Potential probiotic *Lactobacillus casei* KLSA22 producing Lactase for Lactose Intolerant with an antibiotic susceptibility pattern

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Abstract

Lactose is the main sugar in milk and as a result of the main energy source for the infant. Milk contains 4.8% lactose. Lactose is a disaccharide consisting of glucose and galactose. In normal physiological conditions, lactose is hydrolyzed by lactase also known as lactase-phlorizin hydrolase and under its systemic name lactose- galactose hydrolase, which is a brush-border membrane bound enzyme. Lactose intolerance is a form of lactose maldigestion where individuals experience symptoms such as diarrhoea, abdominal cramping, flatulence, vomiting and bowel sounds following lactose consumption. An estimated 30% of the population from the developing countries may suffer from this condition. Lactic acid bacteria (LAB) such as Lactobacillus spp. may help digesting lactose contained in fermented dairy products and this could be beneficial to individuals suffering from lactose intolerance.

As a result, this study also revealed that the effect of antibiotics on Lactobacillus sp. KLSA22 strain which had extraordinary abilities to ferment lactose is able to synthesize lactase under submerged fermentation. Thus enzyme- lactase obtained from L. casei KLSA22 is to be considered as an important part of safety assessment for the treatment of lactose intolerance.

Keywords: Lactase, Lactose intolerance, *Lactobacillus casei*, Probiotics.

Introduction

The example for the manufacture of cheese, yogurt and other fermented milk and some types of butter depends on the activity of starter microbial cultures. Foremost of these are species of *Lactobacillus, Lactococcus, Leuconostoc* and *Streptococcus* which form part of a group commonly referred to as lactic acid bacteria (LAB).

An evidence to previous study on milk products with probiotics is possibly beneficial for digestive health and can improve various digestive problems[1] although milk is a nutrient-dense food and particularly required for a healthy diet which contains essential nutrients such as carbohydrates (lactose), fats, proteins, vitamins and minerals. But many consumers do not enjoy the benefits of milk due to lactose intolerance or maldigestion which is the most common disorder of intestinal carbohydrate digestion in humans.[23]

LAB are the producers of lactase and accomplished in using lactose as the sole source of carbon and energy. Indeed, these microbes are achieving considerable importance in their exploitation and suitability for the production of enzymes on account of development process to therapeutic and industrial importance.

In fact microbial enzymes play a vital role in the biochemical investigation, diagnosis, treatment and monitoring of various dreadful diseases including cancer as the part of enzyme therapy but moreover are preferable due to their rapid production, economic feasibility, high yield, ease of product modification and optimization, regular supply owing to the absence of seasonal fluctuations and rapid growth of microbes on/in inexpensive media.[4,5]

As such, the discovery of new lactase is utmost important but there are few reports on the production of lactase through submerged fermentation (SmF) by employing LAB. Thus far, the selection of an economical and easily available substrate accompanied by a suitable producer bacterium, culture conditions and effective downstream processing is required to reduce the cost of enzyme production.

Material and Methods

Isolation of Lactic Acid Bacteria (Lab): Four different varieties of samples (soil and dairy effluent) were collected in a sterilized container from milk processing fields of Gulbarga, Karnataka, India. All the samples were brought to the laboratory under aseptic conditions and were processed for isolation by serial dilution technique. To isolate LAB, 100 µl of aliquot from each diluted samples were inoculated on lactose agar media composed of (g/ml): lactose 0.5 %, peptone 0.5 %, beef extract 0.3 % and agar 1.5 % adjusted at pH 6.5 ± 0.2; incubated at 37°C for 48 h and the growths of bacterial colonies were observed at every 24 h.

Morphological Study of Lab Isolates: The bacterial white colonies developed on lactose agar medium from different sources were examined for morphological characteristics such as colony characterization, gram staining, motility and sporulation as prescribed by Bergey's Manual of Systematic Bacteriology.[6] The selected prominent white colonies of LAB were sub-cultured and maintained on deMan Ragosa Sharpe (MRS) agar at 4°C for further studies.[7]

Identification of *Lactobacillus Sp.* Klsa22: The molecular study of the isolate signifies the systematic position of the bacterial strain confirmation. The genomic DNA extraction of isolate KLSA22 was performed by the method as described by Brashears et al.[8] The predominantly genomic DNA of the bacterial cells is surrounded by the cell membrane along with cell wall to be disrupted so that DNA is released into the extraction buffer.

Antibiotic Susceptibility Test: The potent isolate KLSA22 strain along with a standard culture *Lactobacillus acidophilus* NCDC11 was employed to detect the antibiotic sensitivity pattern by using disc diffusion method.[6] The cells from 48 h old culture were diluted 1:10 ml in MRS broth and incubated overnight at 37°C. The overnight growth culture (1 %) was overlaid on Muller-Hilton Agar plates using a swab. Multi-antibiotic discs (Icosa G-I Plus-Himedia, India) were placed on seeded plates to allow the diffusion of antibiotics into the medium. The zone of growth inhibition was measured by anti-biogram scale after 24 h at 37°C.

Screening of *Lactobacillus Casei* Klsa22 for Lactase Production: The cultivation of isolated strains and screening of LAB producing lactase was examined by using MRS agar medium containing 1.5 % of lactose (as an additional carbon source) infused with 40 µl of X-Gal (5-bromo.4-chloro.3-indolyl-β-D-galactospyranoside). It is an inert chromogenic substrate for lactase that promotes lactose utilization. Lactase hydrolyzes X-Gal into colorless galactose and 4-chloro-3-bromindigo which forms intense blue precipitate that develops blue colony screening. Further, the screened isolates were subjected for quantitative estimation.

Quantitative Estimation of Lactase Production under Submerged Fermentation By *Lactobacillus Casei* Klsa22: One loopful inoculum of *L. casei* KLSA22 strain was inoculated in 250 ml Erlenmeyer flask containing 100 ml of modified MRS medium composed of (g/ml): Protease peptone (1 %), Beef extract (1 %), Yeast extract (0.55 %), Lactose (2 %), Tri-ammonium citrate (0.2 %), Sodium acetate (0.5 %), K_2HPO_4 (0.2 %), $MgSO_4.7H_2O$ (0.01 %), $MnSO_4.4H_2O$ (0.005 %) and Tween 80 (0.1 ml) adjusted at pH of 6.5 ± 0.2 and fermented for 24 h at 37°C in shaker incubator at 100 rpm. Afterwards, biomass was collected and processed for enzyme extraction.

Results

Isolation of LAB: In the present study, four different varieties of samples were subjected for serial dilution technique to obtain LAB isolates on lactose medium. As a result, total 28 bacterial isolates were obtained as illustrated in table 1.

Morphological Study of Lab Isolates: The total of 28 bacterial white colonies grown on lactose agar medium from different sources were designated as KLSA1 to KLSA28 and were selected to examine for morphological characterization as described in table 1. Based on microscopic observation of gram's staining, all bacterial isolates were identified with gram positive rod-shapes. Amongst 28 isolates, the five isolates were observed as non-motile and non-spore former with potential staining; wherein three isolates (KLSA16, KLSA20 and KLSA24) were found to have short-chain arrangement, while the remaining two isolates (KLSA22 and KLSA27) were observed as long-chain arrangement as depicted in figure 1.

Biochemical and Physiological Properties of Isolate Klsa22: Based on morphological, biochemical and physiological characterization study, the isolate KLSA22 was considered as *Lactobacillus sp.* as per the methods prescribed by Shu et al[9] according to Bergey's Manual of Systematic Bacteriology (Table 2).

Identification of *Lactobacillus Sp.* Klsa22: The isolate KLSA22 was preliminary identified as *Lactobacillus sp.* based on morphological, biochemical and physiological characterization. In contrast, the molecular study of the culture also revealed the species level identification and the systematic position of the bacterial strain confirmation. The electrophoresis study showed that a distinct single band of extracted genomic DNA was visualized on agarose gel under GELDOC confirming its purity as depicted in figure 2. Afterward the purified DNA of isolated strain KLSA22 was amplified the 16S rRNA gene as a template by PCR.

Accordingly, the forward and reverse sequence reactions of the PCR amplicon were conceded in the direction of the sequence retrieved with 1127 bp. The evolutionary study of the isolate revealed a close relatedness to *Lactobacillus casei* with 92 to 100 % similarity (Figure 3). The sequence was submitted to Genbank (SUB1766816) for its acceptance as new strain. Thus, the isolate was confirmed as *Lactobacillus casei* KLSA22 with accession No: KX692282.

Antibiotic Susceptibility Test: The isolated strain *Lactobacillus casei* KLSA22 has shown high sensitivity to clindamycin but moderately sensitivity to gentamycin, linezolid amikacin and tetracycline. Moreover, it was found less sensitive to ofloxacin and penicillin at the same time as the remaining antibiotics were susceptible. In contrast to the standard culture, *Lactobacillus acidophilus* NCDC11 strain showed that most of the antibiotics were susceptible though it was highly sensitive to clindamycin, moderately sensitive to gentamycin and tetracycline but less sensitive to linezolid and amikacin as depicted in figure 4 and table 3.

Screening of Lab for Lactase Production: The selected five isolates (KLSA12, KLSA16, KLSA22, KLSA24 and KLSA27) were screened for lactase producing LAB using modified MRS agar medium. Amongst them, the strain KLSA22 was found of high activity with blue colonies indicating lactose utilization on the medium as shown in figure 5. Thus, screening study confirmed all five strains capable to produce lactase.

Table 1
Morphological study of selected bacterial isolates

Isolates	Colony characteristics	Gram staining	Motility	Endospore staining
KLSA1	Vs, Rd, W, Rf, E, D, O	+ve	+	Sf
KLSA2	S, Rd, W, Rf, E, D, O	+ve	+	Sf
KLSA3	Vs, Rd, W, Rf, E, D, O	+ve	+	Nsf
KLSA4	Vs, Rd, W, Rf, E, D, O	+ve	+	Nsf
KLSA5	S, Rd, W, Rf, E, D, O	+ve	-	Sf
KLSA6	S, Rd, W, Rf, E, D, O	+ve	+	Nsf
KLSA7	Vs, Rd, W, Rf, E, D, O	+ve	+	Nsf
KLSA8	Vs, Rd, W, Rf, Um, D, O	+ve	-	Sf
KLSA9	Vs, Rd, W, Rf, E, D, O	+ve	-	Sf
KLSA10	Vs, Rd, W, Sm, Lb, R, T	+ve	-	Sf
KLSA11	S, Rd, W, Rf, Lb, R, T	+ve	+	Nsf
KLSA12	**M,** Rd, **W, Sm, E, F, T**	**+ve**	**-**	**Nsf**
KLSA13	S, Rd, W, Sm, Um, R, T	+ve	+	Nsf
KLSA14	M, Rd, W, Rf, Un, R, O	+ve	+	Sf
KLSA15	S, Rd, W, Sm, Um, R, T	+ve	+	Sf
KLSA16	**M, Rd, W, Sm, E, D, O**	**+ve**	**-**	**Nsf**
KLSA17	M, Rd, W, Sm, Un, F, T	+ve	-	Nsf
KLSA18	S, Rd, W, Sm, Um, R, T	+ve	+	Sf
KLSA19	S, Rd, W, Rf, E, F, T	+ve	-	Sf
KLSA20	M, Rd, W, Rf, Un, R, O	+ve	+	Sf
KLSA21	S, Rd, W, Sm, Lb, F, T	+ve	+	Nsf
KLSA22	**L, Rd, W, Sm, E, D, O**	**+ve**	**-**	**Nsf**
KLSA23	L, Rd, W, Rf, Um, R, T	+ve	+	Sf
KLSA24	**L, Rd, W, Sm, E, F, O**	**+ve**	**-**	**Nsf**
KLSA25	S, Rd, W, Rf, Un, R, T	+ve	-	Sf
KLSA26	M, Rd, W, Rf, Un, F, T	+ve	+	Sf
KLSA27	**L, Rd, W, Sm, Um, R, O**	**+ve**	**-**	**Nsf**
KLSA28	M, Rd, W, Sm, E, F, T	+ve	+	Nsf

Symbols: Size: S (small, 2.0 mm), Vs (very small, 0.1 - 0.5 mm), M (medium, 3.0 mm), L (large, 4.0 mm); Shape: Rd (round), Ir (irregular); C (color): W (white), C (cream); Surface: Sm (smooth), Rf (rough); Margin: E (entire), L (lobate), Um (umbonate), Un (undulate); Elevation: F (flat), D (drop-like), R (raised); Opacity: O (opaque), T (translucent); +ve (Gram positive bacilli); + (Motile); - (Non motile); Sf (Spore former), Nsf (Non spore former).

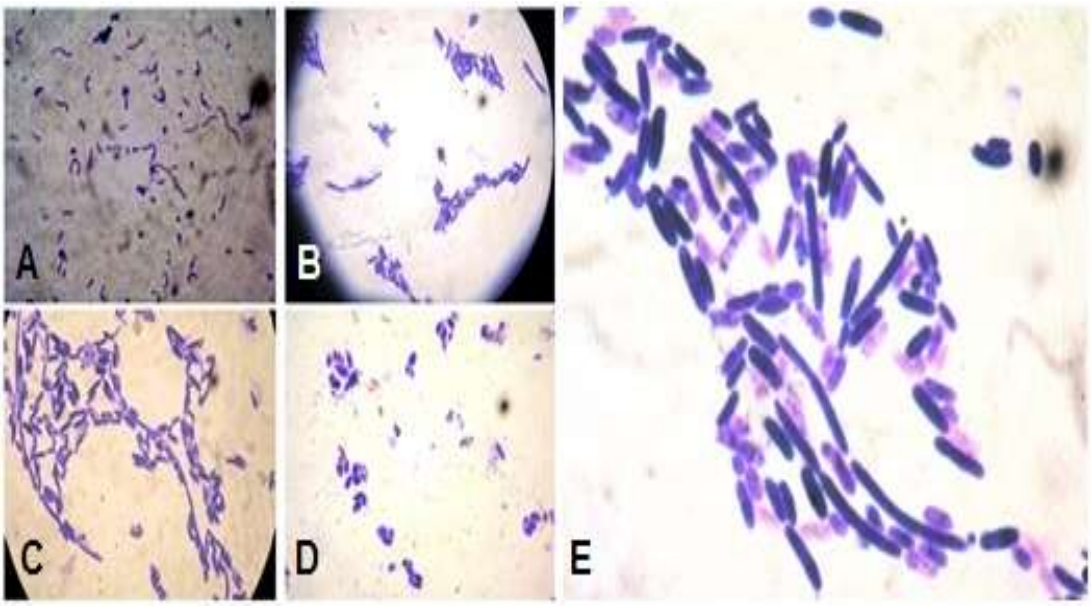

Figure 1: Gram's nature of five bacterial isolates represents A, B, C, D and E viz. KLSA12, KLSA16, KLSA27, KLSA24 and KLSA22 strain respectively.

Table 2
Biochemical and Physiological Characteristics

Test parameters	Isolate KLSA22
Catalase	-
Starch hydrolysis	-
Nitrate reduction	-
Utilization of Sugars	
Arabinose	-
Glucose	+
Lactose	+
Maltose	+
Sorbitol	-
Sucrose	+
Xylose	-

Symbols: + (Positive), - (Negative).

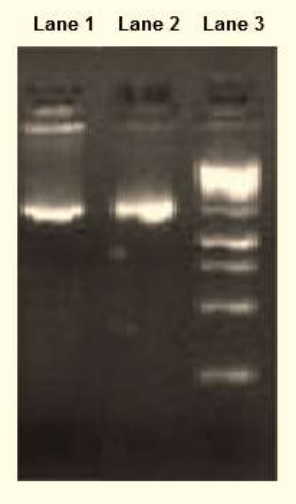

Figure 2: Gel amplicon of genomic DNA showing (Lane 1) Sample strain KLSA22, (Lane 2) Positive control and (Lane 3) Standard marker with 1 kb in size

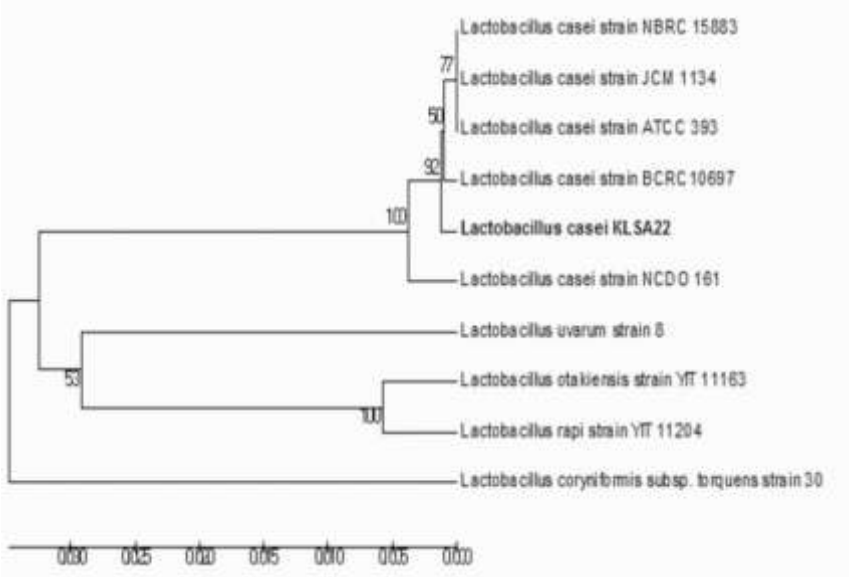

Figure 3: Phylogenetic tree represents evolutionary relationship of KLSA22 strain with aligned sequences of other taxa. The tree was rooted via a neighbor-joining method, numbers within the dendogram signify the occurrence (%) of the branching order in 1000 bootstrapped values (in excess of only 10 values have shown) and bar scale (0.050) denoted substitutions per nucleotide position.

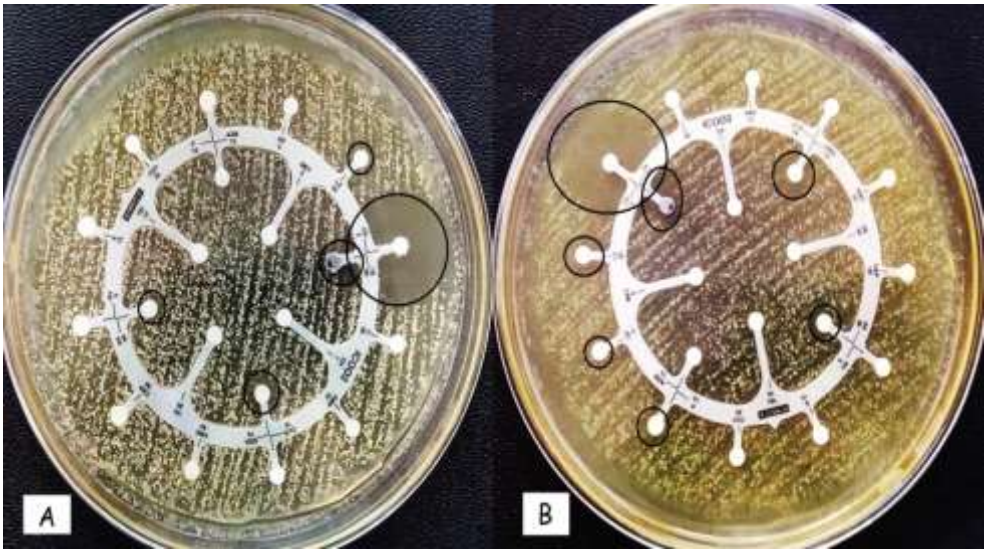

Figure 4: Showing antimicrobial activity of *Lactobacillus* strains against different antibiotics (A) Standard culture *L. acidophillus* NCDC11 and (B) *Lactobacillus sp.* KLSA22

Table 3
Showing antibiotic susceptibility of *Lactobacillus* strains

S.N.	Antibiotics (symbols)	Concentration (µg/disc)	Antibiotic Sensitivity Pattern of Strains	
			Lactobacillus acidophilus NCDC11	*Lactobacillus sp.* KLSA22
1	Cephalothin (CEP)	30	-	-
2	Clindamycin (CD)	2	+++	+++
3	Co-Trimoxazole (COT)	25	-	-
4	Erythromycin (E)	15	-	-
5	Gentamycin (GEN)	10	++	++
6	Ofloxacin (OF)	5	-	+
7	Penicillin (P)	10	-	+
8	Vancomycin (VA)	30	-	-
9	Ampicillin (AMP)	10	-	-
10	Chloramphenicol (C)	30	-	-
11	Oxacilin (OX)	1	-	-
12	Linezolid (LZ)	30	+	++
13	Azithromycin (AZM)	15	-	-
14	Amikacin (AK)	30	+	++
15	Clarithromycin (CLR)	15	-	-
16	Teicoplanin (TEI)	10	-	-
17	Methicillin (MET)	5	-	-
18	Amoxyclav (AMC)	30	-	-
19	Novobiocin (NV)	5	-	-
20	Tetracycline (TE)	30	++	++

Symbols: +++ = Highly Sensitive (≤40mm), ++ = Moderately Sensitive (≥10mm), + = Less Sensitive (≤5mm), - = Resistant (0).

Quantitative Estimation of Lactase Production under Submerged Fermentation by *Lactobacillus Casei* Klsa22: In the present study, the evaluation of bioprocess for lactase production by *Lactobacillus sp.* KLSA22 was carried out under SmF using pure lactose (synthetic substrate) and cheese whey (lactose content). The maximum lactase production was observed with 83.34 IU using 2 % of cheese whey at 48 h time period at static condition. But in contrast, agitation speed at 100 rpm gradually decreased the enzyme activity (Table 4).

Discussion

In accordance with previous reports, all probiotic isolates were sensitive to tetracycline[10]. The strain *Lactobacillus delbrueckii* was sensitive to penicillin and tetracycline reported by Sharma and Singh[11]. Conversely, Sharma et al[12] reported that most of *Lactobacillus spp* showed high level of resistance toward co-trimoxazole and vancomycin, but less sensitive to oflaxacin, penicillin and tetracycline. LAB were sensitive to amikacin and tetracycline previously documented.[13,14]

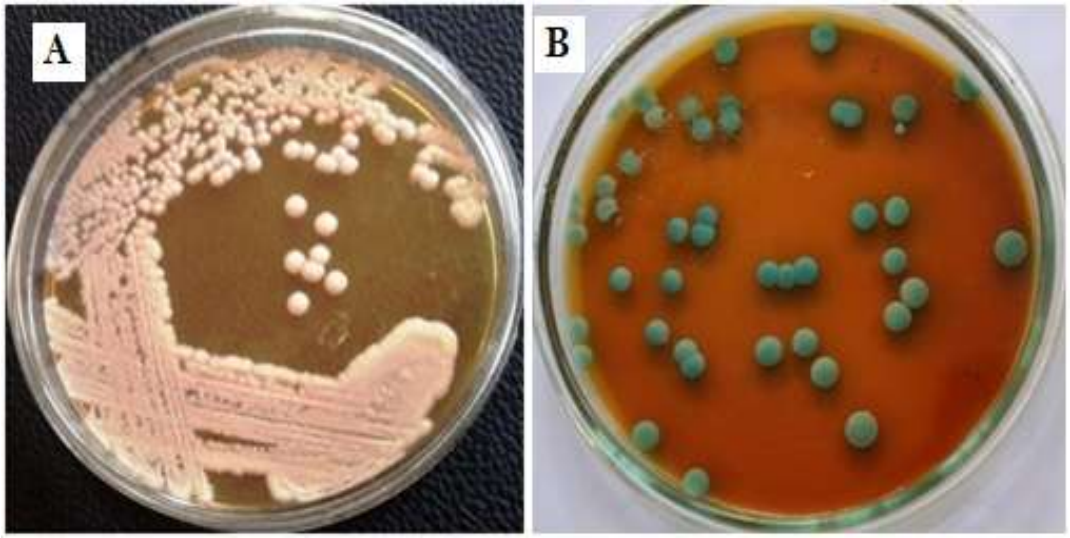

Figure 5: Represents (A) Pure Culture of *Lactobacillus sp.* KLSA22 strain (B) Blue colonies of *Lactobacillus sp.* KLSA22 strain showing Lactose utilization on MRS plate containing X-Gal.

Table 4
Production of lactase through SmF by *Lactobacillus sp.* KLSA22

Fermentation Period (h)	Substrate (%)	Enzyme Activity (IU)	
		Agitation speed at 100 rpm	Static condition
24	Pure Lactose (2 g)	40.17	40.68
	Cheese Whey (2 ml)	40.99	41.77
48	Pure Lactose (2 g)	39.01	69.83
	Cheese Whey (2 ml)	40.73	**83.34**

These reports correlate with the present study revealing that strain KLSA22 showed sensitivity to wide range of antibiotics next to the standard strain *L. acidophilus* NCDC11comparatively.

Conclusion

Potent isolate KLSA22 strain is confirmed genus as *Lactobacillus* and named as *Lactobacillus sp.* KLSA22. As a result, this study also revealed that the effect of antibiotics on *Lactobacillus sp.* KLSA22 strain had extraordinary abilities to ferment lactose that is able to synthesize lactase under submerged fermentation. Thus enzyme- lactase obtained from *L. casei* KLSA22 are to be considered as an important part of safety assessment for the treatment of lactose intolerance.

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