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Genome shuffling of *Lactobacillus rhamnosus* for improved production of lactic acid

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Abstract
Background: The development of bio-based production leads to an increasing demand for rapid engineering of multiple complex phenotypes into a single production host. The approach of genome shuffling is providing a powerful platform for improving multiple complex phenotypes in ill-characterized hosts.
Objectives: The main objectives of this study were to improve acid tolerance and lactic acid production from *Lactobacillus rhamnosus* through genome shuffling.
Methods: In the present study, the genome shuffling was used to improve the acid tolerance of while simultaneously enhancing lactic acid production. A total of 10 yoghurt samples were randomly collected in sterilized glass bottles from farmers directly and were processed immediately for isolation of the lactic acid bacteria. The isolated 5 strains of *Lactobacillus rhamnosus* treated for adaptation of Low pH. Adapted wild-type strain in pH 4.0 medium was then used as the parental, or starter, for genome shuffling**.** All the isolated *Lactobacillus rhamnosus* strains were mutagenized using nitrosoguanidine (NTG) while genome shuffling was carried out using standard method. From the results, it was observed that, the mutants showed a small increase, from 6.1 to 11.2 g/l, in production of l-lactic acid in comparison with the Wild type strain.
Conclusions: In conclusion, genome shuffling successfully improved the tolerance of *L. rhamnosus* towards acid. The research here demonstrated that genome shuffling could greatly accelerate the improvement of important phenotypes of microorganisms by developing their ability to circumvent the extreme process condition.

Keywords: genome shuffling, *Lactobacillus rhamnosus*, lactic acid production, acid tolerance

Introduction

Lactic acid has been widely used in the food, pharmaceutical and cosmetics industries. It has recently emerged as an important source for the production of other chemicals, particularly polylactic acid for biodegradable plastic (Singh, 2006) [12]. Most lactic acid is manufactured by fermentation process as opposed to chemical synthesis since it is environmentally friendly (John. 2009).

Thus, the commercial goal of improving the growth and lactic acid production of microorganisms at acid condition could decrease waste and reduce the cost of production. Moreover, it also reduces potential contamination (Porro 1999; Carlson and Peters 2002; Singh. 2006) [12, 1, 10]. It is of commercial interest for lactic acid production, but the effect of acid stress on the bacterium is a complex and poorly understood process. As a result, it is difficult to improve lactic acid production with rational engineering or direct genetic manipulation. On the other hand, classical strain improvement methods have succeeded in obtaining many industrial strains, but this is a time-consuming and laborious process because of many repeated rounds of random mutation and selection methods, especially for engineering complex phenotypes (Zhang, 2002) [15].

An important objective of a biotechnological research is the engineering of microbial cells for the production of industrially valuable metabolites. The main objective of research into effective technologies is to improve bacteria strains that are able to produce metabolites, and which will find application in industries such as chemical, food, oenology, pharmaceutical, and biofuel (Gong *et al.,* 2009) [3]. To achieve that, a classical strain improvement method is usually employed. This technique is an asexual process ased on sequential random mutagenesis and screening. A promising microorganism is mutagenized to produce a diverse library of random mutants. Screening enables selecting individual strains with an improved relevant phenotype. However, this method seems to be a laborious and time-consuming process, which is its main disadvantage (del Cardayré, 2005; Gong *et al., 2009*) [3, 2], and genome shuffling seems to be a better way of improving industrially important strains. Patnik *et al.*, (2002) used genome shuffling to improve the acid tolerance of a poorly characterized industrial strain of *Lactobacillus*. Scientists in that experiment used classical strain-improvement methods to generate populations with subtle improvements in pH tolerance, and then shuffled these populations through protoplast fusion. They identified new shuffled lactobacilli that grow at substantially lower pH than the wildtype strain does. Moreover, shuffled strains that produced three-fold more lactic acid than the wild type at pH 4.0 have also been identified.

The main objectives of this study were to improve acid tolerance and lactic acid production from *Lactobacillus rhamnosus* through genome shuffling. Thus, in the present study, we applied genome shuffling to improve the acid

tolerance of while simultaneously enhancing lactic acid production.

Materials and Methods

Bacterial Strains and Culture Condition

The bacterial strain *Lactobacillus rhamnosus* MTCC 1408 (obtained from Institute of Microbial Technology, Chandigarh) is used. Stock cultures of the bacteria were stored in MRS medium as 15% glycerol stock at -80ºC (as instructed by Institute of Microbial Technology, Chandigarh). Colonies of *Lactobacillus fermetum* were inoculated into 10ml of different De Ma Rogosa Sharp broth (MRS broth) (Lab M Limited, United Kingdom). The inoculants were incubated by placing the test tubes on a reciprocal shaker and shaking at 220rpm at 3700C for 48h.

Isolation and Identification of *Lactobacillus* Species

A total of 10 yoghurt samples were randomly collected in sterilized glass bottles from farmers directly and were processed immediately for isolation of the lactic acid bacteria. The isolation was performed by the routine microbiological procedure and inoculation on solid medium. Selective media used for cultivation of lactic acid bacteria was MRS (de Man, Rogosa and Sharpe) agar plates. Isolates were identified using standard morphological, cultural and biochemical reactions according to the criteria of Bergey's Manual of Determinative Bacteriology and Howells, (1992).

Measurement of Lactic acid Concentration

The clear supernatants (CFF) were heated with catalase at a concentration of 20unit per ml and then filtered using a membrane filtration technique (0.2µm membrane filters) (Corning Incorporated, Corning 431220, Germany). The resulting filtrate was passed through a chromatography column containing activated silica gel, eluted with n-hexane. Finally, the purity of the lactic acid was estimated using TLC and UV viz spectrophotometer at 333nm against standard lactic acid (Sigma Limited, USA). The purity of the lactic acid, in all cases was found to be above 80%.

Genome Shuffling of Lactobacillus Rhamnosus

Lactobacillus fermentum was mutagenized with Nitrosoguanidine (NTG) to obtain initial mutant library ranging from 3 x 105 – 7 x 109. Colonies from this mutant library were suspended in 10ml of sterilized MRS broth (Mixed peptone 10g/L, Yeast extract 5g/L, Meat extract 10g/L, Glucose 20g/L, Potassium phosphate 2g/L, sodium acetate 5g/L, Magnesium sulphate 0.2g/L, Manganese sulphate 0.05g/L, Tween 80 1.08g/L, Ammonium citrate 2g/L). Genome shuffling was carried out as described by Hida *et al.* (2007) with slight modification. Isolates from NTG treated *Lactobacillus fermentum* were grown in 20ml of MRS broth at 370C for 24h. Cell were harvested by centrifugation at 4000xg for 10minutes at 40C, washed twice with 30ml of 20mm of sodium malate buffer (pH 6.5), containing 0.5M sucrose and 20mM Mgcl2 and treated with lysozyme (10mg/mL in SMM) at 370C for 2h. The protoplast formation was observed with a compound light microscope before being fused by suspending in 10ml of SMM containing 30% NTG and 15% dimethyl sulphoxide (DMSO) and 10mM cacl2. After gentle shaking for 30minute at 00C, the suspension was diluted 10 fold with SMM buffer. Protoplasts were harvested by centrifugation at 3000 x g for 5minutes at 200C and then cultured on MRS Agar (LAB M limited, United Kingdom), Subsequent round of genome shuffling were carried out by repeating the protoplast fusion described above.

Measurement of lactic acid Concentration and Cell Growth in Liquid Culture

Wild type and mutant strain of *Lactobacillus rhamnosus* were each grown in 10ml of MRS broth for 2days at 370C. Each strain was harvested by centrifugation at 220rpm at 370C for 48h. The supernatants were heated with catalase (20units/mL) and then filtered using Millipore membrane filters (Corning Incorporated, Corning 431220, Germany). The resulting filtrates were chromatographed and quantitated as described above.

Results and Discussion

A total 10 samples of yoghurt were collected from local market of Aurangabad City for isolation of *Lactobacillus rhamnosus.* From 10 samples, total 16 isolates were identified, among them, a total of 5 strains of *Lactobacillus rhamnosus* were identified by morphological, cultural and biochemical test used for this study.

Adaptation of to Low pH.

The isolated 5 strains of *Lactobacillus rhamnosus* treated for adaptation of Low pH and it was achieved by modification of the procedure from Patnaik (2002) [8], through a continuous and controlled decrease in broth medium pH 6.2 to pH 5.0 and pH 4.0. For scenarios in which the imposed pH was not tolerated, and the culture steady-state could not be maintained, the culture was replaced with fresh MRS broth of pH lower than 4.0. Adapted wild-type strain in pH 4.0 medium was then used as the parental, or starter, for genome shuffling**.**

The first population of the adapted acid-mutant-library was achieved by slowly decreasing the pH from 6.2 to 4.0. Astable population of was able to grow at pH 4.0, a pH that severely inhibits growth of the wild-type strain. The second population was enriched for acid tolerance of the wild-type strain using UV mutant library selected on MRS-agar-plates at pH 4.0. Ten colonies which had bigger transparent haloes compared to colony diameter were obtained.

The isolated 5 LAB were screened for their acid tolerance. The important criteria for selection LAB as probiotics were acid tolerant at low pH tolerant up to 2%. Among them strain LR9 was found most tolerance to acid at pH 2.

Table 4: Adaptation of to Low pH of *Lactobacillus rhamnosus*

S. No	Code no.	Acid tolerance			
		pH 5	pH 4	pH 3	pH 2
1.	LR2a	R	R	S	S
2.	LR5	R	S	S	S
3.	LR8	R	R	S	S
4.	LR9	R	R	R	R
5.	LR10c	R	S	S	S

From the above table it was obsreved that, the strains LR9 was showen tolerence tp pH 2 to 5, where as Strains LR2a and

LR8 were sensitive to pH 2 and 3 but sensitive to pH 4 and 5. Strians LR10c was only resistant to pH 5 and sensitive to pH 2, 3 and 5. The strains LR9 was found potent pH tolerance strain among the isolated *Lactobacillus rhamnosus*

Being resistant to low pH is one of the major selection criteria for probiotic strains (Quwehand *et al,* 1999; Cakir, 2003). Since, to reach the small intestine they have to pass through from the stressful conditions where the pH can be as low as 1.5 to 2 of stomach, the organism colonizes the epithelium of the lower intestinal tract (Chou and Weimer, 1999; Cakir, 2003). Although in the stomach, pH can be as low as 1.0, in most *in vitro* assays pH 2.0 has been preferred. Due to the fact that a significant decrease in the viability of strains is often observed at pH 2.0 and below (Prasad, *et al,* 1998).

As lactic acid fermentation at low pH (at or below the of lactic acid, pH 3.8) can reduce the cost production and decrease waste, acid tolerance phenotypes meet the performance criteria for industrial strain commercialization. Thus, the strains tolerant to acid conditions are desirable industrial phenotypes (Patnaik 2002) [8]. Environmental tolerance of pH is a complex and yet poorly understood phenomenon, therefore, therefore we applied genome shuffling approach to improve lactic acid production of local. This approach has successfully been used to improve acid tolerance in through fusion between a low-pH-adapted mutant library population and nitrosoguanidine (NTG) mutant-library-population (Patnaik. 2002) [8], and in through UV irradiation and NTG mutagenesis (Wang. 2007). Here, we used an adapted low-pH-mutant library population and an UVirradiation- mutant library population, instead of a nitrosoguanidine (NTG) mutant library population, as the starter of genome shuffling. Since genome shuffling practically mimics the features of natural evolution through recursive genetic recombination, it requires a diverse population of mutants with an improvement of the desired phenotype compared with the wild-type as a starting point (Yu, 2008) [14].

Strain Mutagenesis and Mutant Screening

All the isolated *Lactobacillus rhamnosus* strains were grown in a resting tube containing a 10ml MRS medium at 37 ◦C for 24 h. For NTG mutagenesis, a 0.2 ml culture broth was spread onto the solid MRS with two grains of NTG placed at the center of the plate. The lawn around the inhibition zone was scraped after 48 h incubation and cultivated in liquid MRS for 6 h. The enriched culture was serially diluted in sterile saline and spread on YE agar plates containing 2% CaCO3 at 37 ◦C with 5% CO2 in a CO2 incubator. UV irradiation was performed by exposing *Lactobacillus rhamnosus* wild strains spread on YE agar plates containing 2% CaCO3 directly to UV light at a distance of 20 cm for 30 s. The plates were also incubated at 37 ◦C with 5% CO2 in a CO2 incubator. The fast grown colonies under both conditions which had the bigger transparent haloes compared to colony diameter, were picked off for the shake-flask analysis. The mutants with high l-lactic acid production were selected as the starter for the genome shuffling. The washed cells were suspended in glucose-yeast extract medium and spread onto agar plates 0.6% (v/v) alcohol. This compound inhibits wild type cells since with functional alcohol dehydrogenase activity convert alcohol to the toxic compound acrolein (Pulci *et al.,* 1993). Single colonies obtained on plates were repeatedly transferred to agar plates containing 0.6% (v/v) alcohol to get a stable mutant.

Genome Shuffling to Generate the Acid Tolerant Strains

Genome shuffling is dependent upon the recursive fusion of protoplasts to allow recombination. This recursive strategy permits the obtaining the phenotype of interest quickly. The high frequency of protoplast formation and regeneration is the basis of the efficiency of genome shuffling. Increasing the concentration of mutanolysin in the LPB to 30 _g/ml and lysozyme to 10 mg/ml was necessary to improve the frequency of protoplast formation as judged by osmotic fragility.

The results of the present study also proved that genome shuffling is an effective strategy for generating strains which become more tolerant to the acid and result in the improvement of lactic acid production. However, these previously published results showed that the fusants produced three-fold more lactic acid than the wild-type at pH 2.0. The improvement of lactic acid of our fusant was lower than that of these previous reports.

Therefore, the differences of lactic acid between the shuffled strains and mutated strains are obvious. One of the best performing shuffled strain from F2, F2-2, was selected for the scale-up fermentation. A control experiment was carried out by plating the selected populations of NTG and UV mutants and F1 without exposure to PEG on the YE plates containing 30% and 40% glucose. This was to determine whether acclimatization technique could lead to adaptive growth on high glucose. In contrast to the shuffled strains, no colonies were found on the corresponding plates during the same cultivation period. Otherwise, the protoplasts exposure to PEG, which promotes fusion, generated recombinants on the plates.

The concentrations of lactic acid produced by both the mutant and the wild type of *Lactobacillus rhamnosus* used in this study. Strain LR9 produced the highest concentration of lactic acid among the NTG mutagenized isolates. However, strain LR1-8 and LR-10c were found to have improved production of lactic acid at both first and second genome shuffling protocol. The orders of lactic acid production for the different steps used were as follows; Wild type Strains <UV<NTG< Genome shuffling 1<Genome shuffling 2.

The wild type strain and LR-9 isolates were further examined for both lactic acid production and cell growth in flask culture. The results showed that LR-9 strain had over 4.5 fold increases in lactic acid yield as compared to the wild type after 24h.

In the present study, genome shuffling proved to be an effective strategy for generating the mutants tolerant to the high acid concentration that was considered as one of the extreme process conditions. For lactic acid fermentation, the selection of mutant strains with acid tolerance in *Lactobacillus* (Patnaik *et al.,* 2002) [8] and ammonia tolerance in *Rhizopus* (Miura *et al.,* 2004) have been well studied. As substrate inhibition is one of conventional traits for the batch fermentation of lactic acid, isolating a acid tolerant mutant becomes of considerable importance for industrial applications. Here, we successfully used genome shuffling to achieve increased substrate availability and l-lactic acid production of the *L. rhamnosus*. In our study, the cell growth

Table 5: Production of Lactic acid before and after genome shuffling of *L. rhamnosus*

Observed parameters	*L. rhamnosus* (wild type strain)					*L. rhamnosus* (mutant strain by UV)					*L. rhamnosus* (mutant strain by NTG)					F1: strains from the first round of genome shuffling					F2: strains from the first round of genome shuffling				
	LR2a	LR5	LR8	LR9	LR10c	LR2a	LR5	LR8	LR9	LR10c	LR2a	LR5	LR8	LR9	LR10c	LR2a	LR5	LR8	LR9	LR10c	LR2a	LR5	LR8	LR9	LR10c
Yield of biomass g/g	0.78	0.75	0.78	0.88	0.80	0.80	0.85	0.84	0.86	0.84	0.88	0.85	0.95	0.98	0.95	0.78	0.95	0.88	0.98	0.95	0.90	0.95	0.88	0.98	0.95
Yield of Lactic acid g/g	0.57	0.67	0.51	0.55	0.59	0.60	0.67	0.63	0.70	0.65	0.60	0.69	0.73	0.64	0.88	0.79	0.87	0.77	1.00	0.98	1.17	0.97	0.91	1.33	0.98
Yield of Ethanol, g/g	0.37	0.00	0.36	0.30	0.20	0.33	0.26	0.25	0.30	0.20	0.00	0.36	0.25	0.30	0.00	0.00	0.36	0.25	0.30	0.00	0.00	0.36	0.25	0.30	0.00
Volumetric productivity g/g	2.58	2.38	2.67	2.78	2.80	2.88	2.40	2.77	2.95	2.90	1.95	1.40	1.77	1.95	1.90	1.55	1.40	1.77	1.95	1.90	1.95	1.40	1.77	1.95	1.90

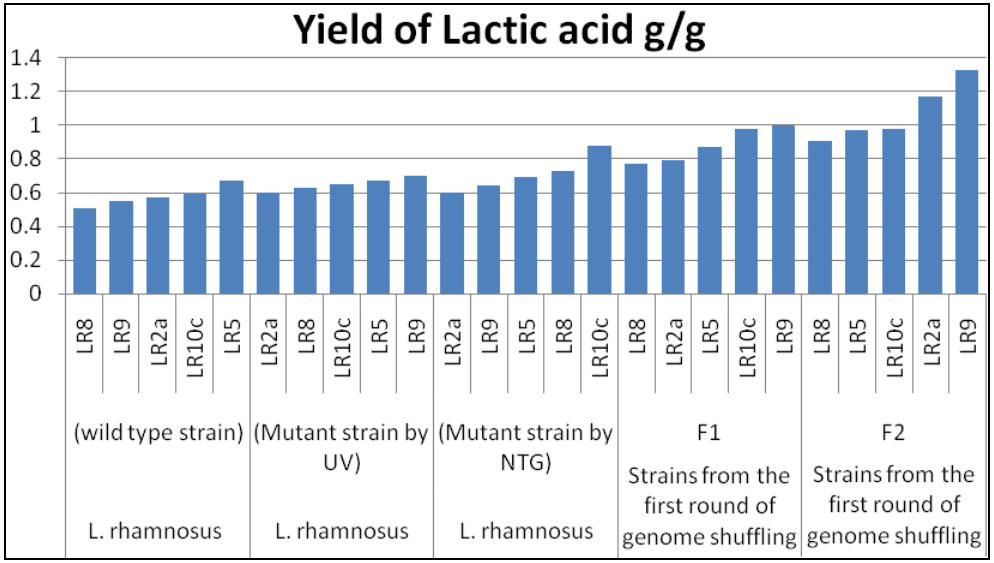

Fig 3: Comparison of wild-type and mutant strains for lactic acid production in YE medium in shake flask. Wild-type of *L. rhamnosus*; UV: UV mutant strains; NTG: NTG mutant stains; F1: strains from the first round of genome shuffling; F2: strains from the second round of genome shuffling

and lactic acid concentration of wild type decreased 33.6% and 41.7% compared with the second round genome shuffled strain, F2-2, at initial acid concentration. Thus the genome shuffled strain active in acid-rich media opens the new avenue for the enhanced production of lactic acid. The technological amelioration of industrial microorganisms leaves infinite room for genome shuffling, because the genome of organism contains the potential to evolve novel functions that will allow it to thrive in alternate environments (Hall, 1999) [4].

Unlike the rational methods for improvement of microbial strains, genome shuffling causes simultaneous changes broadly distributed throughout the genome based on genome plasticity, without the need to know the genome sequence data or network information (Petri and Schmidt-Dannert, 2004) [9]. The high pH-YE plate with2%CaCO3 screening method turned out to be effective for obtaining two phenotypes simultaneously. This result suggests that the residual cells free from protoplast formation and populations without shuffled can be eliminated on a high acid medium. The recursive protoplast-induced mutagenesis had been shown that it could not achieve substantial improvements as genome shuffling in the protoplast populations (Patnaik *et al.,* 2002) [8].

In the present work, genome shuffled LR9 was further examined with high initial acid tolerance ranging from 2 to 4 pH and the corresponding lactic acid yields were all more than 90%. Furthermore, it is noteworthy that F2-2 only produced optically pure l-lactic acid without other side products and avoided using the expensive yeast extract in fermentation medium mentioned in the above cited studies. Yeast extract leads to the highest lactic acid concentrations in a variety of nitrogen sources (Nancib *et al.,* 2001; Rivas *et al.,* 2004) [7, 11]. However, the high cost of yeast extract causes lactic acid fermentation to be economically unattractive because yeast extract was estimated to account for about 38% of the total production cost (Hujanen *et al., 2001*) [6]. Considering the good performance of LR9 the substrate inhibition in lactic acid fermentation was overcome to a great extent in the bioreactor.

Previous work on genome shuffling as a tool for improving production of bioactive constituents has been reported (Zhang *et al.,* 2002; MingHua and Shelley, 2004; Hida *et al.,* 2007) [15, 5]. In the present study, we successfully used genome shuffling to achieve significantly improved production of Lactic acid in *Lactobacillus fermentum.* This observation, clearly demonstrates that, this technique is a powerful means for rapid breeding of organisms with improved attributes of interest (Hida *et al.,* 2007) [5]. The fact that, genome shuffling done over two stages in our study selected improved strains in terms of lactic acid production is in parallel with a previous study, that indicated that a classical breeding approach requires 20 years and approximately one million screens but corroborated the findings of Zhang *et al.,* (2002) [15] who reported that genome shuffling required only a year and 24,000 screens to significantly increase production of a bioactive compounds six fold over a wild type. Nitrosoguanidine treated isolates also showed considerable improvement over the wild type in terms of lactic acid bacteria. This may be an indication that unambiguous mutations in the genome of organism's results in improved production of bioactive compounds (Hida *et al.,* 2007) [5]. Though, it can be concluded that, genome shuffling exist in *Lactobacillus rhamnosus*, there is till need to investigate genome shuffling in isolates mutagenised by other mutagens in order to identify the best mutagens for selecting improved mutants library.

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