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

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ABSTRACT

The aim of this study was to investigate the effect of genome shuffling of *Lactobacillus fermentum* for improved production of lactic acid. *Lactobacillus fermentum* was mutagenized using nitrosoguanidine (NTG) while genome shuffling was carried out using standard method. Results showed that, only four strains (LB1-A, LB-17, LB-21 and LB-24) of the twenty nine strains mutagenized showed improved production of lactic acid as follows 26mg/L, 24mg/L, 40mg/L and 61mg/L as compared with the wild type (15mg/L). Also, after genome shuffling, some strains with improved lactic acid production were selected as follows; LB1-U, LB1-F, LB1-G and LB1-Z from the first experiment and LB-2D, LB – 2F, LB – 2J and LB – 2K from the second experiment. In conclusion, genome shuffling can be seen as an important tool for improving the production of lactic acid.

Keywords: Genome shuffling, *Lactobacillus fermentum*, lactic acid

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INTRODUCTION

Organic acid have extensive uses in food industry as food additives and in the chemical industry as commodity chemicals (Hida *et al.,* 2006). Many organic acids are produced by various micro organisms at sufficient yields for commercial manufacture by fermentation (Dortu and Thonart, 2009; Maklouf, 2006). Lactic acid is one of the most important organic acids that are widely utilized in foods and beverages (Hida *et al.,* 2006; Maklouf, 2006). This acid is enriched in lactic acid bacteria and are produced either through homofermentative or heterofermentative pathway (Mohankumar and Munegalatha, 2011). The importance of this acid in inhibiting the growth of pathogenic microorganisms, maintaining nutritive quality and improving shelf life of foods has also been well documented (Holzapfel *et al.,* 2001; Hirano et al, 2013; Parada *et al.,* 2007). However, industrial fermentation of organic acids using bacteria is a sophisticated process while commercial microorganisms require improvement in multiple factors such as biosynthesis, excretion, acid tolerance and cell viability (Hida *et al.,* 2006). These problems, according to Zhang *et al.* (2007) may be addressed using genome shuffling. Genome shuffling is a novel approach that involved obtaining improved mutants of the wild type through mutation or chemostat mediated adaptation (Hida *et al.,* 2007; MingHua and Shelley, 2004; Zhang et al, 2002). This technique has been used successfully for increasing polyketide antibiotic tylosin in *Streptomyces fradiae* (Zhang *et al.,* 2002) and improved acid tolerance in *Lactobacillus* (Patnaik *et al.,* 2002). In view of this, this study was aimed at investigating genome shuffling in *Lactobacillus fermentum* for improved production of lactic acid.

MATERIALS AND METHODS

BACTERIAL STRAINS AND CULTURE CONDITION

Lactobacillus fermentum used for this study was isolated from local cheese popularly called wara in our previous study (Thomas *et al.,* 2013). 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 370^0C for 48h.

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 fermentum*

Lactobacillus fermentum was mutagenized with Nitrosoguanidine (NTG) to obtain initial mutant library ranging from $3 \times 10^5 - 7 \times 10^9$. 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 37^0C for 24h. Cell were harvested by centrifugation at 4000xg for 10minutes at 4^0C, washed twice with 30ml of 20mm of sodium malate buffer (pH 6.5), containing 0.5M sucrose and 20mM $Mgcl_2$ and treated with lysozyme (10mg/mL in SMM) at 37^0C 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 $cacl_2$. After gentle shaking for 30minute at 0^0C, the suspension was diluted 10 fold with SMM buffer. Protoplasts were harvested by centrifugation at 3000 x g for 5minutes at 20^0C 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 fermentum* were each grown in 10ml of MRS broth for 2days at 37^0C. Each strain was harvested by centrifugation at 220rpm at 37^0C 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

A total of 29 strains of *Lactobacillus fermentum* were used for this study. These organisms were isolated from local cheese in our previous study. The Fig 1 below depict the concentrations of lactic acid produced by both the mutant and the wild type of *Lactobacillus fermentum* used in this study. Strain LB-21 produced the highest concentration of lactic acid among the NTG mutagenized isolates. However, strain LB1-Z and LB-2k were found to have improved production of lactic acid with 72mg/L and 74mg/L respectively 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 <NTG< Genome shuffling 1<Genome shuffling 2.

The wild type strain and LB-2K isolates were further examined for both lactic acid production and cell growth in flask culture. The results showed that LB-2K strain had over 4.5 fold increases in lactic acid yield as compared to the wild type after 24h.

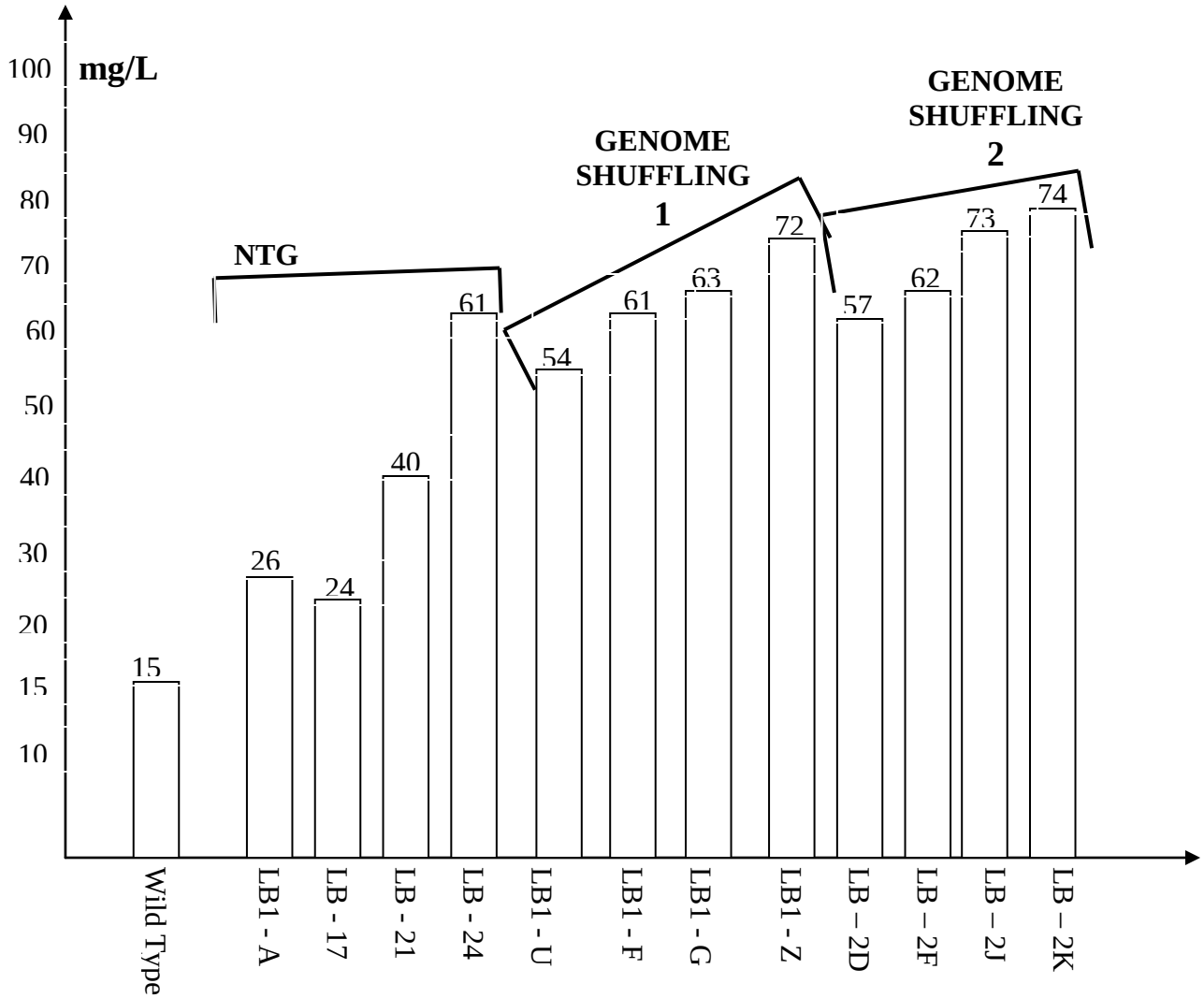

Fig 1. Showing the concentrations of lactic acid produced by the wild and the mutant strains of LAB.

DISCUSSION

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). 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). 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) 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 organisms results in improved production of bioactive compounds (Hida *et al.*, 2007). Though, it can be concluded that, genome shuffling exist in *Lactobacillus fermentum*, 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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