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Research Paper

Screening mutant library of Lactic Acid Bacteria for development of strains with improved physiological traits desired in the dairy fermentation industry

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Abstract

Conventional mutagenesis and mutant library screening has long been used to generate mutant strains of Lactic Acid Bacteria with improved physiological and genetic characteristics. This approach was proved to be successful in the fermentation industry by supplying isolates capable of tolerating operational conditions. Acid development and persistence during dairy fermentation and poor growth are the main hindrances the industry is facing. There in order to alleviate this problem observed in Ethiopian cottage and industrial dairy fermentation, this study was designed with objectives of screening and selecting elite mutant and wild types isolates with improved acid tolerance and elevated growth performance. Hence, mutant library of Lactic Acid Bacteria and their respective wild types were screened for acid tolerance at various temperatures. The Survivors were evaluated for growth performance at 25, 35, 45 and 55 °C and pH 7, 6 and 5. Two mutant isolates (MAS03 and MAB18) and their respective wild type isolates (B6S01 and A6B08) were found be better performing and were selected for growth monitoring test. Acid stress tolerance (pH 3 and 4) was found to be higher at 35 °C and 12 hours of incubation. The growth performance of mutant isolates was found to excel the wild types at pH 7, 6 and 5. Mean Optical Density as high as 8±0.00 were measured at 45 °C and 8th hours by the mutants type MAB18. Similarly at pH 6 Optical density 6.712±0.025 was observed at 35 °C and 12th hour of incubation by the other mutant type MAS03. However, the respective wild types performance at the respective conditions were 2.417±0.021 (A6B08) and 2.999±0.001 (B6S01). The mutant types' lag time was as short as 2 and 4 hours at pH 7 and 6, and 35 and 45°C while it took the wild types 6 to 8 hours. The mutants were found be robust in faster exponential growth, stress tolerance and highly reduced lag time. These traits are desirable industrially; therefore, considering them for cottage and industrial dairy processes could reduce production cost and boost productivity.

Keywords: Fermentation, Growth, Lactic Acid Bacteria, Mutagenesis, Screening, Stress tolerance

Introduction

Lactic Acid Bacteria (LAB) are a large group of gram-positive bacteria predominantly used as starter culture in the manufacture of fermented and functional foods[1-4]. Their role in dairy fermentation is crucial as they are responsible for the production of lactic acid, acetaldehyde, acetic acid and diacetyl which give dairy products longer shelf life, better texture and aroma[5,6]. In addition, LAB in the fermentation process demonstrated antimicrobial effect by secreting bacteriocins that prevent the

proliferation of opportunistic pathogens and spoilage microbes. However, conditions that are present during the manufacturing process of dairy products such as higher operational temperature, aeration, acidic pH, water activity and related factors are observed to affect the performance, stability and even viability of LAB [7,8].

These conditions are mentioned being associated with reduced cell biomass yield as the lag time is longer[9]. In addition lower enzyme activity is manifested during lactic acid production[10] and a relatively reduced anti-microbial agent (bacteriocin) production and increased susceptibility to bacteriophage phage attack are the negative outcomes of the processing conditions inflict on LAB performance[11]. Even though most industrial LAB based fermentation process are carried in buffered mediums or in which the pH is tightly regulated, the operational temperature and the heat generated exceeds the optimal functional range of LAB. In most cases, the industrial dairy fermentation process is characterized by temperature values close to or higher than the optimal range (45 °C) which makes prolonged fermentation process less and less effective along time[12].

Therefore, in order to mitigate the hindrances the dairy fermentation industry has been facing that are attributable to the decrement in performance of LAB, classical methods of generating robust strains has long been utilized. These traditional methods rely on screening and selection elite LAB from their natural habitat (fermented products) on the basis of their relative adaptation and proliferation profile amid the adverse fermentation condition they operate[14,15]. These approaches though effective in generating novel and elite LAB isolates have their own disadvantages[16]. As a result, a once highly productive and effective LAB strain may loose its robustness reducing the quantity and the quality of the production output. In addition, the physiological and genetic basis of natural adaptation is time consuming, often less reproducible and highly resource intensive[8,13,17]. Alternatively genetic engineering precisely modifies, introduces or excises-out genes or parts the DNA associated with adaptation, proliferation and performance of organisms[12, 18]. However, the robustness of the method is often doubted as incorporation of Genetically Modified Organisms (GMO) in dairy, food and beverage industry has not yet get its popular acceptance and there are legal issues associated with it in most [19].

In the contrary, conventional mutagenesis (random mutagenesis) is a widely accepted approach adopted to boost the performance of LAB starter cultures [7,8,12,17,18,20,21].This method is simply initiated by exposing the candidate LAB isolates to physical mutagenic agents such as UV or gamma irradiation or chemicals mutagens (Ethidium bromide, N-Methyl-N-Nitroso-Guanidine, Ethyl Methyl Sulfonate etc.) and introduces diversity[8,22]. Several studies indicated that the robustness of the method in improving tolerance to stressed growth environments and higher potential for increased production of secondary metabolites and other desirable metabolic activities[19,23-25]. The main advantages of conventional mutagenesis are (i) generates food grade strains with reputation of Generally Regarded as Safe (GRAS) (ii) established by taking isolates already at hand or by isolating them from locally available samples, avoiding the disadvantages sought in natural screening of huge LAB library and selection procedure [4,8,12,18-20].

Hence, this study was initiated

- To screen and select acid tolerant LAB from mutant library construct generated by ethidium bromide mutagenesis and wild type LAB isolated form Ethiopian traditionally fermented milk.
- To monitor the growth performance of the acid tolerant mutant LAB relative to the wild type parents
- To point out and set recommendations that favor the development of fermented dairy products using mutant and wild type isolates both in cottage and industrial fermentation condition.

Materials and Methods

Wild and mutant type LAB isolates used

Wild type parents A6B08 and B6S01 belonging to *Lactococcus sp*. and *Lactobacillus sp.* respectively and a total of 80 mutant type isolates (40 isolates from each parental lines) from the previous work of Sewunet et al.[26] were recovered from deep freezer (-80°C) and refreshed on MRS broth (Fluka Analytical, India) media three times.

Screening the wild and mutant type isolates for pH and Temperature Tolerance

Hundred microliters of fresh inoculum of the wild and mutant type isolates was prepared by adjusting the cell concentration spectrophotometrically (Novaspec II, Amersham Biosciences, Inc., UK) to Optical Density (OD) of 0.1- 0.5 at 600nm. The standardized inoculums were inoculated into 5 mL MRS broth (Fluka Analytical, India) with pH set at 3 and 4. The tubes were then incubated aerobically at 15, 25, 55 and 65°C for 24 hs. Following the incubation the tubes were examined visually for turbidity (growth) and isolates that showed turbidity were considered for further study. The method used was adopted from techniques mentioned and with few modifications to attain better results[15,24,25,27-30].

pH and temperature stress test

A factorial arrangement of two acidic pH points (3 and 4) and four incubation temperatures were set. One milliliter of standardized (OD_{600nm}=0.1-0.5) fresh cultures (18 h) of both the wild and mutant type isolates were inoculated in to 50 mL of MRS broth (Fluka Analytical, India) with pH adjusted to 3 and 4 using 0.1N HCl and incubated for 12 h aerobically at the 15, 25, 45, 55 and 65°C with occasional shaking. OD readings were taken at 600nm using spectrophotometer prior to incubation and every two hours after that following the methods mentioned[24,26,27,31,32]. The Survival Ratio (SR) at was calculated using the following formula described by Gibson et al. [33] and Suree et al.[34].

$$\text{Survival Ratio (SR)} = \frac{A_{t_x}}{A_{t_0}}$$

Where: A_{tx}= absorbance at reading 2st, 4th, 6th, 8th 10th and 12th hour of incubation and $A_{t0=}$ absorbance reading at the beginning of incubation.

The survival ratio results were interpreted as:

- SR> 1 indicates bacterial growth despite the preset culture condition
- SR =1 indicates bacterial survival was not affected by the preset culture condition
- SR = 0.5 indicated a loss of half the viable cells present in the culture

Growth at acidic, near neutral and neutral pH and varying incubation temperatures

Factorial arrangement of three pH points 5 (acidic), 6 (near neutral) and 7 (neutral) of MRS broth of 50 Ml were set and inoculated with 1 mL of fresh and standardized cultures of the mutant and wild type isolates. The cultures were incubated at 25, 35, 45 and 55°C for 12h. Growth was monitored spectrophotometrically at 600nm taking measurements at the start of incubation and on the 2nd, 4th, 6th, 8th 10th and 12th h of incubation [24,26,27,31,32].

Statistical Analysis

A factorial completely randomized experimental design was implemented to determine the individual and interactive effect of incubation condition (pH, temperature and time) on survival and growth of the mutant and wild type isolates. The ANOVA procedure was carried out using SAS 9.2 software and means were separated using Tukey's model at 95% confidence interval.

Results

Screening and selection of the wild and mutant type isolates

The screening test for the wild and mutant type isolates revealed the two wild types isolates (A6B08 and B6S01) and two of the mutant types MAS03 (mutant type of A6B08) and MAB18 (mutant type of A6B08) adapted the stress and were able result in turbidity in the medium. Therefore, they were selected for further tests.

Survival of the wild and mutant type isolates at pH 3 and 4

The two wild type isolates used in the study were found to be resistant to the acidic growth condition compared to the mutant type isolates. In addition to maintaining viability, it was observed that the mutant types were relatively proliferating at a significant rate compared to the mutant type isolates. The highest mean SR score (1.039) corresponds to B6S01 (*Lactobacillus sp.*) which indicates a 3.9% rise in population relative to the respective initial figure (Table 1). The two mutant types even though their SR scores confirmed their ability of survival or maintaining maximal viability in the test growth

conditions, there relative performance was lower than their wild type parents. However, MAS03, the mutant type descent of B6S01, was found to be better performing (mean SR=1.005) compared to MAB18, the mutant type descent of A6B08 (mean SR =0.999) (Table 1).

Table 1: Mean Survival Ratio (SR) of the mutant and wild type isolates

Isolate	Genera and Genome status	Mean SR	Effect of incubation temperature		Effect of initial incubation pH		Effect of incubation time	
			Temperature value (°C)	Mean SR	pH value	Mean SR	Time (h) value	Mean SR
B6S01	*Lactobacillus sp.* wild type	1.039[a*]	15	1.014[b]	3	1.016[a]	0	1.000[e]
A6B08	*Lactococcus sp*. wild type	1.017[b]	25	1.013[bc]	4	1.014[a]	2	1.001[e]
MAS03	Mutant type of B6S01	1.005[c]	35	1.050[a]			4	1.031[b]
MAB18	Mutant type of A6B08	0.999[d]	45	1.011[bc]			6	1.002[ed]
			55	1.005[cd]			8	1.009[dc]
			65	1.0[d]			10	1.011[c]
							12	1.051[a]

***means with the same letters are not statistically different at p<0.05**

There was an apparent effect of incubation temperature on the survival ratio values of the isolates. Higher SR values were observed at 35**°**C, at which not only the isolates survived maintained growth despite the preset extreme fermentation conditions. Whereas the least scores belong to 65 °C at which the growth was halted but the survival potential remained positive with no population reduction. However, there was no statistically justifiable difference in the mean SR among 15, 25, 45 and 55 °C in supporting the growth of the isolates in addition to keeping their growth and proliferation intact (Table 1). Nonetheless, the SR values indicated that growth was maintained despite the low pH values the isolates were subjected to. The effect of incubation time on the SR of the isolates tested indicated that, maximal survival and growth being attained at the12th and 4th hs of incubation respectively. There was no statistically significant difference among 0 and 2nd hour incubation and 6th, 8th and 10th hours of incubation (Table 1).

Effect of pH, temperature and incubation time on the growth of the wild and mutant type isolates

The individual and interaction effect of pH, temperature and incubation time on the growth of the mutant and wild type isolates was found to be highly significant on the growth of both the wild and mutant type isolates (p<0.0001). The mean separation ANOVA procedure and comparison indicated that mutants were the best performers compared to their respective wild type parents. However, MAS03, the mutant type the *Lactobacillus sp.* (B6S01), was found to excel the remaining three ingrowth performance (Table 2). The growth performance of the mutant types at pH 7 and 6 excelled the wild type parents (Figure 1, 2 and 3). The highest mean OD values were observed at pH 6 (OD=8±0.00) at 45 °C and 8th h of incubation (Figure 3), and at pH 7 (OD= 6.712±0.025) at 35 °C and 12th h of incubation (Figure 2). Both highest mean OD scores belonging to the mutant types MAB18 and MAS03 respectively.

In the contrary, the respective wild types growth performance at the respective conditions was 2.417±0.021 (Figure 3) and 2.999±0.001 (Figure 2).The lag time at both pH values (7 and 6) and incubation temperatures 35 and 45°C, for mutant types MAS03 and MAB18 was as short as 2 and 4 hs while the wild types took at least to end the lag phase of their growth stage.

Table 2: Mean growth performance comparison of the wild and mutant type isolates

Isolates		Time (h)		Temperature (°C)		pH	
Tukey's grouping	Mean OD	Tukey's grouping	Mean OD	Tukey's grouping	Mean OD	Tukey's Grouping	Mean OD
MAS03[a]	1.537	12[a]	2.126	45[a]	1.923	7[a]	1.493
MAB18[b]	1.513	10[b]	1.825	35[b]	1.563	6[b]	1.394
B6S01[c]	1.036	8[c]	1.644	25[c]	0.656	5[c]	0.702
A6B08[d]	0.699	6[d]	1.033	55[d]	0.643		
		4[e]	0.663				
		2[f]	0.551				
		0[g]	0.537				

***means with the same letters are not statistically different at p<0.05**

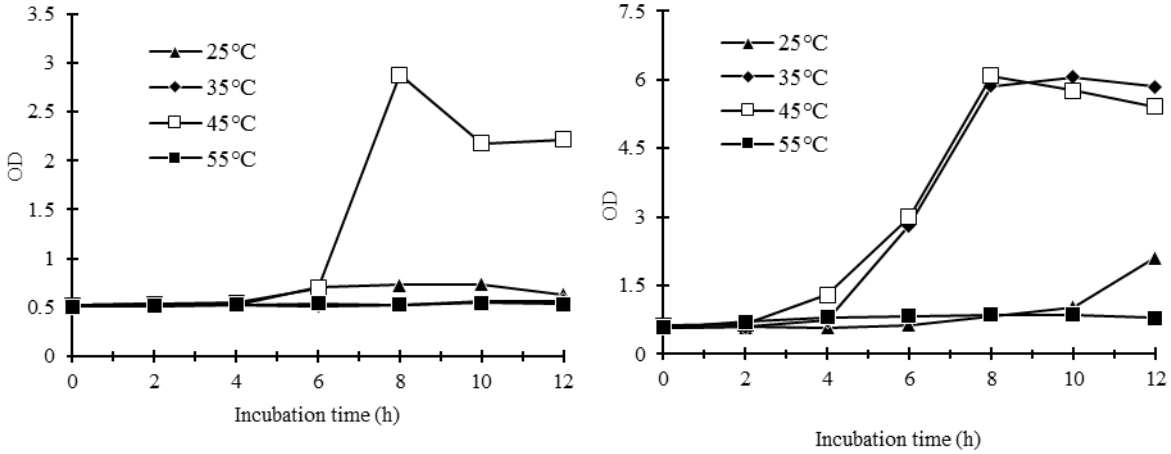

Figure 1: Growth performance of A6B08 (left) and its mutant type MAB18 (right) at pH 7 and four temperature levels

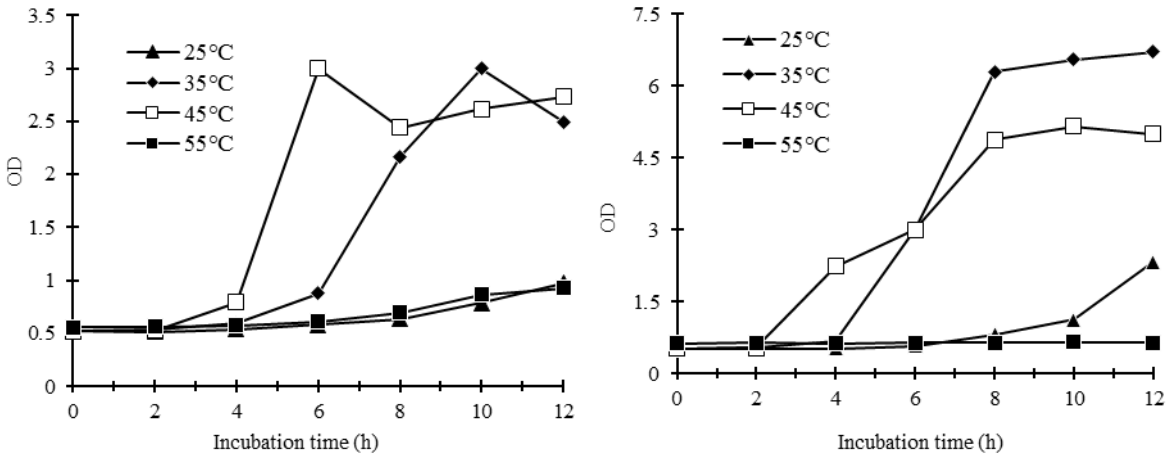

Figure 2: Growth performance of B6S01 (left) and its mutant type MAS03 (right) at pH 7 and four temperature levels

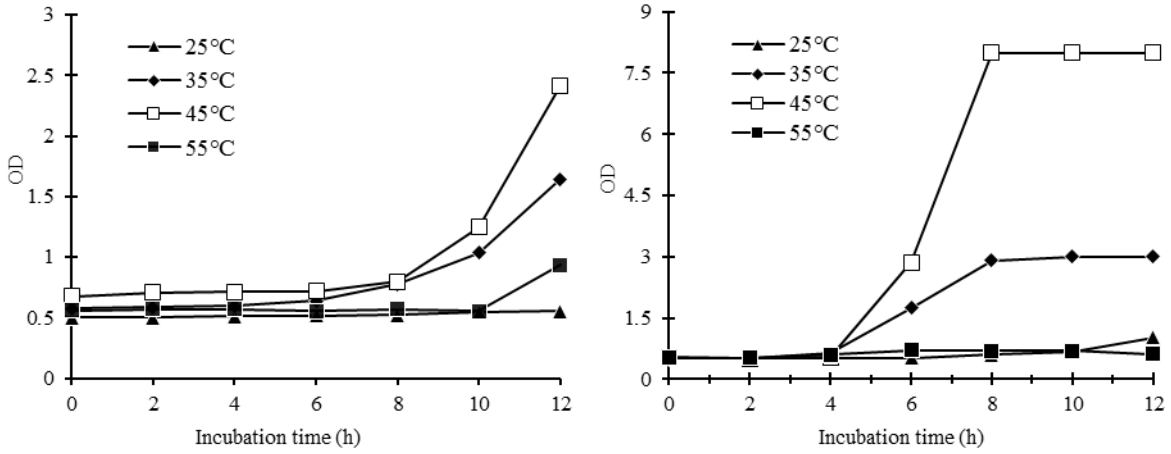

Figure 3: Growth performance of A6B08 (left) and its mutant type MAB18 (right) at pH 6 and four temperature levels

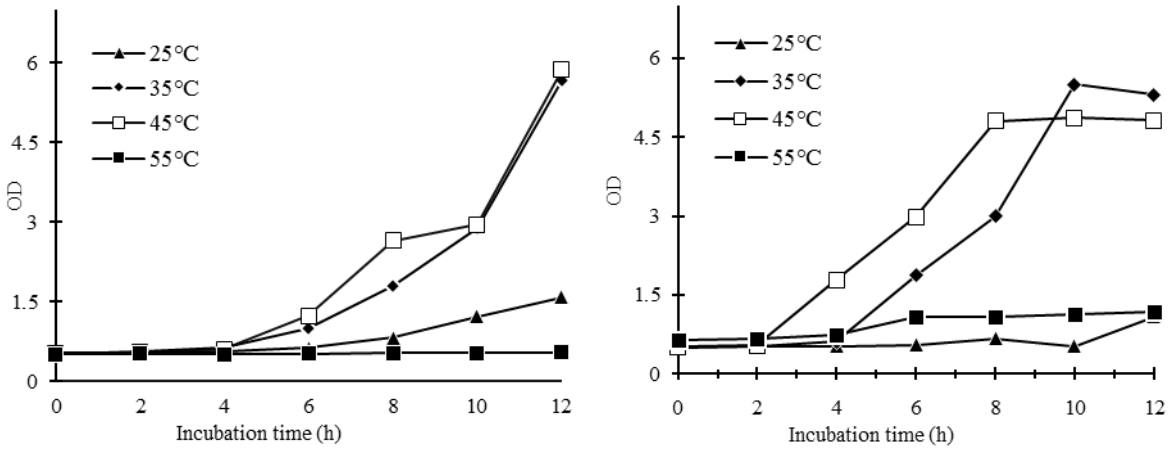

Figure 4: Growth performance of B6S01 (left) and its mutant type MAS03 (right) at pH 6 and four temperature levels

The growth performance of the mutant isolates at pH 5 still excels the wild type counterparts. The highest mean OD values at this pH values were achieved by MAS03, 2.674±0.009, at two incubation temperatures (35 and 45 °C) and 12th h of incubation time (Figure 6). The other mutant was able to attain mean OD of 2.377±0.003 at 45 °C and 12th hour of incubation (Figure 5). However, the respective wild types (B6S01 and A6B08) growth performance was by half below their mutant types with values being 1.158±0.004 at 35 °C and 1.107±0.0135 at 55 °C (Figure 5 and 6). Even though the time it took to enter log phase is longer than the results observed in pH 6 and 7, at pH 5 the mutant were observed to have lesser lag time compared to their wild type counterparts at temperatures.

It was observed that the highest and relatively higher mean OD values were attained at the 35 and 45 °C incubation temperatures. But at the two extreme temperatures (25 and 55 °C) the wild type isolates performed better than the mutant type isolates (Figure 5 and 6). At 55 °C the highest mean OD achieved was 1.107±0.014 at 12th h of incubation by A6B08 (Figure 5). In addition, the same isolate was able to reach OD value of 0.701±0.00 at 25 °C and 12th h of incubation (Figure 5). The mean growth results of the rest isolates at the respective temperatures was barely higher than the initial inoculum size. The majority of pick growth points were reached at 12, 10 or 8 hs of incubation times. However, the mutant isolates were able to reach better or even maxima OD values as early as the 4th h of incubation compared to the wild type isolates. MAS03 was able to score mean OD 2.24±0.003 and 1.794±0.018 at pH 7 and 6 respectively at the 4th hour of incubation (Figure 2 and4). The performance of the respective wild type, B6S01 was found to be 0.79±0.004 and 0.603±0.001 (Figure 2 and 4).

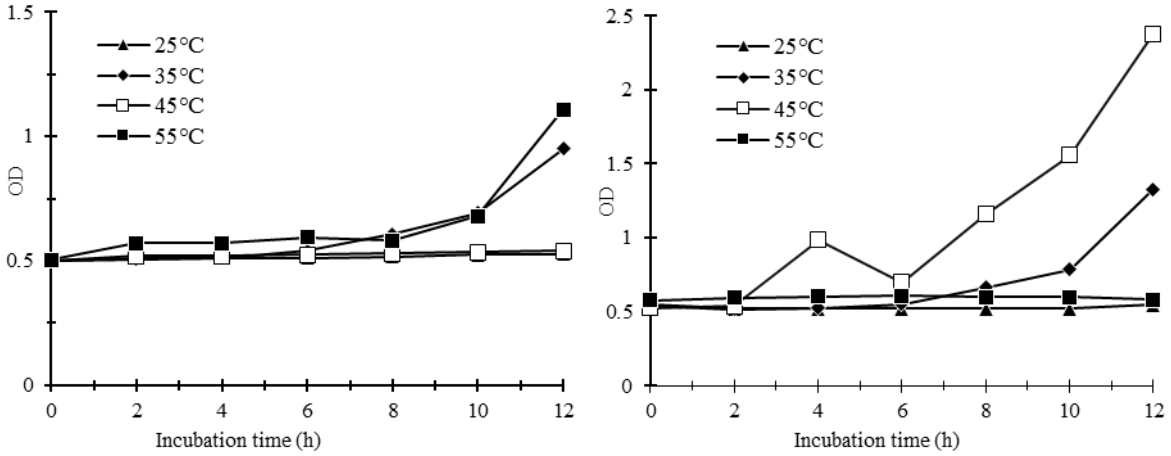

Figure 5: Growth performance of A6B08 (left) and its mutant type MAB18 (right) at pH 5 and four temperature levels

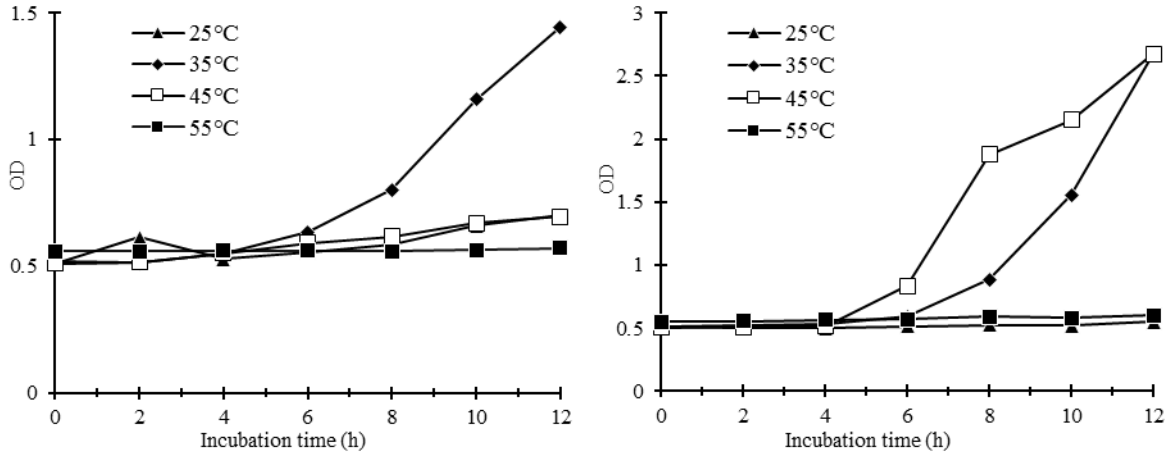

Figure 6: Growth performance of B6S01 (left) and its mutant type MAS03 at pH 5 and four temperature levels

Discussion

The initial screening has led to the rational selection of two mutant types (MAS03 and MA18) and their respective wild types (B6S01 and A6B08). The rest 78 mutant library population were not able to demonstrate growth or better survival. It was suggested that the random mutagenesis induced by ethidium bromide onto the genome of two study mutants has little negative impact on the strains survival. While the 78 mutant populations were unable to perform well compared to the two elite mutant or their wild type parents which could be due to the genomic lesions they sustained. This was confirmed the selected mutants, though lesser in SR values compared to the wild type parents, have shown a measurable survival potential as their wild type parents. However, the rest mutant population were severely affected, hence, segregated, as it was demonstrated with poor stability and SR values. The finding was in accordance with the findings of Saarela et al. [35] in which acid tolerance of two LAB mutants were studied.

The mutant types in this study along with their wild type counter parts were able to endure acidic values as low as 3 and 4. 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 Patnaik et al. [23] 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 Sewunet et al. [26]. However, the survival ratio results recorded at pH 3 and 4 revealed the wild type isolates were slightly better performing than the mutant type isolates. The adaptation potential of the microbes is directly related to shifting their cell

membrane components in response to environmental stress, which was in accordance with the findings and conclusions drawn by related study [36]. In addition, Aline et al. [37] suggested membrane integrity and viability potential at such harsh growth conditions were found to be the most important factors that affect the growth of LAB, supporting the findings made by the current study.

The effect of incubation time, pH (3 and4) and temperature on the survival ratio of the isolates was in accordance with the reports of Sheeladevi et al.[38]. However, the 12th hour survival ratio values excelling the early incubation hours (0, 2 and 4th) are indicative of the fact that the isolates could have resumed and most probably continued to grow had the test incubation time was extended. This could be justified by the findings of Patnaik et al. [23] which suggested the lack of an apparent effect of pH 3 and 4 on survival of the isolates is less justified as the environmental tolerance of pH is a complex and a poorly understood physiological phenomenon. It was observed that, in spite of the low pH environment, there was an increase in growth with an increase in temperature. However, there was a decrease as the temperature exceeds 45 °C, which could be resulted from reduction in the production of stress related molecules at higher temperature values. This finding was supported by a similar study conducted by Asa et al. [39].It was also suggested that the wild type isolate favored the lowest temperature at the lowest pH level, which could be due to it higher potential of producing the cold shock proteins secreted in a pH and temperature stressed environment better than the mutant isolates, as mentioned in the works of the same authors [39].

The growth performance of the mutant isolates at pH values 7, 6 and 5, demonstrated that the mutants developed a potential to proliferate much faster compared to their wild type parents. This result could be attributable to the impact of mutation the mutants acquired during the conventional mutagenesis by ethidium bromide treatment as mentioned in the similar work on LAB [26]. The limiting factors that hinder the growth of Lactic Acid Bacteria at theses pH ranges, are temperature and lactic acid accumulation which is directly proportional to glucose depletion following fermentation by the isolates. This result was in agreement with the works of Sheeladevi et al. [38] which concluded likewise. In this case the mutant isolates were sound to tolerate the mentioned factors and were able to grow faster reaching the highest mean OD as early as the 2nd and 4th h of incubation at 35 and 45°C. This was in contrast to the reports of [40] which stated at these pH conditions the maximum concentration of LAB cells during fermentation decreases at 45°C as the cells start to lose their activity. However, Marzugi et al.[41] reported that lower lag time and strong exponential growth depends on inoculum quality at large. Hence, suggesting that the exceling quality and performance of the mutants relative to their wild type parents could be attributable the mutagenesis procedure and the effective screening and selection scheme adopted as discussed in related works on mutant and wild type LAB [23, 25].

The proliferation of the mutants characterized by strong exponential growth and devoid of a significant lag phase was in contrast to reports of Marzugi et al. and Chramostova et al. [41,42] which suggested such achievement is attributed to the cultivation of cells at 37°C and longer incubation time (17h) compared to the one used in this study. However, in diary fermentation processing in which yogurt is fermented at higher temperatures or close to 43°C. Similarly the processing temperature of most fermented products demand temperature range between 37 to 45°C for maximal microbial action and quality product production as mentioned by Kianoosh et al. [43], consequently, this scenario is usually associated with poorer LAB action. However, the mutants screened and selected were found to experience lower lag phase as low as 2 hours and strong exponential growth even at 45°C. Generally, the improvements and the tendency of not being affected by growth conditions observed has let us to a conclusion that the single or multiple mutations the mutants hosted positively affected one or more genes involved in growth regulation, adaptation, stress tolerance and metabolic pathways. Such positive impact and its detection using effective mutant library screening has long been the core advantage conventional mutagenesis.

Conventional mutagenesis and mutant library screening is therefore proved to be a relatively cheaper, manageable and safer compared to genetic engineering techniques. Mutants generated in this study will guarantee reduced production costs attributable to their strong exponential growth rate (increased cell mass), reduced lag time and stress tolerance. Studies conducted by using this method (conventional mutagenesis and mutant library screening) [8,12,18,22-25,27-31,35,39,44-50] also resulted in similar achievements and the conclusions drawn by the respective reports were in accordance with the ones' made by the current study.

Conclusion

Random mutations incurred during conventional mutagenesis could have multiple impacts both desirable and undesirable. However, careful screening and selection methods could guarantee the maximal harvest of mutants with improved beneficial traits. In this study the two mutant types recovered from a library of 80 mutants proved to have increased growth rate with reduced lag time compared to their wild type parents. Specially their performance at pH 5, 6 and 7 and temperature values of 45 and 55°C will make them preferable and effective in fermentation processes that operate at these conditions. In addition fermentation conditions that demand higher cell density with reduced lag time could be benefiting if these mutant isolates are deployed into action.

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References

1. Lars A., Lactic Acid Bacteria: classification and physiology. 1-67. In: Seppo S. , von W. A. andArthur O., (eds), Lactic acid bacteria: microbiological and functional aspects Marcel Dekker, Inc. , New York, USA, **(2004)**

2. Hammes P.W. and Christian H., The Genera Lactobacillus and Carnobacterium. 320-403. In: Martin D. , Stanley F. , R Eugene , Karl-Heinz S. andErko S., (eds), The Prokaryotes: A Handbook on the Biology of Bacteria Springer Science and Business Media, LLC, USA, **(2006)**

3. Wood J.B. and Holzapfel W.H., The genus Lactobacillus. In: Hammes W. P. andVogel R. F., (eds), The Genera of Lactic Acid Bacteria, Blackie Academic & Professional., UK, London: , **(1995)**

4. Holzapfel W.H. and Wood J.B., The Genera of Lactic Acid Bacteria, London, UK, Publishe **(1995)**

5. Robert W.H., Microbiology and technology of fermented foods, Ames, Iowa, USA:, Publishe **(2006)**

6. Annika M.K. and Marc B., Lactic acid bacteria: microbiological and functional aspects. 249-295. In: Seppo S. , von W. A. andArthur O., (eds), Industrial use and production of lactic acid bacteria, Marcel Dekker, Inc. , New York, USA, **(2004)**

7. Hofvendahl K. , Niel E.V. and Hahn-Haegerdal B., Effect of temperature and pH on growth and product formation of Lactococcus Lactis ssp. Lactis ATCC19435 growing on maltose, Appl Microbiol Biotechnol 51, 669-672, **(1999)**

8. Miguel G. and Sanchez B., Enhancing probiotic stability in industrial processes Microbial Ecology in Health & Disease, 23, 2579-2592, **(2012)**

9. Hofvendahl K. , Niel E.V. and Hahn-Haegerdal B., Effect of temperature and pH on growth and product formation of *Lactococcus Lactis ssp. lactis* ATCC 19435 growing on maltose., Appl. Microbiol Biotechnol., 51(0), 669-672, **(1999)**

10. Luwihan S. , Yulianto W. and Nugraheni E., *Effect of temperature and pH on growth of Lactobacillus acidophillus FNCC 0015 and acceptibility of Noni (Morinda citrifolia) probiotic drink.*, in *The 3rd International Conference of Indonesian Society for Lactic Acid Bacteria: Better Life with Lactic Acid Bacteria: Exploring Novel Functions of Lactic Acid Bacteria*, Faculty of Agricultural Technology, Gadjah Mada University, : Indonesia, Jakarta., 43-46, **(2011)**

11. Siddiqui H. , Masud T. and Muhammad A., Effect of incubation temperature and pH on *Lactococcus lactis ssp*. Diacetylactis and their virulent phages., Pakistan Journal of Biological Sciences, 3(10), 1588-1590, **(2000)**

12. Okafor N., Modern industrial microbiology and biotechnology, United States of America, Publishe **(2007)**

13. Klein-Marcuschamer D. and Stephanopoulos G., Assessing the potential of mutational strategies to elicit new phenotypes in industrial strains, PNAS, 105(7), 2319–2324, **(2008)**

14. Yomano L.P. , York S.W. and Ingram L.O., Isolation and characterization of ethanol tolerant mutants of Escherichia coli KO11 for fuel ethanol production, J Ind Microbiol Biotechnol 20, 132-138, **(1998)**

15. Du C. , Zhang Y. , Li Y. and Cao Z., Novel redox potential-based screening strategy for rapid isolation of Klebsiella pneumoniae mutants with enhanced 1,3-propanediolproducing capability, Appl Environ Microbiol 73(14):4515-4521, **(2007)**

16. Stephanopoulos G., Metabolic engineering by genome shuffling, Nat Biotechnol 20, 666-668, **(2002)**

17. Leroy F. and Vuyst L.D., Functional lactic acid bacteria starter cultures for the food fermentation industry, Trends in Food Science & Technology 15(2004), 67-78, **(2003)**

18. Sarad P., Strain improvement, San Diego, California, USA, Publishe **(2004)**

19. Wilbert S. , Jeroen H. , Willem M.V. and Eddy J.S., Safe use of genetically modified Lactic Acid Bacteria in food: Bridging the gap between consumers, green groups, and industry Electronic journal of biotechnology, 9(4), 424-448, **(2006)**

20. Anderson T.M., Encyclopedia of microbiology. In: Moselio S., (eds), Industrial Fermentation Processes, Elsevier Academic Press. , San Diego, California, USA, **(2009)**

21. Sanchez B. and Margolles A., Selection of a low-acetate producing Bifidobacterium lactis subsp. lactis strain, Appl Environ Microbiol, 78, 3338-3342, **(2012)**

22. Parekh S. , Vinci V.A. and Strobel R.J., Improvement of microbial strains and fermentation processes, Appl Microbiol Biotechnol 54, 287-301, **(2000)**

23. Patnaik R. , Louie S. , Gavrilovic V. , Perry K. , Stemmer W.P.C. , Ryan C.M. and Cardayre S.d., Genome shuffling of for improved acid tolerance, NatBiotechnol, 20(7), 707-712, **(2002)**

24. Sheelendra M.B. and Suresh K.S., High yield of Lactic acid production by Mutant Strain of L. delbrueckii U12-1 and Parameter Optimization by Taguchi methodology, Annals of Biological Research, 3(6), 2579-2592, **(2012)**

25. Wang Y. , Li Y. , Pei X. , Yu L. and Feng Y., Genome-shuffling improved acid tolerance and L-lactic acid volumetric productivity, JBiotechnol., 129(3), 510-515, **(2007)**

26. Sewunet A. , Melaku A. and Ameha K., The effect of ethidium bromide mutagenesis on morphological characteristics and physio - biochemical performances of Lactic Acid Bacteria isolated from traditionally fermented Ethiopian cow milk, Int J Pharm Bio Sci, 7(1), 183-192, **(2016)**

27. Ismail S. , Ahmed E. and Willard B., Mutagenesis by ethidium bromide and N-methyl-N'-nitro-N-nitrosoguanidine in off-flavour compound producing strains of Streptomyces, J. Biosci. , 23(5), 595-600, **(1998)**

28. Sebastian M. , Sri W. , Retno I. and Ending S.R., Mutation technique for increasing the production of antibacterial Lactobacillus plantarum PGR-2, Indonesian food and nutrition progress., 5(2), 73-78, **(1998)**

29. Yu L. , Pei X. , Lei T. , Wang Y. and Feng Y., Genome shuffling enhanced Llactic acid production by improving glucose tolerance *Lactobacillus rhamnosus*, J. Biotechnol., 134(12),154-159,**(2008)**

30. Nikolaos E., Random Mutagenesis Methods for In Vitro Directed Enzyme Evolution, Current Protein and Peptide Science, 11(1), 91-100, **(2010)**

31. Ismaila S. , Sudi D. , Nanditta D. and Ali-Dunkrah U., Mutagenesis and Selection of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* for Potential use as Starter Culture, The Journal of American Science, 4(3), 80-87, **(2008)**

32. Sebastian M. , Sri W. , Retno I. and Ending S., Mutation technique for increasing the production of antibacterial *Lactobacillus plantarum* PGR-2, Indonesian food and nutrition progress, 5(2), 73-78, **(1998)**

33. Gibson G.R. , Rouzaud G. , Brostoff J. and Rayment N., *An evaluation of probiotic effects in the human gut: microbial aspects*, in *Final Technical report for FSA project ref G01022*, Food Standards Agency United Kingdom, 1-22, **(2005)**

34. Suree N. , Saranya P. and Thitirut J., Screening and identification of lactic acid bacteria from raw seafoods and Thai fermented seafood products for their potential use as starter cultures, Songklanakarin, J. Sci.Technol, 34(3), 255-262, **(2012)**

35. Saarela M.H. , Alakomi H.L. , Matto J. , Ahonen A.M. and Tynkkynen S., Acid tolerant mutants of Bifidobacterium animalis subsp. Lactis with improved stability in fruit juice, Food Sci. Technol., 44, 1012-1018, **(2011)**

36. Hossein N. , Stenzel M. and Britz D., Effect of growth at low pH on the cell surface properties of a typical strain of *Lactobacillus casei* group, Iranian journal of microbiology, 2(3), 144-151, **(2010)**

37. Aline R. , Marielle B. and Catherine C., Fermentation pH influences the physiological-state dynamics of *Lactobacillus bulgaricus* CFL1 during pH-controlled culture, International journal of microbiology, 2(0), 80-110, **(2011)**

38. Sheeladevi A. and Ramanathan N., Lactic acid production using Lactic Acid Bacteria under optimized conditions, International Journal of Pharmaceutical & Biological Archives, 2(6), 1686-1691 **(2011)**

39. Asa L. and Torkel W., Lactic Acid Bacteria as probiotics, Current issues on intestinal microbiology. , 7(0), 73-90, **(2005)**

40. Goksungur Y. and Guvenc U., Continuous Production of Lactic Acid from Beet Molasses by *L. delbrueckii* IFO 3202, J. of Chemical Eng. Biotechnology, 69, 399-404, **(1997)**

41. Marzugi N.E., Elenshasy H.A., AbdMalek R., Othman Z., Sarmidi M.R. and Abdul-Aziz R., Optimization of cell mass production of the probiotic strain Lactobacillus lactis in batch and fed-batch culture in pilot scale levels. 873-79. In: Medez-Vilas A., (eds), Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, Formatex Research Centre, Badajoz, Spain **(2010)**

42. Chramostova J., Romana M., Ivana L., Erik P., Jan D. and Irena N., Influence of Cultivation Conditions on the Growth of Lactobacillus acidophilus, Bifidobacterium sp., and Streptococcus thermophilus, and on the Production of Organic Acids in Fermented Milks, Czech J. Food Sci., 32(5), 422-429, **(2014)**

43. Kianoosh K., Parinaz T. and Negin A., Effect of Process Variables on the Probiotic and Starter Culter Growth in Sinbiotic Yogurt Beet, RRJFPDT, 3(2), **(2015)**

44. Betoret E., Betoret N., Vidal D. and Fito P., Functional foods development: trends and technologies, Trends Food Sci. Technol. 22(0), 498-508, **(2011)**

45. Chand P., Aruna A., Maqsood A. and Rao V., Novel mutation method for increased cellulase production, J. Appl. Microbiol., 98(2), 318-23, **(2005)**

46. Michaeel B., Kees B. and Lubbert D., Mutational analysis of exopolysaccharide biosynthesis by Lactobacillus sakei 0-1, FEMS Microbiology Letters 169(1998), 241-249, **(1998)**

47. Nomoto K., Kiwaki M. and Tsuji H., Genetic modification ofprobiotic microorganisms, Hoboken, New Jersey, USA, Publishe **(2009)**

48. Sadia J., Muhammad A., Munir A. and Haq N., Strain improvement through UV and chemical mutagenesis for enhanced citric acid production in molasses-based solid state fermentation, Food Biotechnol., 24(2), 165-179, **(2010)**

49. Zhang D.X., Perry K., Vinci V.A., Powell V.A., StemMer W.P.C. and Cardayre S.D., Genome shuffling leads to rapid phenotype improvement in bacteria, Nature, 415(6872), 644-646, **(2002)**

50. Francoise L., Papa F., Marie P., Frederique C. and Regis B., Influence of temperature, pH and NaCl concentration on the maximal growth rate of Brochothrix thermosphacta and a bioprotective bacteria Lactococcus piscium CNCM I-4031, Food Microbiology, 31(12), 222-228, **(2012)**