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THE EFFECT OF ETHIDIUM BROMIDE MUTAGENESIS ON MORPHOLOGICAL CHARACTERISTICS AND PHYSIO - BIOCHEMICAL PERFORMANCES OF LACTIC ACID BACTERIA ISOLATED FROM TRADITIONALLY FERMENTED ETHIOPIAN COW MILK

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ABSTRACT

Mutagenesis has been used to generate highly productive and stress tolerant variants of microbes. In this study a random mutagenesis using a chemical mutagen, ethidium bromide, was carried out on Lactic Acid Bacteria (LAB) isolated from Ethiopian traditionally fermented cow milk. Twenty colonies of LAB were isolated and out of which two isolates (B6S01 and A6B08) with least mean pH scores were selected and characterized to genus level. After being mutagenized with four different concentrations of ethidium bromide (0.5, 1.0, 1.5 and 2.0 $g.L^{-1}$), 80 survivors were selected and screened. Accordingly, four mutants with the least mean pH scores were selected and characterized. The two wild type isolates, (B6S01 and A6B08) with mean pH 4.50 ± 0.03 and 4.52 ± 0.02 respectively, were identified as *Lactobacillus spp*. and *Lactococcus spp* respectively. The mutagenesis affected the sugar fermentation pattern and acidification potential of the mutants. The mutant types MAS03 and MAB18 were now able to ferment arabinose and fructose, and galactose respectively. Lower mean pH values were associated with ethidium bromide at 0.5 $g^{-1}L$ and 1.5 gL^{-1} concentration significantly ($p<0.01$). However, ethidium bromide at 2.0 gL^{-1} concentration resulted the least pH value, 4.20 ± 0.03, on the mutant MAB37. The effectiveness of the mutagenesis was demonstrated by better acidification potential and unique array of sugar fermentation. Utilization of these identified mutants in dairy processing could reduce production cost and enhance productivity.

KEY WORDS: *Acidification; Characterization; Ethidium Bromide; Lactic Acid Bacteria; Mutagenesis. Screening*

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INTRODUCTION

Mutagenesis has been used to generate improved, highly productive and stress tolerant strains benefiting the humankind and ensuring its welfare. Particularly in the food processing, pharmaceutical, agricultural and industrial sectors, the application of improved mutant variants revolutionized the conventional production and processing schemes. The apparent boost in the quality and quantity of microbial products harvested (e.g. Lactic acid, Insulin, antibiotics etc) and the reduction in production cost proved the effectiveness of mutagenesis[1, 2, 3]. There are many kinds of mutagenesis techniques such as Random mutagenesis, PCR based genome shuffling and side directed mutagenesis, transformation of any type, genetic engineering etc. Among these random mutagenesis techniques employing physical and chemical mutagens are advantageous in food processing, as their resulting mutant types are of food grade, Generally Regarded as Safe (GRAS). Hence, alleviating the public from the fear associated with the safety of genetically modified organisms (GMO) based products for consumption [4], [1], [5]. Reports have indicated that random mutagenesis using physical mutagens (Ultraviolet and Gamma irradiation) and chemical mutagens like ethidium bromide (EB), N-Methyl-N'-Nitroso-Guanidine (NMNNG) and others were found effective in generating improved LAB and other microbes [6, 7, 8, 10]. Moreover, different authors demonstrated the potential of improved mutants for their tolerance to stressed growth environments and higher potential for increased production of secondary metabolites and other desirable metabolic activities [6, 7, 8, 9, 10]. 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. However, numerous environmental factors limit their metabolic activities and deteriorate their growth. Growth conditions characterized by relatively higher or lower temperatures and mediums with acidic pH were associated with reduced cell biomass yield [11]. Similarly, [12] asserted that lower enzyme activity during lactic acid production is associated with reduced bacteriocin production. It was also reported that LAB susceptibility to phage attack is higher at lower pH and higher temperatures values [13]. Isolation, screening and selection of LAB to attain better performing isolates applicable to fermentation and other industrial processes have been practiced for long period of time. However, due to the resource intensiveness and technical difficulties mutagenesis was sought to be a shortcut to serve the same cause [14, 5]. Apart from other mutagenesis techniques (PCR based and genetic engineering), have been widely used to generate food grade and GRAS LAB that enhance productivity at lesser costs [1, 14]. Ethiopia holds tremendous potential for dairy development and production. Currently, it hosts the largest livestock population in the African continent. The total cattle population of the country is estimated to be 53.99 million of which, the female cattle constitutes 55.48 % of the total [15]. It was also estimated that, in Ethiopia about 3.804 billion liters of cow milk is produced annually [15]. However, the dairy processing is generally limited to fermentation of raw milk to produce traditional milk products such as *ergo* (locally fermented milk in Ethiopia), *kibe* (butter), and *ayib* (cheese). These products are produced using traditional techniques without a defined starter culture. Rather, through spontaneous fermentation or back slopping, whose product is neither predictable nor controllable [16, 17]. Consequently, there is a great need to isolate, screen, characterize and measure the performance of local LAB in fermented milk products for the development of starter culture that will endure the traditional fermentation process in Ethiopia [17, 16]. There is a potential to develop starter cultures that function effectively in varying fermentation conditions by screening form naturally occurring potent isolates. However, it will be resource intensive and technically difficult to manage collection, storage, analysis and inference of huge number of isolates and microbial culture [14]. Considering the poor economic status of the country, importing commercial starter cultures from abroad and delivery to households will not be feasible. Taking into account, the mentioned ground facts it is crucial to identify relatively better performing LAB isolates and enhance their starter function using mutagenesis. This approach will not only be successful in screening and achieving better performing isolates from locally available LAB, but also generates variants with enhanced productivity. Adopting such a feasible, resource effective and none hi-tech infrastructure demanding biotechnological tool in Ethiopia and other developing countries will pave the way towards better productivity in the dairy processing and other sectors [10, 5]. Mutant screening techniques that considered the traditional processing scheme and uncontrolled environment they will be operating in will guarantee a better quality product and a step forward to a modernized processing industry [18, 5].Therefore, this study was conducted with objectives to isolate better performing LAB from traditionally fermented cow milk, mutagenize the respective isolates and screening them for enhanced traits. As an ultimate goal, generate better performing mutant variants compared to their wild type parents that will improve the traditional dairy processing sector of Ethiopia.

MATERIALS AND METHODS

Isolation of Lactic Acid Bacteria

A total volume of 1.2 L milk sample milked from healthy cows was collected from Holetta Agricultural Research Center (HARC) and Biftu Berga Dairy Production and Processing Farmers Cooperative Union (BBDPPFCU) at Holetta. One hundred milliliter milk sample was randomly collected from 12 jars (6 samples from each collection site) using clean and detergent free 250 mL glass bottles. The contents of the bottles were mixed and homogenized in 2 L glass beaker by gentle shaking. The beaker was sealed with aluminum foil and tightened with scotch tape. After two days (48 h) of fermentation the curd was dispersed by gentle stirring as it is practiced traditionally. Following a tenfold serial dilution, 100 µL of the contents

of the tube sample was spread plated on de Man Regosa and Sharpe (MRS) agar (pH 6.0 ± 0.2 at 25°) (Fluka Analytical, India) and M17 agar (pH 6.9 ± 0.2 at 25°C) (Oxoid, UK), both supplemented with 0.5% (w/v) $CaCO_3$ (Merck, Germany) to differentiate acid producers from the rest. After 48 h of aerobic incubation at 37°C, the resulting colonies were counted using colony counter from plates bearing 30 to 300 c.f.u.mL^{-1} [19]. A total of twenty colonies bearing the very features of LAB and with visible clearance zone (resulting from the reaction of acid produced and $CaCO_3$) were picked (Lars, 2004). The isolates were re-cultured on the respective media until pure (uniform) colonies were maintained. After ensuring the purity of the isolates their respective agar slant cultures of MRS and M17, and broth cultures of MRS with glycerol were prepared for long and short term preservation at -80 °C and 4°C respectively.

Screening isolates for pH reduction ability

Hundred micro liters of standardized fresh (18 h culture) broth cultures with OD_{600nm} adjusted to 0.1 to 0.5, corresponding to McFarland standard of 0.5 and CFU ml^{-1} of 10^5 to 10^8, were inoculated into 5 mL MRS broth (pH 6.0 ± 0.2 at 25°C) incubated for 48 h at 37°C [20, 21]. Broth pH measurements were conducted on the 24th and 48th h of incubation.

Ethidium Bromide (EB) mutagenesis and screening of mutants

Mutagenesis was carried out according to the method of [10] with modifications. Two hundred micro liters of standardized fresh cultures of the isolates were used to inoculate 10 mL of MRS broth in 8 falcon tubes and grown their late logarithmic phase at 37ºC. Cells were harvested by centrifugation 10,000 rev.min^{-1} for 10 min at 4°C and the sediment was washed twice by re-suspending it in equal volume of normal saline (10 mL) followed by centrifugation at the same speed and length of time. The washed pellets of the two isolates in 8 separate tubes were then re-suspended in 10 mL of ethidium bromide solution with concentration of 0.5, 1.0, 1.5 and 2.0 g.L^{-1} and incubated at room temperature on shaker (120 rev.min^{-1} for 30 min). Cells were washed three times, re-suspended in normal saline and serially diluted. After spread plating on nutrient agar (Oxoid, India), plates were incubated for 48 h aerobically at 37°C. For each of the two parental lines, 10 colonies from each of the four respective EB concentrations were randomly picked from plates bearing colony counts of 50 to 100 c.f.u. mL^{-1} [3],i.e. [(2 parental lines) (4 concentration levels of EB) (ten colonies each) = a total of 80 colonies)]. The mutants were screened for their pH reduction ability in MRS broth by measuring the pH at 24th and 48th h of incubation. The best performing four mutants were selected and characterized.

Morphological characterization of the wild and mutant types

Cell morphology

Gram reaction was used to determine the morphology, arrangement and gram reaction pattern of the isolates following the method suggested by [19].

Colony morphology

The relative colony size, shape and color of the isolates were determined after culturing the purified isolates on MRS and M17 media [22].

Biochemical Characterization of the wild and mutant types

Catalase test

Using sterile inoculating loop, samples were taken from their respective slants and mixed thoroughly with 2 to 5 drops of 3% (v/v) H_2O_2 on clean grease free microscope slides. The slides were examined for the presence or absence of bubble formation [19].

Carbohydrate fermentation

Carbohydrate fermentation was performed in MRS broth (Fluka Analytical, India) containing glucose (20 gL^{-1}), which served as positive control, and re-constituted MRS broths each containing each of the 12 test sugars (sorbitol, galactose, xylose, sucrose, maltose, arabinose, cellulose, inositol, rhamnose, fructose, mannitol and lactose) at a rate of 20 gL^{-1}. Bromocresol purple (BCP) (Riendel De-Haen, Germany) was added (0.02 gL^{-1}) as pH indicator to detect acid production following the fermentation of the respective sugars. BCP has a purple alkaline color and a yellow acidic color within pH range of 6.8 to 5.2 [19]. Inverted Durham's tubes were inserted to detect gas (CO_2) production [23, 24, 19]. Hundred micro litters standardized (OD = 0.1 – 0.5) fresh cultures of the isolates were inoculated into 5 mL^{-1} MRS broth and were incubated at 37°C for 48 h aerobically. Non-inoculated MRS broths with BCP were used as a negative control. Following the incubation, the tubes were examined for broth color change, turbidity and gas accumulation in the inverted Durham's tubes.

Hydrogen sulfide and gas production from glucose fermentation

The hydrogen sulfide and gas production from glucose fermentation test was carried out using Triple Sugar Iron Agar (TSI) (pH 7.4 ± 0.2 at 25°C) (Himedia, India) with phenol red pH indicator. Phenol red (pH range 8.4 to 6.8) changes color from red (alkaline color) to yellow (acidic color) following the acidification of the due to the fermentation of the sugars incorporated (sucrose, lactose and glucose) [19]. The butt and the slant of the media were stabbed and streaked respectively with 100 µL of standardized fresh broth cultures. Non inoculated TSI agar slants were used as negative controls. After incubation at 37°C for 48 h aerobically the tubes were examined for cracking, color change and blackening, traits attributable to gas production, acid production and H_2S production respectively [24, 19].

Physiological Characterization

Growth at cardinal temperatures (15 and 45°C) test for the wild type isolates

Growth at cardinal temperatures, 15 and 45°C [22] was carried out in MRS broth containing BCP. Hundred micro liters of standardized fresh broth cultures were used to inoculate 5 mL^{-1} broth and cultures were incubated at 37°C for 24 h. Growth was monitored by visually inspecting changes in color to BCP and turbidity.

Salt tolerance of the wild and mutant types

Three levels of salt (NaCl) concentration (2, 4 and 6.5 $g.L^{-1}$) were used to determine salt tolerance in MRS broth supplied with 0.02% (w/v) BCP. Five milliliters of broths were inoculated with 100 μL of fresh and standardized broth cultures. The tubes were incubated at 37°C for 24 h and tolerance was assessed if the indicator changes color (purple to yellow) and turbidity due to bacterial mass (precipitate or flocculate) is detected. Salt free MRS broth inoculated with the isolates and culture free MRS broth with the respective salt concentrations were used as positive and negative control respectively [24].

Identification of the wild type isolates

Identification of the wild type isolates was made by considering the morphological, biochemical and physiological characterization test results and classification of the isolates was made to genus level. The main activities involving characterization (morphological and biochemical) and pH reduction tests were all run in three replications both for the wild type isolates and mutant types. Positive and negative controls were also included in the same manner when feasible.

Statistical analysis

A factorial Completely Randomized Design (CRD) was implemented to determine the effect of isolate, incubation time and the mutagen's (ethidium bromide) concentration on MRS broth pH reduction. Generalized Linear Model (GLM) was implemented and Tukey's post-hoc test was used for mean separation. All the data were analyzed using SAS version 9.2.

RESULTS

An average bacterial population of 4.9×10^8 c.f.u. mL^{-1} (log = 8.683**)** and 6.73×10^8 c.f.u.mL^{-1} (log **=** 8.825) was counted on MRS and M17 agar plates respectively. Out of the twenty isolates randomly picked, two isolates, B6S01 and A6B08, reduced the MRS broth pH (6.2 ± 0.2 at 25°C) to mean pH of 4.50 ± 0.03 and 4.52 ± 0.02 respectively after 48 h of fermentation; hence they were selected for further study and mutagenesis (Table 1).

Mutagenesis, screening and selection of the mutant isolates

After being mutagenized with four levels of EB concentration, the resulting mutant types of were initially screened for pH reduction potential in MRS broth (pH 6.2 ± 0.2 at 25°C). Some of the mutants exhibited faster growth compared to their parental lines and other mutant types. This was confirmed by the rapid yellowish discoloration of the broth and development of highly dense turbidity. Lower mean pH reduction results were observed associated with 0.5 $g.L^{-1}$ and 1.5 $g.L^{-1}$ EB treatments (Table 2 and Fig. 1). However, mutant MAB37 survived the 2.0 $g.L^{-1}$ EB treatments with the lowest observed pH reduction value of 4.20 ± 0.03. The other mutant type MAB18 survived the 0.5 $g.L^{-1}$ EB treatment with lowest pH reduction point of 4.24 ± 0.05. These low pH-scoring isolates were the mutant types of the A6B08's parental line. Among the mutants of B6S01, MAS03 and MAS28 were the two best performing mutant type isolates that scored the least mean pH reduction values, 4.34 ± 0.12 and 4.37 ± 0.05, respectively. These mutant isolates were product of 0.5 and 1.5 $g.L^{-1}$ EB treatment of their respective wild type parent. As it was observed in the wild type isolates (Table 1), least pH reduction scores of the mutants were observed at 48^{th} h of incubation compared the 24^{th} h one. Considering the lowest pH reduction test values they scored, MAB37 and MAB18 descendants of the wild type A6B08, and MAS03 and MAS28, the mutant types of B6S01 were selected and characterized.

Morphological and biochemical characterization of the wild and mutant type isolates

Colonies both on MRS and M17agar plates, all appeared smooth, raised convex, circular and creamy (milky) white in color with a relatively slight size difference (very small to large). The Gram staining test result indicated both wild type isolates were gram-positive but with different cellular shapes. The isolate B6S01 was found to be rod (bacilli) shaped and with cell arrangement pattern ranging from dispersed single cells to forming short bacilli chains. It also forms of small clusters but large clusters were rare. Whereas, A6B08 was found to be cocci shaped and its cellular arrangement ranges from often dispersed single cells to dyads and tetrads. It was also appeared to form small and large dense clusters and rarely seen to form short to long chains (chains of 8 - 10 cells seldom seen). The cell morphology, gram-staining pattern and the colony morphology of the mutants remained unaltered, bearing relatively similar appearances of their respective wild type parents. However, the mutant types were observed to retain crystal violet firmly and appeared relatively darker than their respective wild type parents. The occurrence persisted even under relatively extended washing (rinsing) to decolorize the stain (crystal violet). The cocci shaped wild type, A6B08, was able to ferment and grow only in broth supplied with common hexoses and disaccharides in dairy products and food (glucose, lactose, maltose and sucrose). Moreover, its ability to ferment arabinose is unique to it and the B6S01's mutant type MAS03. With the exception to arabinose, the rod (bacilli) shaped wild type isolate, B6S01 was able to ferment all the sugars A6B08 could ferment. In addition, it could ferment the common sugar in milk products, galactose, and rare sugars in food and dairy products (sorbitol, inositol and rhamnose). However, none of the wild type isolates were capable of fermenting the most

abundant complex carbohydrate cellulose in addition to xylose and fructose. In turn, the mutant type isolates demonstrated varying patterns of sugar fermentation relative to their wild type parents. The mutant MAS03 has gained a new potential of fermenting arabinose and fructose unlike its wild type parent, B6S01. Similarly, the other mutant, MAB18, has gained a new potential of fermenting galactose which its wild type parent, A6B08 could not utilize; nonetheless, it has lost the ability to ferment manitol and arabinose (Table 3). Similarly, MAB37, the mutant type of A6B08 lost the ability to utilize manitol and arabinose. MAS28, other mutant type of B6S01, was unable to ferment the hexose sugars (sorbitol, galactose and manitol) and the rare sugars in food and dairy products (inositol and rahamnose) compared to its wild type parent. However, like their wild type parents, none of the mutants were capable of fermenting cellulose and xylose. Even though they could ferment one or more of the sugars incorporated in the TSI medium, none of the wild and mutant type isolates produced gas and hydrogen sulfide. This was confirmed by yellowish discoloration of the purple media with no blackening and cracking which could have been a clue for hydrogen sulfide and gas production respectively. The salt tolerance result indicated that B6S01 was able to grow intensively in all salt levels (2, 4 and 6% w/v) whereas the cocci shaped isolate (A6B08) could only tolerate 2% (w/v) NaCl concentration.

Identification of the isolates to a genus level

The results of morphological (cell and colony), biochemical and physiological tests in the current study is presented in table 4. The two isolates where identified to their respective genus and this result is in line with different authors [25, 26, 27, 28, 29]. Accordingly, the cocci isolate A6B08 belongs to *Lactococcus spp.* while the rod shaped isolate, B6S01, belongs to *Lactobacillus spp*.

DISCUSSION

The average LAB population determined in the traditionally fermented milk sample was in agreement with previous reports of [30, 31]. However, the low count of the *Lactobacillus spp*. observed in the present study could be due to the fact that the microbial population of milk product fermented for days less than five and at lower temperatures including room temperature (~25°C) is predominated by elevated number of *Lactococcus spp.* [30, 31]. The morphological characteristics of the wild type isolates were found to match the typical colony and cell morphology features of LAB reported by [32]. Similarly, the biochemical characteristics were in accordance with previous reports [25, 26, 27, 28, 29]. Morphologically the mutants maintained the colony and cell appearance of their wild type parents. However, the manner they retain crystal violet firmly could be the effect that mutagenesis posed on their membrane integrity and repair system. The MRS broth pH reduction test revealed mutant LAB variants with significantly ($P< 0.01$) better acidification potential compared to their respective naturally occurring parental lines. This was sought to be attributable to the enhanced growth rate of the mutants and their enhanced catabolic ability in converting the sugar in the medium (glucose) to lactic acid [33] yet without being affected by the developing acidic environment compared to their parental lines. The adaptation and proliferation potential of microbes in stressed or in gradually stress developing environments is directly correlated with the shifting in their cell membrane components and the types and levels of expression of stress induced proteins involved in the balance, regulation and maintenance of membrane integrity [25, 34, 35]. Thus, it was suggested that mutation experienced by the regulatory genomic regions responsible for the mentioned traits (better to state the traits) resulted in improved expression of the stress induced protein and other genes involved in the mentioned process either directly. In addition, it may also affected the metabolic and physiologic mechanisms by strengthening the stress tolerance capability of the mutant variants. Previously conducted studies on Ethiopian fermented dairy products indicated that the average least pH observed on spontaneously fermented cow milk was 4.3. The figure did not exclude the contribution of coli forms, which could ferment lactose to acid (by coding for beta-lactamase), hence contributing to the reduced pH of the sour milk samples [30, 36]. Therefore, adopting the technique used in this study and considering the resulting mutant variants with improved acidification potential in the traditional fermentation process will lead to a boost in product harvest and safer product by eliminating threats posed by pathogenic and spoilage microbes [5]. The mutagenesis did not affect the main metabolic pattern the mutants utilize carbohydrates; hence they remained homofermentive like their wild type parents. However, the unique positive (gain of new trait not detected in the wild type parent) and negative (loss of a trait active in the wild type parent) features observed in sugar fermentation pattern were in accordance with the report of [7]. In general, the loss of existing traits and the gain of new ones, is attributable to the effect of mutagenesis on multiple genes involved in the biosynthesis of enzymes used for the utilization of the sugars or regulatory mechanisms associated with metabolic pathways [33]. But specifically, according to the detailed sugar (carbohydrate) metabolism regulation systems presented in [37], utilization of sugars in growth medium is regulated primarily by global regulatory proteins such as Catabolic control protein A (CcpA) and the ATP dependent HPr protein. These regulatory proteins play a crucial role in an inducer-exclusion mechanism and carbon catabolite repression, which controls the major catabolic pathways and glycolytic flux. CcpA protein conjugated with the co repressor form of HPr (Ser-P-HPr) was found to bind specific DNA sequences called catabolite responsive elements (*cre*). *Cre* sites could be located within the promoter regions or transcription initiation sites overlapping with catabolite regulated genes. The binding to CcpA in these regions, depending on where it did, acts an activator or a repressor promoting Carbon Catabolite Activation (CCA) or repression (CCR), respectively [37, 38]. Therefore, it was

suggested that the mutagenesis procedure in this study affected the biding sites of CcpA turning it to CCA or CCR, initiating or repressing the synthesis catabolic enzymes wherein the mutant isolates gained new potential of fermenting sugars or lost their catabolic traits respectively. In addition, specific lines of local regulatory proteins (LacI, LysR, AraC, GntR, DeoR, BglG etc.), found widely distributed among the genome of the LAB genera affect the catabolic fate of sugars in the medium. The effect of these regulatory proteins was proved to determine the level of expression of genes involved in fructose, galactose and xylose assimilation in lactococci [37, 38]. Taking into account this fact, it was suggested that one or more of the mentioned regulatory proteins might have been affected positively or negatively by the mutagenesis, especially in isolates that have lost or gained traits to ferment galactose and fructose (MAB18, MAS38 and MAS03). The simple and feasible mutagenesis technique utilized in this study generated mutants that could utilize galactose, the main sugar responsible for 'post-acidification' in dairy based fermented products like cheese and yoghurt [4]. Post-acidification is a condition where by the fermented dairy product will sustain continued acidification due to the fermentation of leftover sugar (mainly lactose and galactose) to lactic acid. The phenomenon results in 'browning' of cheese and textural changes in yoghurt. Most LAB involved in fermentation of milk take in lactose and utilize glucose while excreting galactose [4, 39]. Attempts were made to engineer LAB which could metabolize lactose or galactose to complete or nearly complete acidification or rerouting the metabolic path towards the production of more pH-neutral components like alanine [4, 40]. However, the use of the mutant variants generated by this study (MAB18 and MAS03) in the processing scheme will alleviate the mentioned problem and will contribute in towards longer shelf life of dairy products still maintaining the desired textural and sensory parameters [4]. The statistical incoherence observed between the degree of effect and concentration of EB was in contrast with the recommendations given by [7] that higher concentration could result in greater effect and generate better performing mutants. The randomness of impacts (positive, negative and null) of the mutagenesis procedure posed on the performance the mutant type isolates relative their wild types confirmed the unpredictability of induced random mutagenesis [41].

Table 1
Mean 24 h and 48 h pH reduction test result of the isolates

No.	Isolate	Mean pH ± S.D of MRS Isolates		No.	Isolate	Mean pH ± S.D of M17 Isolates	
		24 h incub.	48 h incub.			24 h incub.	48 h incub.
1	A6B01^{d*}	6.40 ± 0.02	5.51 ± 0.02	11	B6S01^{d}	4.64 ± 0.01	4.50 ± 0.03
2	A6B02^{a}	6.36 ± 0.04	7.55 ± 0.01	12	B6S02^{bcd}	4.74 ± 0.19	4.60 ± 0.13
3	A6B03^{f}	4.83 ± 0.03	4.60 ± 0.00	13	B6S03^{cd}	4.74 ± 0.04	4.52 ± 0.02
4	A6B04^{c}	6.38 ± 0.04	6.65 ± 0.04	14	B6S04^{bcd}	4.80±0.024	4.54 ± 0.01
5	A6B05^{c}	6.50 ± 0.00	6.50 ± 0.02	15	B6S05^{bc}	4.93 ± 0.03	4.63 ± 0.03
6	A6B06^{b}	6.43 ± 0.08	6.85 ± 0.05	16	B6S06^{b}	4.80 ± 0.05	4.77 ± 0.02
7	A6B07^{b}	6.50 ± 0.03	6.68 ± 0.01	17	B6S07^{bc}	4.77 ± 0.02	4.74 ± 0.02
8	A6B08^{g}	4.68 ± 0.02	4.52 ± 0.02	18	B6S08^{a}	5.18 ± 0.08	4.92 ± 0.02
9	A6B09^{e}	5.02 ± 0.02	4.77 ± 0.02	19	B6S09^{bc}	4.80 ± 0.00	4.65 ± 0.01
10	A6B10^{c}	6.46 ± 0.03	6.48 ± 0.01	20	B6S10b^{cd}	4.70 ± 0.03	4.64 ± 0.02
MSD**		0.058		**MSD**		0.154	

***Where: *abcdefg**, Means of isolates with the same letters are not significantly different at α=0.05,*
****MSD = Minimum Statistical Difference, incub = Incubation***

Table 2
Mean pH reduction values of mutant types following ethidium bromide (EB) treatment

No.	Parental lines	EB (% w/v) treatment	Sub-Grand Mean pH ± S.D. of Mutant types	
			24 h incubation	48 h incubation
1	A6B08	0.5^{a}	4.483 ± 0.284	4.452 ± 0.308
2		1^{c}	5.043 ± 0.481	5.043 ± 0.326
3		1.5^{b}	4.625 ± 0.243	4.395 ± 0.196
4		2^{d}	4.893 ± 0.519	4.68 ± 0.393
5	B6S01	0.5^{a}	4.695 ± 0.330	4.37 ± 0.131
6		1^{c}	5.193 ± 0.373	4.891 ± 0.335
7		1.5^{b}	5.087 ± 0.438	4.997 ± 0.431
8		2^{d}	5.324 ± 0.223	5.106 ± 0.165
R^2		0.978479		
CV(%)		1.513221		

Where:: abcd* letters of the same type have no statistically justified difference

Table 3

Sugar fermentation results of the wild type parents and their respective mutant types

No.	Sugar	A6B08	Mutant types of A6B08		B6S01	Mutant types of B6S01	
			MAB37	MAB18		MAS28	MAS03
1	Sorbitol	- ve	- ve	- ve	+ ve	- ve	+ ve
2	Galactose	- ve	- ve	+ ve	+ ve	- ve	+ ve
3	Xylose	- ve	- ve	- ve	- ve	- ve	- ve
4	Sucrose	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
5	Maltose	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
6	Arabinose	+ ve	- ve	- ve	- ve	- ve	+ ve
7	Cellulose	- ve	- ve	- ve	- ve	- ve	- ve
8	Inositol	- ve	- ve	- ve	+ ve	- ve	+ ve
9	Rhamnose	- ve	- ve	- ve	+ ve	- ve	+ ve
10	Fructose	- ve	- ve	- ve	-ve	- ve	+ ve
11	Mannitol	+ ve	- ve	- ve	+ ve	- ve	+ ve
12	Lactose	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
13	Glucose*	+ ve	+ ve	+ ve	+ ve	+ ve	+ ve
14	Negative control**	- ve	- ve	- ve	- ve	- ve	- ve
Ratio		**6/13**	**4/13**	**5/13**	**10/13**	**4/13**	**11/13**

Where: *Glucose=growth (turbid broth, a flocculating or precipitating bacterial cell mass) and color change of BCP indicator (purple to yellow) in broth supplied with glucose was used as a positive control for fermentation of the rest sugars and those capable are marked '+', **Negative control= MRS broth free of any of the cultures was used as a negative control and fermentation tubes bearing appearances close or similar to it are marked '-', meaning no turbidity, no precipitate and no flocculating mass detected, and the original BCP color (purple) remained unaltered.

Table 4

Morphological, biochemical and physiological characterization results of the isolates

Test		Isolates	
		A6B08	**B6S01**
Cell morphology characterization	Shape	Cocci	Rod
	Cell arrangement	single cells, diads to tetrads	single cells to short chains
Colony morphology characterization	Color	White	White
	Shape	C*	C
	Relative size	VS** to S***	S
Sugar fermentation	Sorbitol	- ve	+ ve
	Galactose	- ve	+ ve
	Xylose	- ve	- ve
	Sucrose	+ ve	+ ve
	Maltose	+ ve	+ ve
	Arabinose	+ ve	- ve
	Cellulose	- ve	- ve
	Inositol	- ve	+ ve
	Rhamnose	- ve	+ ve
	Fructose	- ve	- ve
	Mannitol	+ ve	+ ve
	Lactose	+ ve	+ ve
	Glucose	+ ve	+ ve
Growth	2% NaCl	+ ve	+ ve
	4% NaCl	- ve	+ ve
	6.5% NaCl	- ve	+ ve
	15°C	+ ve	+ ve
	45°C	+ ve	+ ve
Catalase reaction		- ve	- ve
Gas production from glucose fermentation		- ve	- ve
H_2S production		- ve	- ve

C = circular, **VS = very small, S = Small, '-ve'= no growth, no fermentation or no activity observed, '+ve' growth, fermentation or activity observed***

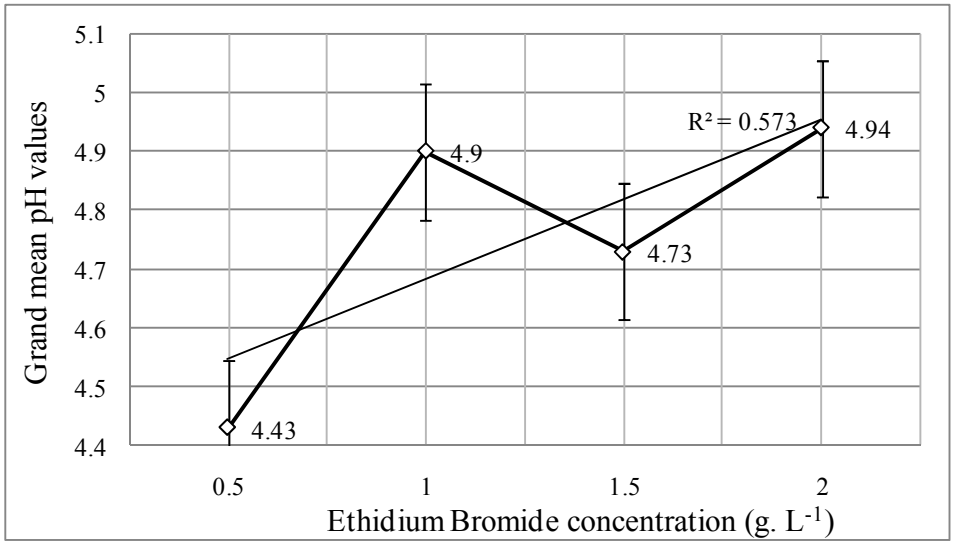

Figure 1
Relationship of ethidium bromide treatment concentration and Grand mean pH reduction values of 80 mutant isolates

CONCLUSION

This study practically demonstrated the effectiveness of EB mutagenesis in inducing genetic changes and generating improved LAB. Its implementation could be a resource effective approach through which locally available LAB could be fine-tuned to isolates with highly adaptive and productive traits. The mutant isolates generated by this study were capable of reducing the pH of the medium they were grown in better than their wild type parents. Their utilization in the dairy fermentation, especially in the Ethiopian traditional milk processing practice, will transform the production scheme to an efficient sector by shortening the lengthy fermentation time and product loss due to contamination (rapid colonization associated with the acidification of milk stock hinders the proliferation of spoiling microbes, contaminants and opportunistic pathogens). Due to their newly acquired and some enhanced traits, mutants generated by this study could also be utilized in variety of food, beverage and pharmaceutical processes where lactose is not the only sugar to be fermented. There in a need for the detailed genomic study which helps in understanding the nature of the induced mutation, the types and mechanisms of genes associated with stress that could serve as potential inputs in cloning, transformation and engineering strategies.

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