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COMPARATIVE STUDY OF WILD AND MUTANT STRAIN OF LACTOBACILLUS RHAMNOSUS L43 FOR PRODUCTION OF BACTERIOCIN.

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ABSTRACT:

Random Strain Improvement methods influence the growth of culture Lactobacillus rhamnosus L43 strain isolated from marine water sample from Thirumullavaram beach, Kerala. and the antibacterial performance. Strain Improvement was carried out using UV radiation (1 min -10 min.), Sodium Azide (1 to 10 ppm) and Ethidium Bromide (1 to 10 mg/ml). Out of these, Ethidium Bromide was not found to be significant for the strain improvement and Sodium Azide treated mutant had shown highest antibacterial activity against Escherichia coli 1687 (26 mm) than UV treated mutant (21.5) and wild strain (18.5 mm). The mutant has shown the highly significant p value < 0.05 and standard deviation was found to be 2.70 by ANOVA. The Sodium Azide treated mutant of 6 ppm was used for production process because this mutant had shown 40.80 % improvement in bacteriocin production in terms of antibacterial activity.

Key words: - Lactobacillus rhamnosus L43, Sodium Azide, Ethidium bromide, Escherichia coli (1687)

INTRODUCTION:

Lactic acid bacteria (LABs) are industrially important organisms used for the production of dairy products like yoghurt, cheese, buttermilk and kefir. Apart from having a preservative effect by inhibiting the growth of spoilage microorganisms through the production of organic acids, bacteriocin and consumption of nutrients, LABs also improve organoleptic properties of the product by producing metabolites that can enhance taste and texture (Adimpong, et. al.,2012). The global dairy industry is constantly exploring new ways to improve products to fulfil consumers' demand for improved taste and texture or the reduction of additives, sugar, fat or overall calorie content. This pushes the boundaries of microbial performance and requires the constant development of new starter cultures with novel properties. Wild type strains may have properties unique to the industry, but to fully exploit their potential, specific improvements are often required. In other cases it might be needed to reduce or eliminate an unwanted property. In addition, it can be of interest to improve strains which already have established industrial applicability.

Random mutagenesis (classical strain improvement) has been used extensively in the food industry (Johnson et. al., (2014). This approach is based on the introduction of random mutations into the genome of interest, characterization of a large subset of variants, and selection of strains with the desired property for further use. Despite many successes, the method is generally hampered by the fact that, apart from the desired mutation, many unintended mutations which could have a negative impact on performance are introduced.

In this review we will illustrate the use of the abovementioned methods with examples from the literature and our own laboratories and elaborate on the importance of natural strain improvement techniques. Focus will be on Lactobacillus rhamnosus L43 primarily production of bacteriocin and used for dairy fermentations.

MATERIALS AND METHODS:

2.1 Strain Improvement

The effect of mutation on Lactobacillus rhamnosus L43 and thereby the improvement of strain was studied. For mutation strain was exposed to physical mutagen (UV rays) and to chemical mutagens (Sodium Azide and Ethidium Bromide) (Venkatanagaraju and Divakar 2013; Sewunetet al., 2016)

2.1.a Physical mutagenesis by UV radiation:

1 OD of inoculum of Lactobacillus rhamnosus L43in MRS broth used for dilution. The inoculum was diluted to give 10-6 dilution and exposed to UV radiation (254 nm) at a distance of 20 cm for 1 min. to 10 min. 0.1 ml of suspension of last dilution from each exposure was plated on MRS Agar plate by spread plate technique and incubated with control plate at 300C for 24 h (Kamal et al., 2003). Irradiation with UV light was performed in a dark room and incubation also done in a dark in order to minimize the photo reactivation effects. After incubation, number of colonies were counted and percent survival curve for each plate was plotted. The mutant colonies were selected from the plates above 50% survival, replica plate technique was performed using 0.3% bile salt containing MRS agar and the developed mutant colonies inoculated in MRS broth (pH 3) for conformation of probiotic ability. The mutant colonies were transferred on bile salt containing MRS agar and used for testing its antibacterial activity against Escherichia coli(1687) was 18.5 mm

2.1.b Chemical mutagenesis by Sodium Azide:

1 OD inoculum was diluted to give 10-6 dilution and plated on MRS agar plates containing varying concentration and incubated at 300C for 24 h. After incubation, number of colonies were counted and percent survival curve for each plate was plotted. The mutant colonies were selected from the plates above 50% survival, replica plate technique carried in 0.3% bile salt containing MRS agar and the mutant colonies inoculated in MRS broth (pH 3) for conformation of probiotic ability. The mutant colonies grew on bile salt containing MRS agar and used to study antibacterial activity against Escherichia coli was 18.5 mm

2.1.c Chemical mutagenesis by Ethidium Bromide:

The strain Lactobacillus rhamnosus L43 was grown to late logarithmic phase of growth in MRS broth. The cells were harvested and washed twice with sterile 0.9% NaCl solution and 1 mg/ml to10 mg/ml of Ethidium bromide were added to 2 ml of each cell suspension. The mixture was kept in incubator for 30 min respectively. The treated cells were incubated in 10 ml MRS broth, washed twice and re-suspended in 0.9% NaCl solution and after serial dilution it was spread on MRS agar plates and incubated at 300C for 48 h. Then mutants were isolated (Gawalet al., 2002). After incubation, number of colonies were counted and percent survival curve for each plate was plotted. The mutant colonies were selected from the plates above 50% survival, replica plate technique was performed with 0.3% bile salt containing MRS agar and the mutant colonies were inoculated in MRS broth (pH 3) for conformation of probiotic ability. The mutant colonies subcultured by growing on bile salt containing MRS agar and used for bacteriocin assay.

2.1.dStatistical analysis:

Each experiment and determination was done in duplicate. The data were examined by one-way ANOVA using MINITAB 14 at a level of significance of $p< 0.05$.

RESULT & DISCUSSION:

3.1 Strain improvement of Lactobacillus rhamnosus L43

The effect of mutation on antibacterial activity of Lactobacillus rhamnosus L43 and an improvement of strain was studied. For mutation,

the strain was exposed to physical mutagen (UV rays) and to chemical mutagens (Sodium Azide and Ethidium Bromide).

3.1.a(i) UV Treatment:

Effect of UV radiation on survival was expressed in terms of % survival and % mortality. For this purpose after incubation, number of colonies were counted. Figure 1 shows the UV survival curve for the investigated organisms.

From the data it was observed that the number of colonies decreased as UV exposure increased. The colonies survives after 3 min. to 9 min., exhibit round creamy colonies and colony size also increased as compared to wild type strain. The colonies were considered as mutants, colonies survived above 50% of survival curve (above LD50 dose). The LD50 mortality was found very close to 4 min. of UV treatment (Figure 1). The mutants were selected and studied for screening test of probiotic properties.

3.1.a(ii) To study the effect of pH 3 and 0.3% bile salt on mutant isolates:

The mutant isolates were found to be resistant to low pH in presence of turbidity and tolerance to bile salt shown the colonies on replica plate from master plates of UV treatment. The 5 min. and 6 min. treated plate containing mutant colonies for screening test of probiotic properties. Mutant isolate from 5 min. and 6 min. UV exposure out of these, mutant from 6 min. had shown the highest antibacterial activity against Escherichia coli as 21.5mm. Photoplate 1 and table 1 represents the result of antibacterial activity of mutant compared with wild strain. It observed that mutant obtain from 6 min. UV exposure had exhibited remarkable antibacterial activity was more than that control as antibacterial activity of wild strain. The mutant isolates has shown the significant p value < 0.05 and standard deviation was found to be 1.976 by ANOVA.

Similar work was carried by Singhviet al., (2009) and observed that UV mutagenized Lactobacillus lactismutant RM2-24, produced lactic acid with high productivity. Similar result obtained by Joshi et al., (2009) for UV improved strain Lactobacillus lactis.

3.2 a(i) Sodium Azide Treatment:

The colonies from the plates showing above 50% survival were examined for the post mutation effect as antibacterial activity. Figure 2 shows the Sodium Azide survival curve for the investigated mutant organism.

From the data, it was observed that the number of colonies decreased as the concentration of Sodium Azide was increased. The colonies survived in presence of Sodium Azide concentration 6 ppm, 8 ppm, have round creamy colony and colony size decreased as compared to wild type colony. These colonies were considered as mutants i.e. colonies survived above 50% of survival curve (above LD50 dose) were considered as mutants. LD50 obtained as 4.2 ppm. The mutants were selected and studied for screening test of probiotic properties.

3.2.b(ii) To study the effect of pH 3 and 0.3% bile salt on mutant isolates:

The mutant isolates were found to be resistant to low pH in presence of turbidity and tolerance to bile salt obtained the colonies on replica plate from master plates of MRS containing concentration of Sodium Azide. The 6 ppm and 8 ppm treated plate containing mutant colonies for screening test of probiotic properties. Mutant SA-5 isolate from MRS containing 6 ppm concentration of Sodium Azide has shown the highest antibacterial activity against Escherichia coli as 26.5 mm. Therefore, mutant strain (SA-5) selected for further studies. Plateplate 2 and table 2 represents the result of antibacterial activity of mutant compared with wild strain. The mutant isolates has shown the significant p value < 0.05 and standard deviation was found to be 2.76 by ANOVA.

3.3.a: Chemical mutagenesis by Ethidium Bromide:

In this experiment, the colonies from the plates showing above 50% survival were examined for the post mutation effect on antibacterial activity. Table 3 shows the average number of colonies for each plate. Figure 3 shows the Ethidium Bromide survival curve for investigated mutant.

From the data, it was observed that the number of colonies decreased as the concentration of Ethidium bromide was increased. The colonies that survived on exposure to ethidium bromide concentration of 1 mg/ml to 10 mg/ml had the small creamy colonies but not change in colony size as compared to wild type strain. The LD50 dose was found to be 2.8 mg/ml. The colonies above 50% of survival curve (above LD50 dose) were considered as mutants. The mutants were selected and studied for screening test of probiotic properties.

3.3.b To study the effect of pH 3 and 0.3% bile salt on mutant isolates:

The mutant isolates were found to be resistant to low pH in terms of turbidity but had not shown the colonies on replica plate of MRS with 0.3% bile salt from master plates of MRS. It was observed that Ethidium bromide treated cells of Lactobacillus rhamnosus l43 had lost antibacterial activity, hence unable to show any improvement in isolated mutants (photoplate 3).

Strain Improvement was carried out using UV radiation (1 min-10 min.), Sodium Azide (1- 10 ppm) and Ethidium Bromide (1 to 10 mg/ml). Out of these, Ethidium Bromide was not found to be significant for the strain improvement and Sodium Azide treated mutant had shown highest antibacterial activity against pathogen (26 mm) than UV treated mutant (21.5) and wild strain (18.5 mm). The Sodium Azide treated mutant of 6 ppm was used for production process because this mutant had shown 40.86% improvement in in terms of mm. Sodium Azide treated mutant (SA10 - Sodium Azide treated mutant no.5) used for further studies.

The above findings were in accordance with the findings of Saarelaet al., (2012) in which acid tolerance of two LAB mutants were studied. According to Sewunetet al., (2016), the random mutagenesis induced by Ethidium Bromide onto the genome of two study mutants has little negative impact on the strains survival.

The mutant types in this study were able to endure acidic values as low as. Such strains are the prime candidates in numerous industrial fermentation process as they can reduce the production cost. This potential benefit was also supported by Patnaiket al., (2002) in which the authors were able to generate mutants with improved acid tolerance meeting the requirements of industrial fermentation. Thus, proving the effectiveness of the natural selection and mutant library screening procedure used in this study. The success of this finding was shared and found to be a similar study conducted by Sewunetet al., (2016). Conventional mutagenesis and mutant library screening is therefore proved to be a relatively cheaper, manageable and safer compared to genetic engineering techniques.

CONCLUSION

In strain improvement, Sodium Azide treated mutant had shown highest antibacterial activity against selected pathogen, hence the Sodium Azide treated mutant of 6 ppm (SA-5) was used for production process. This mutant had shown 42.86 % improvement in bacteriocin production.

The genetic improving protocol such as mutagenesis is an elementary part of process development, generally working as reduction of production costs. The result obtained in the present investigation confirmed that Sodium Azide treatment is an important tool in strain improvement for increasing production of bacteriocin as compared with UV-mutagenesis and Ethidium bromide.

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Table 1: Antibacterial activity and protein content of mutants probiotics

Sr. No.	Min.	Antibacterial activity (mm)	Protein conc. mg/ml
1	Control	18.5	6.2
	6 min.		
2	1	16	4.2
3	2	20	8.2
4	3	21	10.5
5	**4**	**21.5**	**11.6**
6	5	19	8

Table 2.: Antibacterial activity and protein concentration of Sodium Azide mutants

Sr. No.	Ppm	Antibacterial activity (mm)	Protein conc. mg/ml
	6 ppm		
1	1	21	10
2	2	20.5	10.5
3	3	18	6.8
4	4	22.6	10.5
5	**5**	**26**	**11.06**

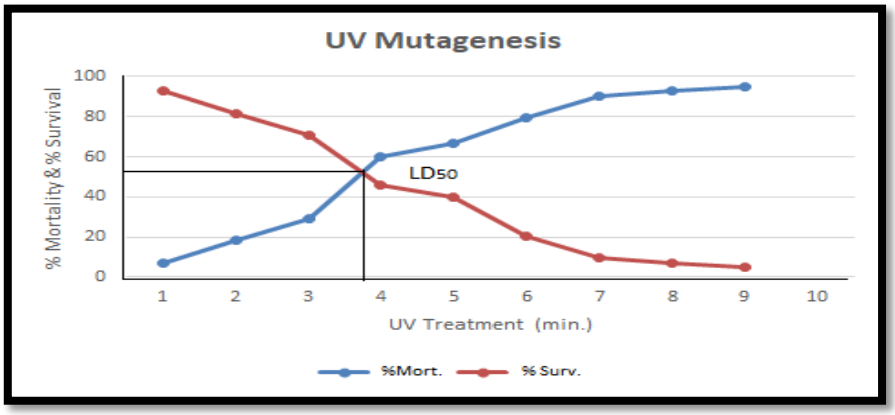


Fig. 1: Survival curve of UV treated *Lactobacillus rhamnosus L43*

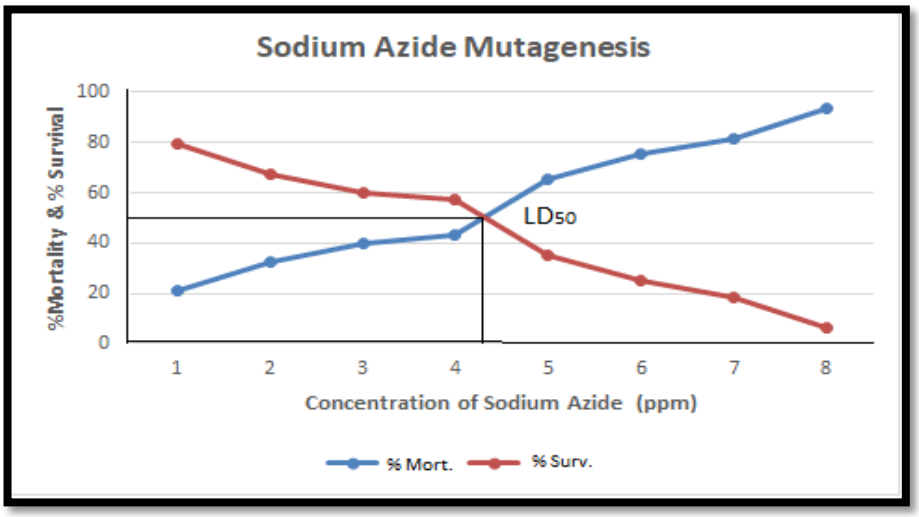


Fig.: 2: Sodium Azide treatment survival curve of *Lactobacillus rhamnosus L43*

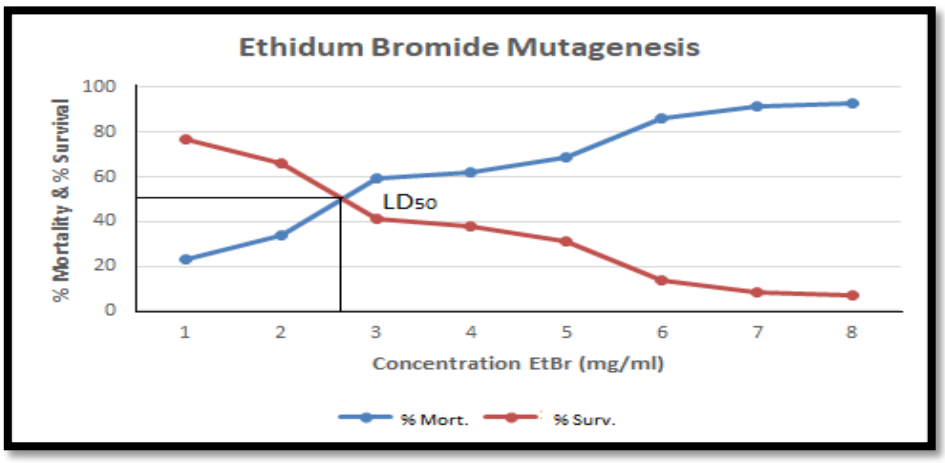
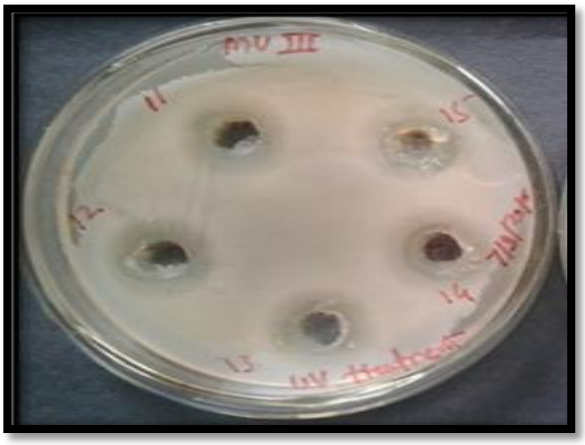
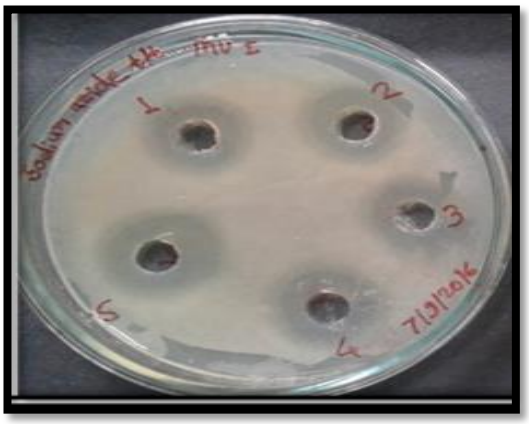


Fig. 3: Survival curve of Ethidium Bromide treated *Lactobacillus pentosus* B25



Phtoplate 1: Control **Antibacterial activity of UV treated Mutants**



Photoplate 2: Antibacterial activity of Sodium Azide Treated mutants

Photoplate 3: Antibacterial activity of Ethidium Bromide Treated mutants