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Bio-hydrolysis Study of Lactose in Produced Yoghurt by β-Galactosidase from *Lactobacillus fermentum*

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Authors' contributions

This work was carried out in collaboration among all authors. Author OSE conceived and designed the experiments, performed the experiment and processed and analyzed the data and wrote the manuscript. Author EFU supervised the research, analyzed the data, interpreted the data and revised the manuscript. Author ACE co-supervised the research and revised the manuscript. Author CG analyzed the research design and methodology, interpreted the data. Author OEH co-supervised, revised the manuscript, guided the experimental design, performed the experiment and processed the data. All authors read and approved the final manuscript.

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ABSTRACT

The present study investigated the physicochemical and biohydrolysis of lactose in conventional and locally produced yoghurt drinks, respectively. Physicochemical properties of the conventional yoghurt in the presence of the locally produced one were: pH 3.0; 4.0, titratable acid 1.14; 1.04, conductivity 2751.54, 2204, viscosity 71.11; 112.14, TDS 1788.5; 1433, TSS 781; 654, TS 3532.54; 2087, Mg 11.06; 34.19, Ca 28.76; 45.21, TOC 155.23; 145.23 and TOM 190.93, 178.63 for yoghurt sample I and II respectively. Lactose concentrations of the respective yoghurts were: 2.12 and 1.79 Mg/ml. β-galactosidase was produced from isolated *Lactobacillus* sp under submerged fermentation system after 12 days of incubation. 60% ammonium sulphate was found suitable for

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precipitation of proteins with highest enzyme activity, the precipitate was desalted through reverse osmosis using dialysis bag. Optimum biohydrolysis was achieved after 20 hours of incubation with residual sugar concentration of 0.51 mg/ml while the control experiment had 0.93 mg/ml of the residual sugar. Optimum pH and temperature of hydrolysis were: 5.0 and 70°C respectively. Rate of hydrolysis proceed linearly as the enzyme concentration increases from 0-20% v/v. the present study has shown exclusively the nutritional components of yoghurts drinks commercially sold in the market, it went further to show hope for the metabolic compromised individuals with intolerance to lactose as the enzyme lactase produced from non noscomial organism showed great potentials of hydrolysis.

Keywords: Biohydrolysis; lactose; β-galactosidase; physicochemical; residual sugar.

1. INTRODUCTION

Dairy foods among those of yoghurts and other dairy fermented foods are the outcome of curd formed during the fermentation reaction of milk sugar (lactose) a disaccharide of glucose and galactose in the milk and fermenting bacterial enzymes under certain conditions (Philip et al., 2017). The fermentation process of this food is done in absence of atmospheric oxygen. During the making of yogurt, the milk sugar is hydrolysed by starter bacterial culture enzyme β-galactosidase [1-9]. This enzyme is an inducible biocatalyst synthesized by *Lac Z* genes of the clustered structural genes of lactose operon in the presence of inducer allolactose at the promoter region of the operon and lactose [10]. "In the course of the action of the bacterial enzyme on the lactose, lactic acids and acetaldehyde are produced which in turn lowers the pH of the milk causing it to have a sour taste" [11-21].

The produced lactic acid among others acts on milk protein (casein and other digestive proteins present) to give yoghurt its characteristic texture, flavor and other sensory/organoleptic features [22-25].

"Yoghurt takes the advantages of the demerits of milk especially from animal origins and provides an opportunity to increase the longevity (shelf life) of milk and preserve its nutritional constituents for human consumption owing to its relatively low pH values. In addition, yoghurt is a functional food that contains probiotics, prebiotics and symbiotics plants antioxidants, vitamins, linolenic acid, essential fatty acids, soluble fibres and vitamins" (Elsanhoty et al*.,* 2009).

"Yoghurt production involved the standardization and pasteurization (sterilization) of liquid flowing milk to be fermented with mixed starter cultures of *Lactobacillus* sp. and *Streptococcus* sp" [26-34]. "Yoghurt has high nutritional and therapeutic properties that promote health in human body particularly for a variety of gastrointestinal conditions and in preventing antibiotic-associated diarrhea" (Bamishaiye, 2011).

"It is of suggestion that human consumption of yoghurt containing *Lactobacillus acidophilus* helps prevent *vulvovaginal candidiasis*. Higher nutritional value of these products has been attributed to the increased production of certain nutrients and to the pre-hydrolysis of major milk components by lactic starter cultures, rendering them more digestible" [35-43].

In the world population today, large fraction of approx. 0.1% in every community (WHO, 2010) of the populace are intolerant to milk and dairy products; this is attributed to metabolism and inborn errors in which there is a poor or non – secretion of β-galactosidase within the intestinal epithelial cells needed for hydrolysis of lactose (milk sugar) (Philip et al., 2017). "Lactose has a low relative sweetness and solubility, and excessive lactose in large intestine can lead to tissue dehydration due to osmotic effects, poor calcium absorption due to low acidity, and fermentation of the lactose by microflora resulting in fermentative diarrhea, bloating, flatulence, blanching and cramps, and watery diarrhea" [44-59]. "Furthermore, lactose is a hygroscopic sugar and has a strong tendency to absorb flavours and odours and causes many defects in refrigerated foods such as crystallization in dairy foods, development of sandy or gritty texture, and deposit formation" [60].

2. MATERIALS AND METHODS

2.1 Study Design

The present study was carried out in three distinct stages:

a. Isolation of the bacteria strains from whey effluent and its identification and characterizations
b. Production of β-galactosidase from the probiotics under optimized conditions and its purifications.
c. Determination of initial lactose contents of the yoghurt drinks and application of β-galactosidase for the hydrolysis study.

All materials comprising of chemicals, reagents use in the present study were of analytical grade and were products of BDh, sigma Aldrich Bristol. The equipment are all of good working conditions and standardized at each use.

2.2 Isolation of Strains of *Lactobacilli* the from Dairies

From the stock dairy waste water solution, ten folds serial dilutions were carried out and from the 10^{-4} to 10^{-6} dilutions, innoculum were plated out on a media as described by Ezeonu et al. [61].

2.3 Preparation of Media and Plate Pouring

Differential media De-man ragoshia sharpie (MRS) was prepared according to the manufacturer description (64.5 g in 1000 ml of distilled water). Also, antifungal agent of azole derivative was added to the broth prepared. The prepared broth was sterilized by autoclaving for 15 minutes at 121^0C and 15 psi pressure.

2.4 Inoculations of Folds of Diluted Dairy Waste Water on the Prepared Plate and Subsequent Sub Culturing

From the 10^{-1} to 10^{-6} fold dilutions, inoculations were carried out on the prepared nutrient media and the differential media (MRS) around flame. After the inoculation, the inoculated plates were incubated for 3-4 days at 38^0C using the incubator for colonies growth. All morphological contrasting colonies were purified by repeated streaking and sub-culturing on separate plates. This process was continued till pure bacteria colonies were obtained.

2.5 Microscopic Features of the Isolated Bacteria

Three day old pure cultures were examined both under the microscope and by physical examinations. The colour, texture, spores and growth patterns were also observed.

2.6 Bacteria Identification

The three days old pure cultures were used in preparing microscopic slides. Prior to the microscopic examinations, basic microbiology investigation (gram staining) was carried out on the colonies to distinguish the gram positive bacteria from the gram negative ones.

Identification was carried out by relating features and the micrographs to “Atlas of Bacteriology” (Barnett and Hunters, 1972).

Biochemical test of each of the organisms were carried out as follows:

- Sugar fermentation tests
- Nitrogen digestion test
- Specific enzyme identifications

2.7 Molecular Identification of *Lactobacilli* sp.

Genomic DNA (gDNA) from the selected isolate was obtained using the QIA amp DNA Mini Kit. The 16S rDNA gene was amplified by RT-PCR (the conditions for the amplification stated below) using the forward (5'-AGTTTGATCATGGTCAG-3') and reverse (5'-GGTTACCTTGTTACGACT-3') primers. The amplified DNA sequence was compared to the Gen Bank database of National Center for Biotechnology Information (NCBI) using the BLAST program (Kumar et al., 2016).

2.8 Production of β-galactosidase from the Probiotics (*Lactobacilli* sp.)

Submerged fermentation technique was used for β-galactosidase production from *Lactobacilli sp.*as described by Allam et al. [62]. The whole setups were sterilized at 121°C/ 15psi for 20 minutes using the electronic autoclave (Uday Burdon’s Patent Autoclave, India).

2 ml of the thirty-sixth (36^{th}) hour standardized microbial suspensions were inoculated into each of the sterile conical flasks containing the nutrient broth. They were incubated at pH 6.0 and at 37°C for forty eight hours (48 hours). Samples were drawn at the end of the incubation period, each drawn sample was filtrated using the muslin cloth of pore size 2 mM and the filtrate (crude extracts) was used to assay for β-galactosidase activity.

2.9 Yoghurt Production

Yoghurt drinks was produced using the standard recipe as described by Corbishely (2003).

2.10 Determination of Physicochemical Properties of the Yoghurt Drinks

Physicochemical properties of the yoghurt drinks were determined as described by ATSDR [63]. The following tests to be conducted include:

Yoghurt pH Profiling Test
Yoghurt Conductivity Test
Total Dissolved Solid (TDS) Contents of the Waste Water
Total Solids (TS) (Gravimetric Method)

$$\text{Total solids} = \frac{\text{mg.total solids X 1000}}{\text{mL of sample}}$$

2.11 Estimation of Residual Lactose Concentration

This was achieved by measuring the lactose content of the yoghurt drinks using the phenol-sulphuric acid assay method as described by Dubois et al. [64]. The absorbance of the characteristic yellow orange color was measured at 490 nm. Glucose concentrations (appearance test) were to be determined by the glucose-oxidase method using the kit Glucose PP (Analysa).

2.12 Lactose Hydrolysis Studies

This was carried out using β-galactosidase produced from *Lactobacillus* sp. as described by Allam et al. [62]. Hydrolysis medium contain the yoghurt measured out, the enzyme solution adjusted at specific pH and temperature.

2.13 Optimization of the Hydrolysis Process

2.13.1 Effect of incubation days

Conical flasks (250 ml) containing 50 ml of the yoghurt drinks was incubated at pH 6.0 and at 37°C for eighty four (84) hours. Briefly, 5 ml of β-galactosidase solution was dispensed into each of the conical flasks containing the yoghurt. Aliqout of sample was drawn at every 12 hours till the 84th hour of incubation. Lactose and glucose concentrations respectively were determined as described in section above.

2.13.2 Effect of pH

As described above, each conical flask containing 50 ml of the yoghurt was incubated at pH of 4.0-8.0 in the range of 1.0 unit progressions. Initial pH of the dairy broth was adjusted using 2% HCl v/v and 0.1 M NaOH w/v. They were incubated till the optimum day of sugar hydrolysis. Lactose and glucose concentrations respectively were determined as described in section above.

2.13.3 Effect of temperature

As described in section above, each conical flask containing 50 ml of the produced yoghurt was incubated at 40°C-80°C. They were incubated at the optimum pH and till the optimum day of sugar hydrolysis. Lactose and glucose concentrations respectively were determined as described in section above.

2.13.4 Effect of β-galactosidase concentrations

As described in section above, each conical flask containing 50 ml of the produced yoghurt were dispensed with 5, 10, 15 and 20 ml of both crude and purified β-galactosidase respectively. They were incubated at the optima pH, temperature and day of sugar hydrolysis. Lactose and glucose concentrations respectively were determined as described in section above.

2.14 Statistical Analysis

Data gotten from the present study was analysed using Microsoft excel sheets and analysed variable was compared using ANOVA from SPSS.

3. RESULTS AND DISCUSSION

Pure colonies of *Lactobacillus* on MRS agar plate. The colony forming unit as standardized using the Macfarland standard solution was 3.8 x 10^8 CFU/ml. this shows high heterotrophic activity of bacteria isolates in the whey effluent.

Basic morphological and biochemical techniques respectively were used to identify the isolate as a *Lactobacilli* genre organism (Table 1). Basic morphological features of the bacteria showed that strains of *Lactobacilli* are rod shaped, non sporulating and non motile creamy coloured bacteria; biochemically, they are obligate gram positive, starch hydrolyzing and lactic acid

forming organisms with optimum growth at 27-39^{o}C.

These findings corroborate with that of basic manual for organisms isolations and identifications written by Ezeonu et al. [61]. Molecular test (16S rDNA) was used to identify the pure isolates of *Lactobacilli*. Electrophoretogram of the amplified genome of *Lactobacilli* using RT-PCR showed a typical band at 750 bp. Kumar et al. (2016) reported that band size of 650-800 are typical of bacteria Fig. 2). *Lactobacillus fermentum was* identified after the genomic sequencing with ascribed NCBI accession number of HMO35543.1 (Fig. 3).

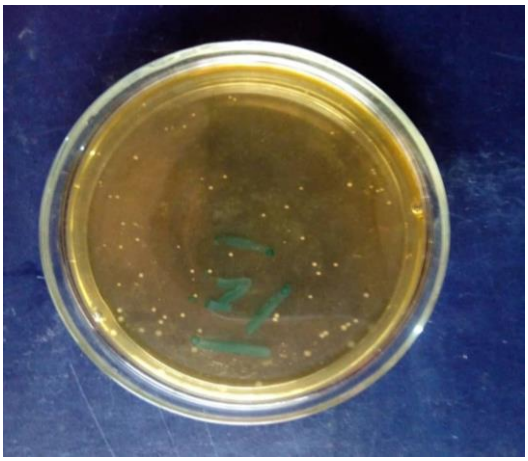

Plate 1. Pure colonies of *Lactobacilli* sp. on MRS culture agar plate

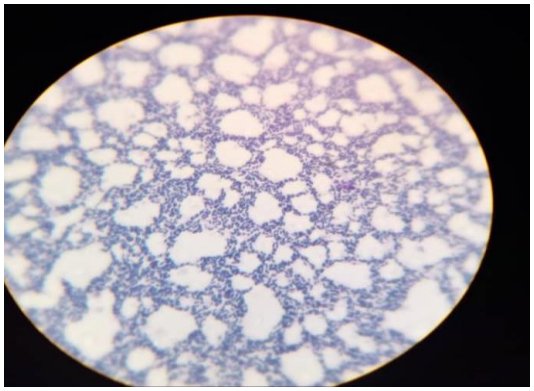

Fig. 1. Micrograph of stained strains of *Lactobacilli* sp. using lactophenol blue dye under the objectives of light microscope x100 objectives

Table 1. Basic morphology and biochemical features of strains of *Lactobacilli* sp. isolated from the mould bread

Morphology	Biochemical Tests
Gram positive Rod shape	
Non-sporulating	Starch hydrolysis (+)
Smooth and raised colony	Heamolysis (-)
Cream colour	Catalase (-)
Non-motile (no flagella)	Lactic acid formation (+)
	Temperature range (30-40 **ºC)**
	Urea hydrolysis (-)
	Glucose fermentation (+)
	Hydrogen sulphide (-)
	Indole utilization (-)
	Gelatin hydrolysis (+)

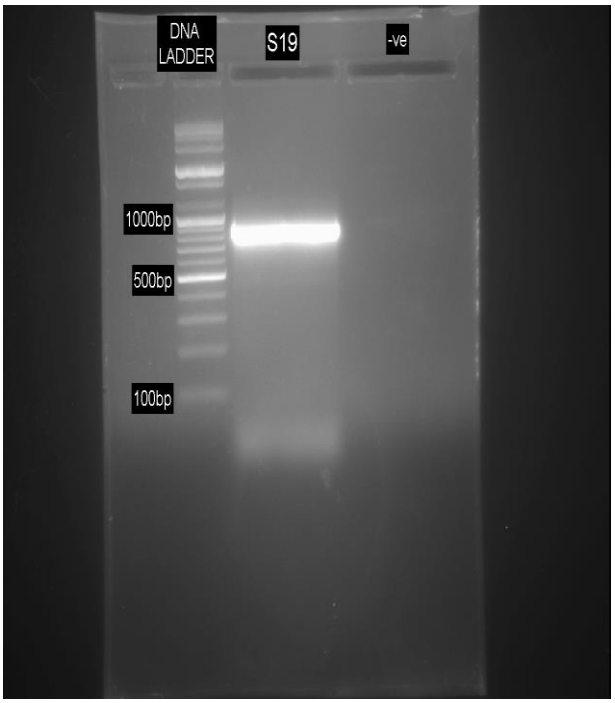

Fig. 2. Electrophoretogram of the amplified genomic DNA viewed on a UV trans-illuminator

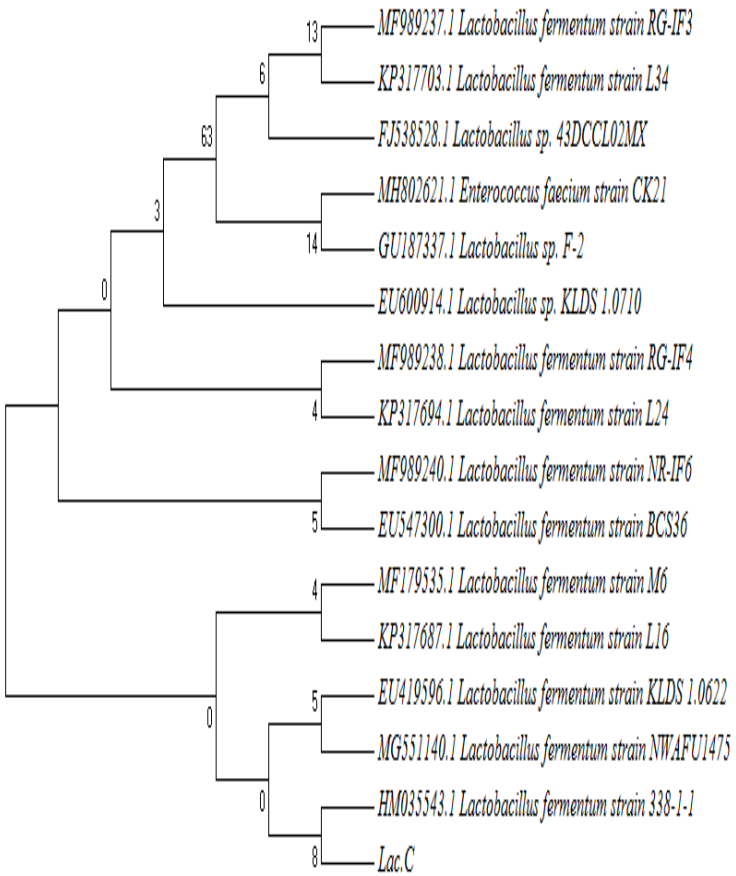

Fig. 3. Phylogenic evolutionary relatedness of strains of *Lactobacilli acidophilus* obtained using NCBI BLAST tools

Effect of incubation day(s) on the production of β-galactosidase from *Lactobacilli fermentum* showed that the highest β-galactosidase activity and protein concentration were obtained on the 12th and 7th day of the eighteen days of production respectively. Enzyme production at these observed days (12 and 7) respectively is evident of catabolite inducement of the substrate present in the fermentation media to the organisms for higher protein production [62]. *Lactobacilli* a starter culture bacterium for the enzyme production is a known probiotic in food industries, they are known to be fastidious in nutrient requirements and growth and as such have relatively large lag period with their prebiotic before switching on their clusters genes (*Lac-operon*) (Cohn and Monod, 1951). This is evident in the result as peak β-galactosidase activity was seen in the last phase of the tri-phasic curve. Akcan (2011) reported day 8 as the peak production day of β-galactosidase activity produced from *Bacillus licheniformis* ATCC-12759 in submerged fermentation system.

60% ammonium sulphate saturation was found suitable to precipitate protein with highest β-galactosidase activity (97.54 µmol/min) (Fig. 5). Samoshina and Samoshin (2005) in their study on fungal β–galactosidase michaelis constants ration reported an optimum precipitation of protein with highest β–galactosidase activity at ammonium sulphate salt saturation of 60% at precipitation pH of 5.5. Oluwaniyi et al (2016) reported the precipitation of β–galactosidase from *Kluveroyces lactis* isolated from a yoghurt waste site at 65% saturation of ammonium sulphate and at pH 7.0.

3.1 Pictures of some of the Conventional Yoghurt from Aba, Abia State

Analysis of the physicochemical properties of the conventional and plant based yoghurt drinks sampled revealed different physical and chemical make-up of the variant dairy drinks (Table 2). Produced yoghurt drink showed the following: pH 4.0, conductivity 2204 $\Omega^{-1}cm^{-1}$, total acid 1.04 mg/ml, viscosity 112.14mm, total dissolved solid 1433mg/ml, total solid 2087mg/ml, total suspended solid 654 mg/ml, Ca and Mg concentration (a factorial of the liquor hardness) of 45.21 and 34.19 mg/ml respectively while total organic carbon (oxidizable carbon) (TOC) and total organic matter content (TOM) are 145.23 and 178.63 mg/ml respectively. Low pH value and total acid concentrations of yoghurt drinks can be attributed to insitu compositions of dairy drinks and starter cultures used for the fermentations as most fermenting bacteria used during yoghurt production are obligate acidophile [62]. Conductivity of the yoghurt drinks shows the exchangeablility of dissolved ions in the liquor and other physical attributes like TS, TSS and TDS showed the presence of dissolved solid substances. Hardness of every flowing liquid is a factor of dissolve minerals of calcium and Magnesium. The presence of higher concentrations of these ions (Ca and Mg) relatively showed the conventional nutritive mark quality of dairies as good source of calcium for bone and teeth formation. Conventional yoghurt used for the comparative study showed similar characteristics to the produced drinks but was seen to be relatively acidic with higher titratable acid content and much ions accruing to its high conductivity value. The produced yoghurt contains higher amount of calcium and magnesium compared to the control experiment.

Lactose concentrations of the yoghurt drinks (Table 3). Residual lactose of the corresponding yoghurts as shown in the table revealed the higher amount of lactose in the conventional yoghurt than in the produced sample drinks. Fermentation of lactose by the probiotics depends on the concentrations of the prebiotics (lactose) and the prevailing physiologic conditions of the fermentation medium.

Biohydrolysis studies of the lactose in the yoghurt using the produced β-galactosidase was carried out for 84 hours. Optimum hydrolysis of the sugar progresses as the incubation hour progresses. More of the sugar was seen hydrolyzed in the treatment system infused with the β-galactosidase than in the control experiment. Remaining lactose confirmed in the fermentation medium was found to be 0.51 and 0.93 mg/ml in the test and control experiments respectively. It was observed that peak biohydrolysis was seen at pH 5.0 and 70°C respectively with residual sugar contents of 0.513 and 0.452 mg/ml respectively. Allam et al. [62] in their study on production of β-Galactosidase and their activity in probiotics stated that the enzyme is a strict acidophile and quiet resilient in at higher temperatures. This is seen evident in their activity that peaked at the respective conditions of hydrolysis. Effect of enzyme concentrations (%v/v) on the hydrolysis of the sugar (%w/v) in the yoghurt drinks was seen in progression of the enzyme concentrations from 5-20 %v/v.

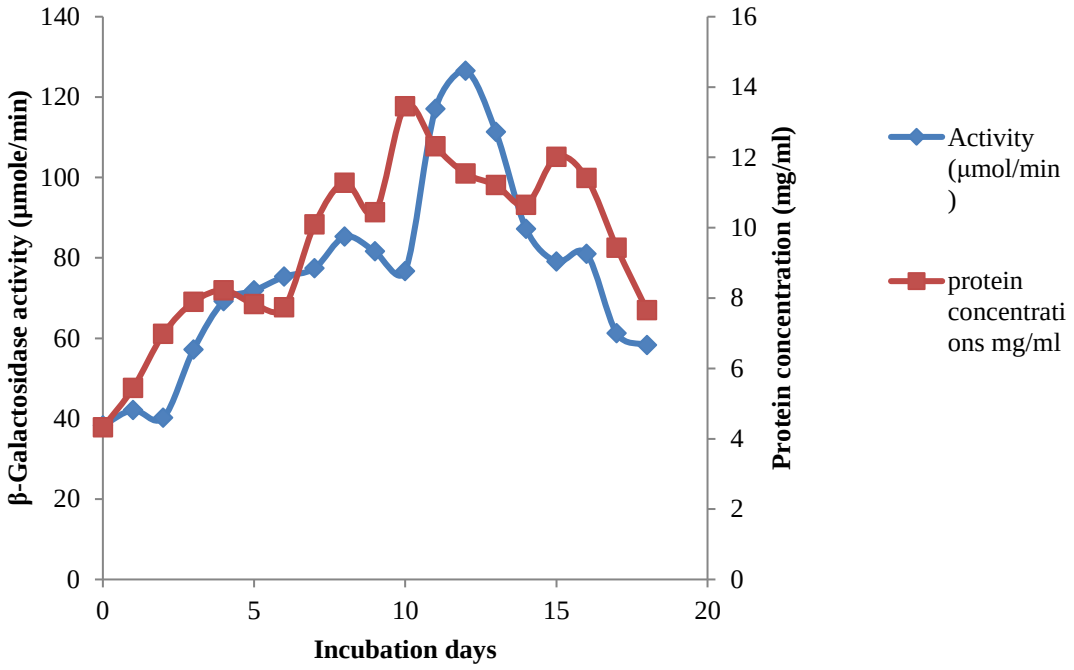

Fig. 4. Production of β-galactosidase from *Lactobacillus fermentum*

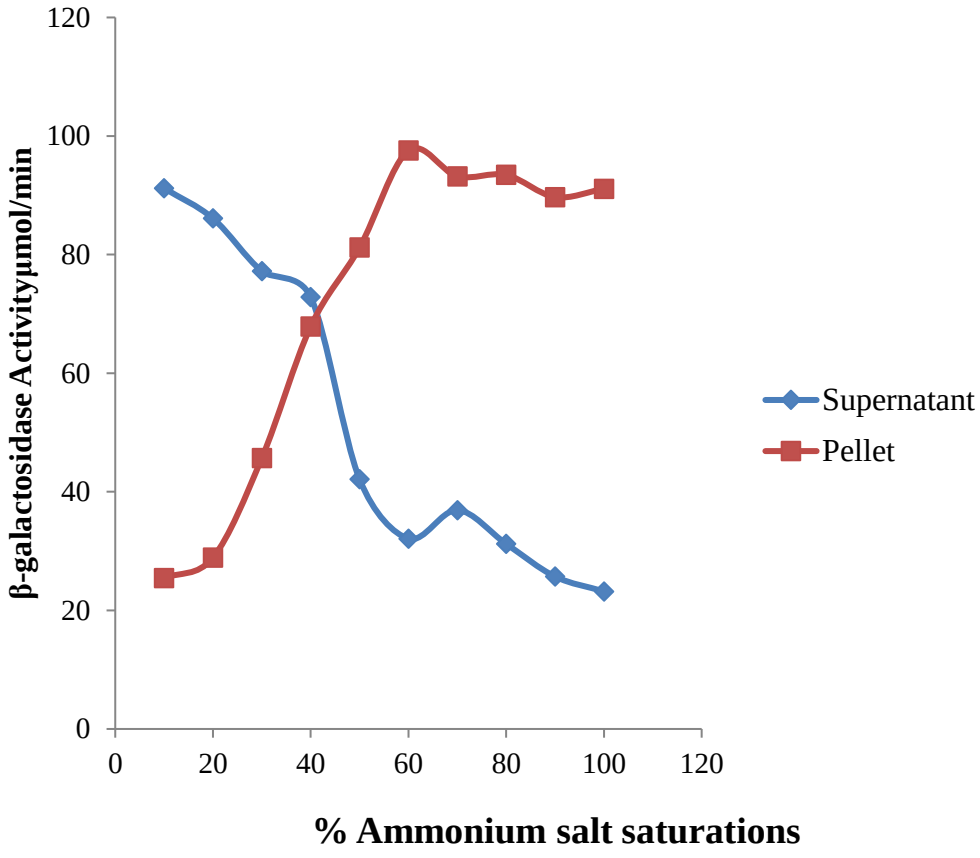

Fig. 5. Ammonium sulphate precipitation of the protein from the crude extract

Fig. 6. Pictures of the conventional yoghurts

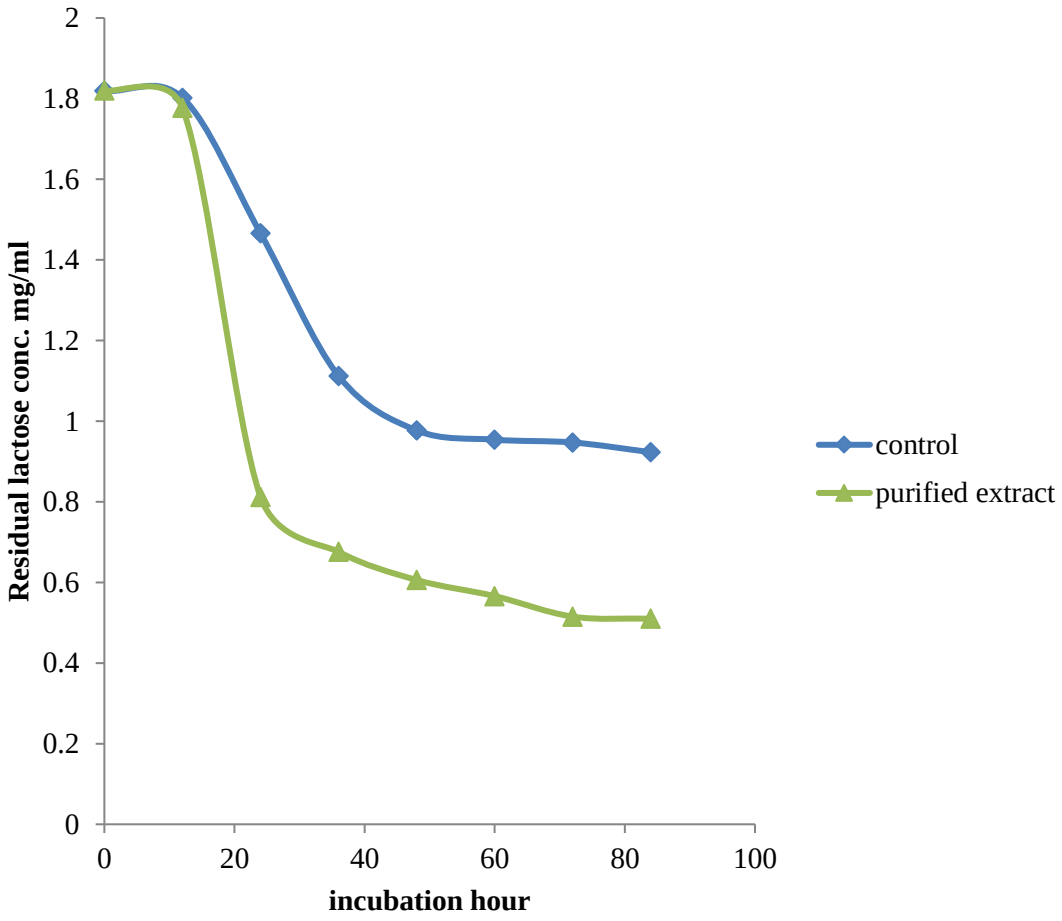

Fig. 7. Effect of incubation time on hydrolysis of yoghurt drink containing lactose (mg/ml)

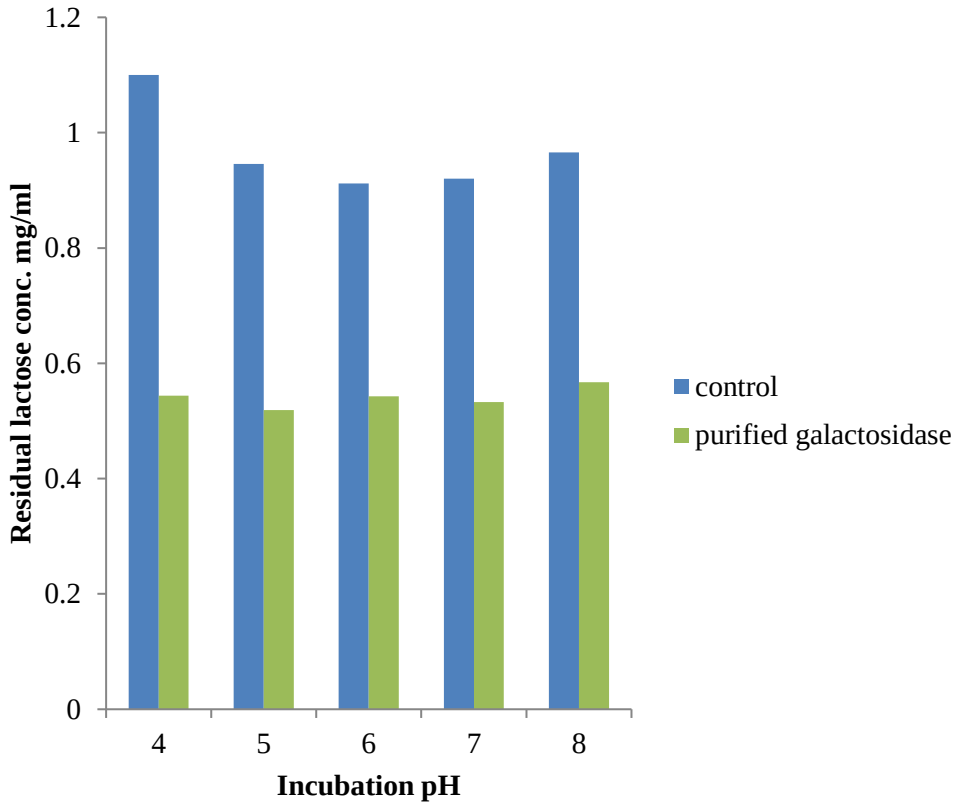

Fig. 8. Effect of incubation pH on hydrolysis of yoghurt drink containing lactose (mg/ml)

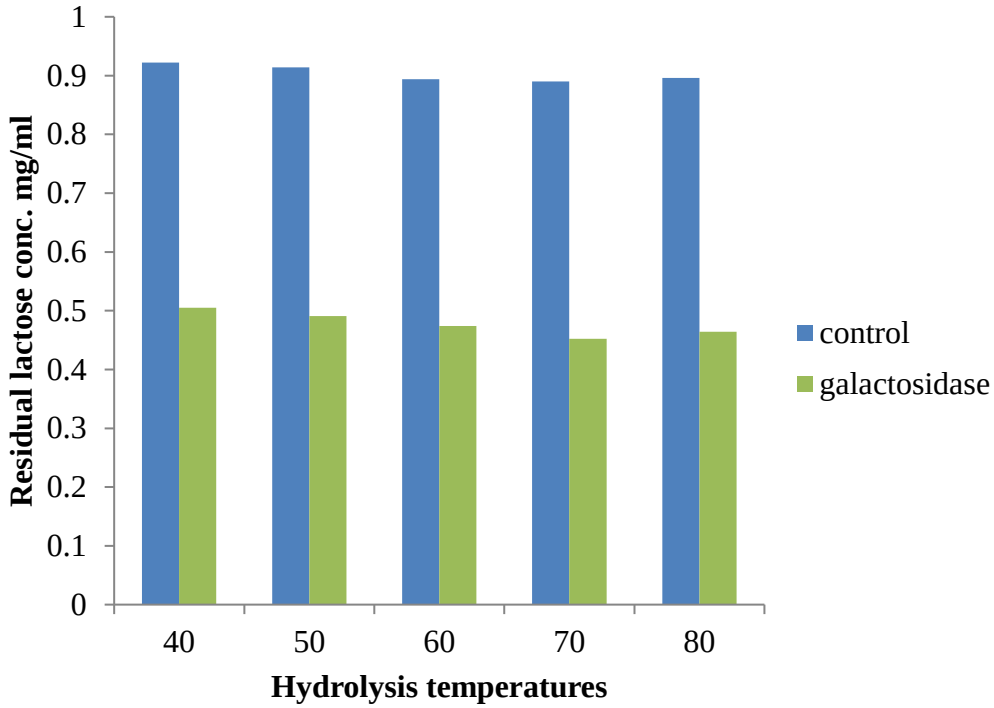

Fig. 9. Effect of incubation temperature on hydrolysis of yoghurt drink containing lactose (w/v)

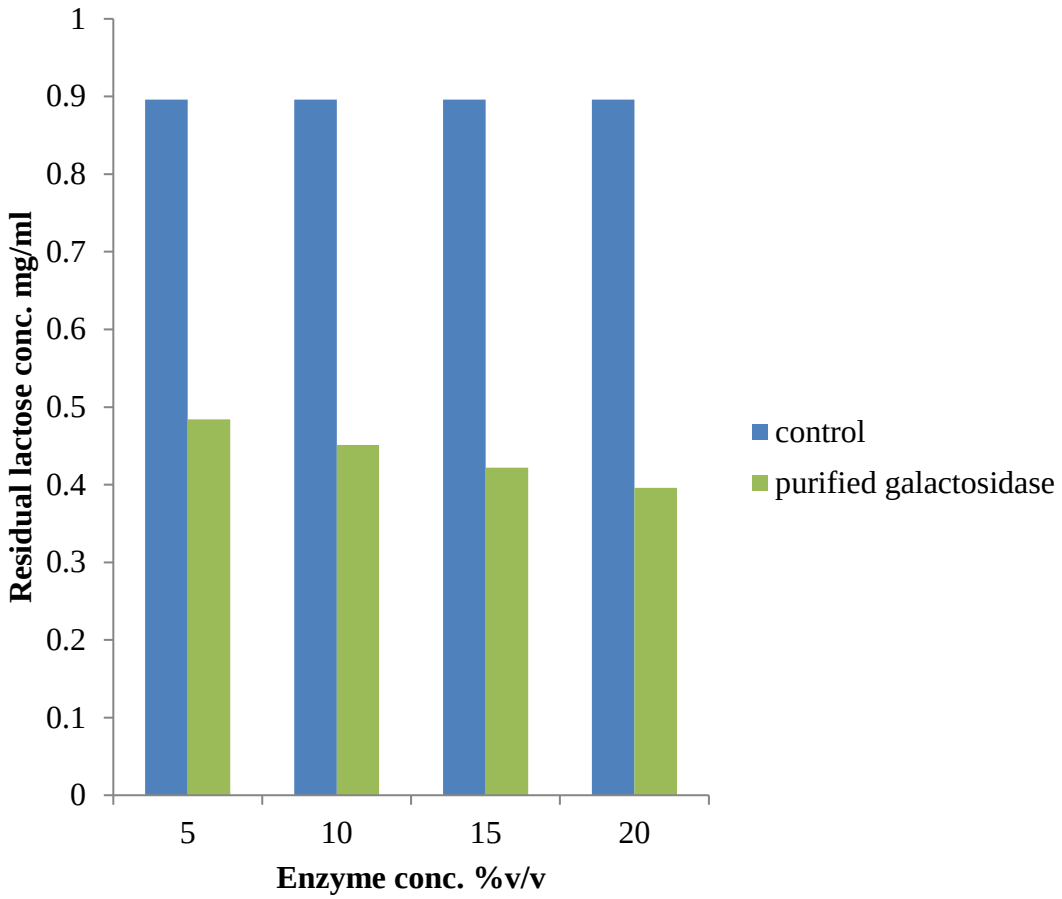

Fig. 10. Effect of enzyme concentrations on hydrolysis of yoghurt drink containing lactose (w/v)

Table 2. Physicochemical properties of yoghurt drinks

Physiochemical parameters	Conventional yoghurt 1	Yoghurt sample
pH	3.0	4.0
TTA	1.14	1.04
Conductivity $\Omega^{-1}cm^{-1}$	2751.54	2204
Viscosity	71.11	112.14
TDS (g/ml)	1788.50	1433
TSS (g/ml)	781	654
TS (g/ml)	3532.54	2087
Magnesium (mg/ml)	11.06	34.19
Calcium (Mg/ml)	28.76	45.21
Total Organic Carbon (TOC) mg/ml	155.23	145.23
Total Organic Matter mg/ml	190.93	178.63

Table 3. Lactose concentrations of the yoghurt drinks

Yoghurt drinks	Lactose composition (Mg/ml)
Yoghurt drink 1	2.12
Produced Yoghurt	1.79

N=3

4. CONCLUSION

Overall picture of yoghurt (both conventional and locally made) on quality assessment needs emphasis on quality control during processing and storage. Also standardization of milk for yoghurt manufacture should be observed to meet legal standards and adjustment of yoghurt mix should approach the standard of the yoghurt package label. This study has shown that there are variations in the quality of yoghurt drinks made from milk derived wholly from plant in terms of physicochemical and other nutritional relevance. β- galactosidase on advocacy should be incorporated in every yoghurt produced wholly from animal based dairies for efficient hydrolysis of the sugar which present a big health challenge for lactose intolerance patience.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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