermal heating. Temperature of the microbial suspension after MW treatment at 90 W did not go beyond 15°C; while using MW at 450 W (2 min), temperature reached 50.10 ± 0.41°C; at 270 W (2 min) temperature reached 29.50 ± 0.50°C, and at 270 W (5 min) it was 67.80 ± 0.62°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 (including control) were put in ice for 5 min to nullify any variations in initial temperature. Test organism was immediately (in less than 5 min) inoculated onto BHI or MRS agar for *S. mutans* and *L. plantarum* respectively, following MW treatment. Incubation was made at 35°C under static condition for 48 h.

Lactic acid estimation: Lactic acid was estimated using the photometric method described by Kimberley and Taylor (26) with some modification (27). Briefly, after measuring the growth at 625 nm, culture broth was centrifuged (Nüve NF 800 R) at 7500 rpm for 15 min, and the resulting supernatant was used for lactic acid estimation. One mL of this supernatant (after appropriate dilution) was mixed with 1 mL of 20% $CuSO_4$ (HiMedia) solution and 8 mL water, followed by addition of 1 g of calcium hydroxide (CDH, Delhi). This mixture was incubated at room temperature for 30 min, followed by centrifugation at 7500 rpm for 15 min. One mL of the resulting supernatant was mixed with 0.05 mL of 4% $CuSO_4$ solution, followed by addition of 6 mL concentrated H_2SO_4 (Merck, Mumbai). After proper mixing the reaction mixture was placed

in a boiling water bath for 5 min, followed by cooling and addition of 0.1 mL of *p*-hydroxy diphenyl reagent [1.5% *p*-hydroxy diphenyl (HiMedia) in 95% ethanol]. It was allowed to stand at room temperature for 30 min, and then in a boiling water bath for exactly 90 seconds. After cooling OD was measured at 560 nm, and lactic acid concentration was calculated from a standard curve obtained using pure lactic acid (HiMedia) at 10-30 μg/mL.

Screening for mutants : Following the MW treatment of *L. plantarum* suspension, the treated inoculum was streaked on MRS agar plate, and incubated at 35°C for 48 h. After the incubation 3 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 MRS agar plate. Daughter populations thus generated from a single parent colony were then inoculated into the MRS broth, followed by incubation at 35°C for 48 h under static condition. After incubation, lactic acid estimation was made for all the MW treated inoculums. Then the plates corresponding to the MW treatment yielding higher lactic acid were selected for further experiments. Subculturing was done from the plates of overproducing mutant(s), and daughter population resulting from each subculturing was checked for its lactic acid production (in comparison to the wild type), upto 10 generations. Similar screening was done with *S. mutans* for reduced lactic acid production, using BHI as the growth medium.

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

Lactobacillus plantarum: *L. plantarum* was given three different MW treatments viz. 270 W for 2 min, 270 W for 5 min, and 450 W for 2 min. When the MW treated inoculum corresponding to 270 W (5 min) was plated on MRS agar plate, no growth appeared after incubation, suggesting that due to high temperature cells were killed during MW treatment. From the plates corresponding to remaining two MW treatments, three colonies (designated as A, B, and C) were picked from each plate, and streaked on to a new MRS agar plate (one plate from each colony). Inoculum made from the resulting growth was inoculated into MRS broth, followed by lactic acid estimation after incubation. Four of the six selected colonies were found to produce significantly higher lactic acid compared to the wild type control (Table 1). Interestingly, despite considerable change in the lactic acid producing ability of the MW treated cultures, no notable change occurred with respect to growth and pH (except a minor reduction in pH for one of the colonies picked from 270 W treatment). This indicates that in the lactic acid overproducing cultures, per cell lactic acid production and/or excretion was much higher than the wild type control.

From these four overproducing colonies, we selected the three (viz. the colonies at serial number 3, 5, and 6 in Table 1) showing highest magnitude of lactic acid overproduction. These overproducing colonies were designated as VHVS_LP, VHVS_LP2, and VHVS_LP1 respectively. Lactic acid production in these three colonies (i.e. in their daughter populations) was studied up to 10 generations, to investigate whether they retain the trait of lactic acid overproduction, or revert back to parent phenotype. Results of this study over multiple generations are presented in Table 2.

Among the three selected colonies, at the selection stage VHVS_LP2 registered maximum lactic acid overproduction (15.47 times higher than wild type) followed by VHVS_LP1 (9.47 times higher than wild type) and VHVS_LP (9.15 times higher than wild type). VHVS_LP2 could maintain significant lactic acid overproduction only till 2nd generation, and experiments with it

were discontinued after 3rd generation. VHVS_LP1 could maintain lactic acid overproduction till 7th generation. VHVS_LP was able to maintain lactic acid overproduction till 10th generation, however the magnitude of overproduction kept on shrinking over generations (Fig 1). Though at the 10th generation VHVS_LP showed much lesser overproduction (6.66%) as compared to the overproduction at the selection stage (815.78%), this relatively smaller overproduction was still statistically significant (p<0.05). This mutant was confirmed for its identity by subjecting it to 16 s rRNA sequencing, and this sequence was submitted to GenBank with accession no. KJ690777 (http://www.ncbi.nlm.nih.gov/nuccore/KJ690777.1).

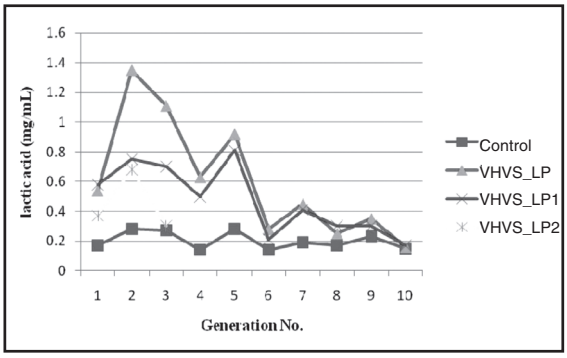

Fig. 1. Lactic acid production over multiple generations of overproducing mutants of *L. plantarum*
Control: wild type strain (with no MW exposure)
VHVS_LP, VHVS_LP1, and VHVS_LP2 are the overproducing mutants obtained after MW exposure.

Throughout these experiments the mutants showed considerable variation with respect to lactic acid production in comparison to the wild type, but there were no such major variations with respect to pH and growth, in general. This indicates that the observed lactic acid production resulted from increased production and/or secretion of lactic acid in the mutant cells, without growth potential of the mutants getting much affected. One of the bottlenecks in the way of industrial lactic acid production is the physiological demand of keeping the pH relatively higher (between 5 and 7) (19). It may be possible that the mutants obtained in this study were perhaps capable of continuing lactic acid production for some more time, even after the pH went below 5. Additionally, the mutants might be producing lower amount of by-products such as succinic acid, making diversion of more sugar flux possible into lactic acid synthesis.

Table 1. Effect of MW exposure on pH, growth and lactic acid production in *L. plantarum*

No.	MW treatment (Power and time duration)	Colony code	pH (Mean±SD)	% change compared to control	Growth (OD_{625}) (Mean±SD)	% change compared to control	Lactic acid (mg/mL) (Mean± SD)	% change compared to control
1.	0 min (control)	-	4.50±0.01	0.00	0.55±0.001	0.00	0.19±0.00	0.00
2	270 W (2 min)	(A)	4.47±0.07	-0.66	0.55±0.002	0.00	0.79±0.28	315.78
3		(B)	4.45±0.07	-1.11*	0.55±0.002	0.00	1.74±0.26	815.78*
4		(C)	4.46±0.03	-0.88	0.55±0.002	0.00	0.75±0.09	294.73*
5	450 W (2 min)	(A)	4.47±0.02	-0.66	0.55±0.001	0.00	1.80±0.16	847.36**
6		(B)	4.52±0.00	0.44	0.55±0.001	0.00	2.94±0.00	1447.36**
7		(C)	4.53±0.03	0.66	0.55±0.000	0.00	0.17±0.00	-10.52**

*p<0.05; **p<0.01; minus sign indicates a decrease over control

Table 2. Lactic acid production over multiple generations of selected overproducing mutants of *L. plantarum*

Colony designation	pH (Mean ± SD)	% Change compared to control	Growth (OD_{625}) (Mean ± SD)	% change compared to control	Lactic acid (mg/mL) (Mean ±SD)	% Change compared to control
			1st Generation			
Control	4.26±0.007	0.00	0.819±0.062	0.00	0.17±0.01	0.00
VHVS_LP (270W/2 min; A)	4.23±0.00	-0.70	0.709±0.018	-3.55	0.54±0.03	217.64**
VHVS_LP2 (450W/2 min; A)	4.08±0.00	-4.22	0.700±0.0141	-14.53*	0.37±0.09	117.64
VHVS_LP1 (450W/2 min; B)	4.27±0.00	0.23	0.575±0.0381	-29.80*	0.58±0.03	241.17**
			2nd Generation			
Control	4.27±0.00	0.00	1.59±0.09		0.28±0.04	0.00
VHVS_LP	4.24±0.00	-0.70*	1.47±0.01	-8.55	1.35±0.09	382.14**
VHVS_LP2	4.22±0.00	-1.17	1.65±0.01	3.77	0.68±0.01	142.85**
VHVS_LP1	4.29±0.00	0.46*	1.52±0.00	-4.41	0.75±0.12	167.85*
			3rd Generation			
Control	4.21±0.04	0.00	1.54±0.00	0.00	0.27±0.00	0.00
VHVS_LP	4.14±0.00	-1.66	1.46±0.007	-4.88**	1.11 ±0.26	311.11*
VHVS_LP2	4.13±0.00	-1.90	1.21±0.0141	-21.43**	0.30±0.14	11.11
VHVS_LP1	4.14±0.02	-1.66	1.51±0.0424	-1.95	0.70±0.21	159.25
			4th Generation			
Control	4.47±0.00	0.00	1.76±0.000	0.00	0.14±0.03	0.00
VHVS_LP	4.39±0.01	-1.78*	1.75±0.007	-0.56	0.63±0.05	350.00**
VHVS_LP1	4.44±0.00	-0.67*	1.70±0.007	-3.12**	0.50±0.07	257.14*
			5th Generation			
Control	4.14±0.00	0.00	1.40±0.00	0.00	0.28±0.02	0.00
VHVS_LP	4.11±0.00	-0.72*	1.57±0.014	12.14**	0.92±0.03	228.57*
VHVS_LP1	4.15±0.00	0.24	1.50±0.028	7.14*	0.81±0.12	189.28*
			6th generation			
Control	4.18±0.01	0.00	1.66±0.00	0.00	0.14±0.03	0.00
VHVS_LP	4.18±0.02	0.00	1.68±0.02	1.20	0.28±0.01	100.00*
VHVS_LP1	4.18±0.01	0.00	1.66±0.25	0.00	0.21±0.01	50.00
			7th generation			
Control	3.96±0.00	0.00	1.42±0.000	0.00	0.19±0.01	0.00
VHVS_LP	3.91±0.00	-1.26*	1.43±0.014	0.70	0.45±0.03	136.84*
VHVS_LP1	3.90±0.00	-1.51	1.36±0.007	-3.87	0.41±0.05	115.78*
			8th generation			
Control	4.13±0.01	0.00	1.58±0.02	0.00	0.17±0.00	0.00
VHVS_LP	4.06±0.01	-1.69*	1.56±0.00	-1.26	0.25±0.07	47.05
VHVS_LP1	4.06±0.01	-1.69*	1.53±0.042	-3.16	0.30±0.14	76.47
			9th generation			
Control	4.11±0.00	0.00	1.65±0.014	0.00	0.23±0.07	0.00
VHVS_LP	4.03±0.00	-1.94**	1.65±0.014	0.00	0.35±0.07	52.17*
VHVS_LP1	4.10±0.00	-0.24	1.60±0.00	-3.03*	0.30±0.14	30.43
			10th generation			
Control	4.22±0.00	0.00	1.6±0.00	0.00	0.15±0.04	0.00
VHVS_LP	4.20±0.01	-0.47	1.64±0.00	2.50*	0.16±0.01	6.66*
VHVS_LP1	4.22±0.00	0.00	1.60±0.00	0.00	0.17±0.03	13.33

*$p<0.05$; **$p<0.01$; minus sign indicates a decrease over control; VHVS_LP2 could maintain significant lactic acid overproduction only till 2nd generation, and hence experiments with it were discontinued after 3rd generation.

Table 3. Effect of low power MW (90 W) on pH, growth and lactic acid production of *S. mutans*

MW treatment (min)	Colony code	pH (Mean ± SD)	% change compared to control	Growth (OD_{625}) (Mean ± SD)	% change compared to control	Lactic acid (mg/mL) (Mean ± SD)	% change compared to control
0 (Control)	-	4.45±0.04	0.00	0.30±0.00	0.00	0.62±0.02	0.00
2	(A)	4.43±0.02	-0.44	0.35±0.04	15.21	0.48±0.00	-22.58*
	(B)	4.41±0.01	-0.89	0.34±0.00	10.35**	0.31±0.05	-50.00**
	(C)	4.41±0.01	-0.89	0.33±0.02	9.38	0.33±0.00	-46.77*
4	(A)	4.41±0.01	-0.89	0.33±0.04	7.76	0.16±0.04	-74.19**
	(B)	4.42±0.06	-0.67	0.34±0.02	11.32	0.17±0.03	-72.58**
	(C)	4.45±0.04	0.00	0.29±0.01	-6.14	0.34±0.05	-45.16*
6	(A)	4.44±0.02	-0.22	0.25±0.01	-18.77*	0.41±0.03	-66.12**
	(B)	4.42±0.01	-0.67	0.29±0.01	-3.23	0.23±0.00	-62.90**
	(C)	4.63±0.03	4.04**	0.19±0.00	-38.51**	0.20±0.00	-67.74**

*p<0.05; **p<0.01; minus sign indicates a decrease over control

Streptococcus mutans: *S. mutans* was exposed to MW at 90 W for three different time durations viz. 2 min, 4 min and, 6 min. Following the MW treatment, two BHI agar plates were streaked from each of the MW treated vials. From the plates corresponding to each MW treatment three colonies (designated as A, B, and C) were picked, and streaked on to a new BHI agar plate. Inoculum made from the resulting growth was inoculated into BHI broth, followed by lactic acid estimation after incubation. Out of these randomly selected 18 colonies, nine were found to produce significantly lower lactic acid compared to the wild type control (Table 3; data shown only for the colonies producing lesser lactic acid). For a *S. mutans* strain to be a good probiotic candidate, in addition to reduced lactic acid production it should also be having better growth potential than the wild type strain. The criteria of reduced lactic acid production was fulfilled by all the 9 isolates listed in Table 3, but only the isolate designated as (2B) was capable of growing better (10.35% higher growth) than the wild type, and hence it was selected for further experiments. Additionally one more isolate (2A) was also selected for further study; though not statistically significant, it registered the highest percentage increase in growth. Better growth or increased colonisation potential can make any particular strain better suited for probiotic applications (22), as upon use as a probiotic they are expected to compete with the pre-existing natural flora.

The two selected *S. mutans* isolates showing lesser lactic acid production were designated as VHVS_SM and VHVS_SM1, and their lactic acid production was investigated over multiple generations, whether they retain the trait of reduced lactic acid production. VHVS_SM retained its capacity to produce lesser lactic acid than wild type till 10th generation (Fig 2), along with a higher growth potential (Table 4). VHVS_SM1 could retain the trait of lesser lactic acid production only till 2nd generation, and hence experiments with this isolate were discontinued after third generation. Though the selected mutants had different growth and lactic acid production pattern than wild type, no major changes were noted with respect to pH of the culture broth at the time of harvest.

In this study two mutants have been obtained using MW mutagenesis. First one (VHVS_LP) was a superior producer of lactic acid, and another one (VHVS_SM) was inferior

Table 4. Lactic acid production over multiple generations of selected underproducing mutants (VHVS_SM, and VHVS_SM1) of *S. mutans*

Colony designation	pH (Mean ± SD)	% Change compared to control	Growth (OD_{625}) (Mean ± SD)	% change compared to control	Lactic acid (mg/mL) (Mean ±SD)	% Change compared to control
			1st Generation			
Control	4.50±0.03	0.00	0.42±0.07	0.00	0.16±0.01	0.00
VHVS_SM (2 min; A)	4.51±0.01	0.22	0.33±0.05	-21.43	0.09±0.02	-43.75*
VHVS_SM1 (2 min; B)	4.52±0.02	0.44	0.44±0.09	4.76	0.13±0.03	-18.75*
			2nd generation			
Control	4.33±0.09	0.00	0.10±0.00	0.00	0.27±0.01	0.00
VHVS_SM	4.36±0.00	0.69	0.21±0.00	108.86**	0.17±0.00	-37.03**
VHVS_SM1	4.38±0.01	1.15	0.20±0.01	90.65*	0.15±0.03	-44.44*
			3rd generation			
Control	5.53±0.00	0.00	0.67±0.00	0.00	0.24±0.01	0.00
VHVS_SM	5.57±0.01	0.72	0.70±0.00	3.53*	0.13±0.01	-45.83*
VHVS_SM1	5.55±0.00	0.36	0.71±0.00	4.86*	0.24±0.04	00.00
			4th generation			
Control	4.57±0.01	0.00	0.67±0.01	0.00	0.24±0.01	0.00
VHVS_SM	4.63±0.01	1.31*	0.70±0.01	4.74	0.13±0.02	-45.83*
			5th generation			
Control	5.61±0.00	0.00	0.83±0.12	0.00	0.24±0.04	0.00
VHVS_SM	5.64±0.03	0.53	0.70±0.01	-15.55	0.16±0.02	-33.33
			6th generation			
Control	5.37±0.02	0.00	0.53±0.01	0.00	0.27±0.01	0.00
VHVS_SM	5.39±0.09	0.37	0.67±0.03	26.72*	0.19±0.01	-29.62*
			7th generation			
Control	4.49±0.08	0.00	0.39±0.01	0.00	0.24±0.01	0.00
VHVS_SM	4.64±0.05	3.34	0.56±0.00	44.05**	0.18±0.01	-25.0*
			8th generation			
Control	4.55±0.01	0.00	0.43±0.02	0.00	0.26±0.02	0.00
VHVS_SM	4.55±0.01	0.00	0.53±0.01	23.48**	0.18±0.00	-30.76**
			9th generation			
Control	4.79±0.00	0.00	0.58±0.00	0.00	0.25±0.01	0.00
VHVS_SM	4.80±0.01	0.2	0.64±0.01	10.99**	0.18±0.01	-28.0*
			10th generation			
Control	4.55±0.00	0.00	0.68±0.00	0.00	0.28±0.00	0.00
VHVS_SM	4.57±0.02	0.43	0.71±0.01	4.41*	0.24±0.01	-14.28*

*p<0.05, **p<0.01; minus sign indicates a decrease over control
VHVS_SM1 could retain the trait of lesser lactic acid production only till 2nd generation, and hence experiments with this isolate were discontinued after third generation.

producer of lactic acid. These two were mutants of wild type strains of *L. plantarum* and *S. mutans* respectively. In both cases the magnitude of alteration in lactic acid production of the mutants was higher at the selection stage, and this magnitude underwent a reduction over generations, i.e. the mutation got partially (but not completely) reverted towards the parent phenotype. Other mutants (VHVS_LP1, VHVS_LP2, and VHVS_SM1) obtained reverted back to the parent phenotype much faster. Literature contains reports indicating reversible nature of MW effects, as well as, those suggesting the MW induced mutations to be stable. Kothari *et al*. (15) reported mutagenic effect of MW radiation on exopolysaccharide production in *Xanthomonas campestris,* however the xanthan overproducing mutants were shown to revert back to the parent phenotype. Pasiuga *et al*. (28) reported disappearance of low-level MW induced effects after few generations in *Drosophila melanogaster.* MW treatment might have a profound effect on mutation repair system of a cell for initial few generations, but thereafter the repair system may restore its efficiency. Exploitation of MW mutagenesis resulting in genetically stable mutants has also been reported by few workers. Lactic acid overproducing mutants of *Lactobaciilus rhamnosus* using MW radiations (2450 MHz; 400 W for 3 min) were obtained by Lin *et al*. (14), and these mutants were found to be stable for up to 9 generations. Li *et al.* (29) claimed *Kleibsella pneumoniae* mutants with superior nitrogen fixing and P-solubilising ability, obtained through MW (250 W for 36 s) mutagenesis, to be genetically stable.

Altered lactic acid production observed in the mutants reported in this study may be due to the effect of MW on lactic acid producing and/or secreting machinery of the cell. Bollet *et al*. (30) has reported alteration in the cell membrane permeability on account of MW treatment. Lactate is transported across the cell membrane of *S. mutans* as lactic acid in an electroneutral process independent of metabolic energy, and has important bioenergetic implications for the cell (31). As lactic acid production is important for the *S. mutans* cells from a bioenergetic point of view, even partial loss of the ability to produce lactic acid may not be favourable for them, and the mutants may tend to restore the lactic acid producing capacity typical of the wild type strain.

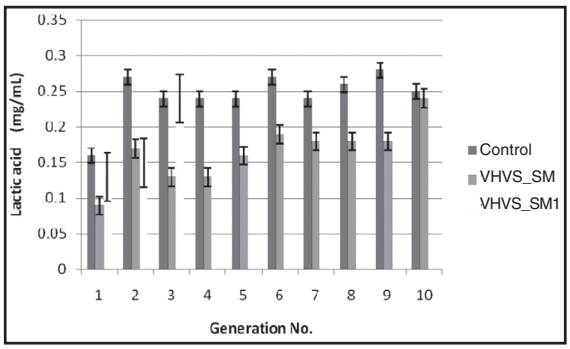

Fig. 2. Lactic acid production over multiple generations of the underproducing mutants of *S. mutans*

Control: wild type strain (with no MW exposure)
VHVS_SM and VHVS_SM1 are the underproducing mutant strains obtained after MW exposure.

Among the possible targets on which MW might have acted giving rise to mutation(s) are genes coding for lactate dehydrogenase, *ldh* open reading frame (ORF), etc. It is possible that MW treatment might have introduced multiple mutations in the genome of the mutant(s) obtained, all of these mutations may not be directly responsible for the observed modification in the lactic acid producing ability of our mutants. While comparing the lactic acid overproducing *L. rhamnosus* strains mutated by MW irradiation with the parent strains through AFLP analysis, Lin *et al*. (14) identified 51 bands of genomic DNA from mutated strains which were distinct from the parent strain, and predicted 45 genes with possible mutations. Among these 45 genes, three (malate/lactate dehydrogenase, pyruvate kinase, and NAD-dependent aldehyde dehydrogenase) were related to L-lactic acid production. Mutations in these and other such proteins involved in pyruvate metabolism can affect lactic acid production.

Acidogenicity has been proposed as the major virulence trait of the odontopathogenic bacterium *S. mutans* (32). Under conditions of sugar excess lactic acid is the major end-product of glycolysis by this bacterium. *S. mutans* strains with partial loss of lactic acid production may be able to still maintain somewhat acidic environment in the oral cavity restricting growth of other pathogens, but they may not produce so much of lactic acid which may lead to demineralization of the teeth enamel.

Conclusion

This study shows that MW radiation can be used as an effective mutagenic agent. During the MW treatments from which VHVS_LP and VHVS_SM were obtained, temperature did not go too high (not beyond 30ºC). Thus it can be said that the mutations observed were largely owing to the MW-specific athermal effect. These mutants should be subjected to further investigation including transcriptome and proteome analysis, so that mutations responsible for altered lactic acid production can be identified. Mutation(s) thus identified as responsible for lactic acid overproduction may then be introduced in industrial strains of lactic acid bacteria, paving the way for economically more favourable production of lactic acid. On the other line, mutation(s) identified as responsible for reduced lactic acid production can help in construction of useful effector strain(s) for use in replacement therapy of dental caries.

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