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Molecular Screening and Identification of Lactobacillus acidophilus Producing β-Galactosidase

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Abstract: β-galactosidase producing *Lactobacillus* was isolated from dairy industrial waste water collected from Rumuekini, Rivers state. Physicochemical properties of the diary effluent showed the following: pH (5.5); Conductivity (845 $\Omega^{-1}cm^{-1}$); Dissolved oxygen (6.24 mg/ml); Biochemical oxygen demand (5.22 mg/ml), Total oxidizable carbon contents (72.6 mg/ml), Total organic matter content (89.30 mg/ml) and Temperature (37.67°C). Total solids, suspended solid and total dissolved solids of the waste water were: 618.84, 82.65 and 536.19 mg/ml, respectively. The basic morphological features of the bacteria showed that the isolate are: rod shaped (*Bacilli*), non sporulating and non motile bacteria; biochemically, they are obligate gram-positive, starch hydrolyzing and lactic acid forming organisms with optimum growth at temperature of 25-40^{o}C. Electrophoretogram of the amplified genome of *Lactobacilli* showed a typical band at 750 bp. *Lactobacillus acidophilus* was identified after the genomic sequencing with ascribed NCBI accession number of JX255677. p-NPG and o-NPG used in the nutrient broth in the presence of the bacteria showed positive test for β-galactosidase production with yellow colouration after three days of incubation, however the colouration was found intense in broth infused with p-NPG. Optimum bacteria growth was observed at 36th hour of incubation with 2ml of the microbial suspension containing the highest heterotrophic counts of the organism (5.4 x 10^{8} CFU/ml). Highest β-galactosidase activity and protein concentration were obtained on day 12 (126μmol/min) and day 13 (0.72 mg/ml) of the incubation at pH 6.0.

1. Introduction

β-Galactosidase (EC 3.2.1.23) is a 464-kDa homo-tetramer intracellular/extracellular protein responsible for hydrolysis (through galactosylation and degalactosylation) and transgalactosylation of β-D-galactopyranosides (such as lactose) and is widespread in nature (Jacobson *et al.*, 2014). The crystal structure of the enzyme was initially determined as monoclinic crystal form with the four

peptides in the asymmetric unit (Matthews, 2015). Within each monomer, the 1023 amino acids form five well-defined structural domains which include: Domain 1 which is a jelly-roll type barrel, Domain 2 and Domain 4 are fibronectin type III-like barrels**,** Domain 5, a β-sandwich and Domain 3 the central is a TIM-type barrel (Jacobson *et al.,* 2014).

β-galactosidase can be sourced from: bacteria, fungi, strains of the actinomycetes, plants, animals and human tissues (Husain, 2010). Probiotic strains of Lactobacilli are major producers of β-galactosidase with high applications in food industries (lactose hydrolysis) and environmental monitoring (whey treatment). Lactose is the major milk sugar; a large population suffer from lactose intolerance because of genetic disorder resulting in the inability to produce β-galactosidase for hydrolysis of lactose to glucose and galactose. Such individual will require exogenous β-galactosidase for hydrolysis of lactose content of their diets (Cohn and Monod, 1951).

The huge demand for β-galactosidase has necessitated the need for an improvement in the enzyme production and an economic way(s) of its reusabilities using cheaper support materials. Lactose intolerance is a major problem requiring the hydrolysis of lactose in the intestine. Growing interests and huge demands of β-galactosidase has necessitated an improvement in the enzyme production and an economic way(s) of its reusabilities using less cost support materials.

Screening of bacteria isolates with potentials of β-galactosidase production is very crucial to meet its economic demand and utilizations (Akcan, 2011). The present study has the objectives of utilization of: evaluation of the physicochemical properties of the dairy effluent, microbiology and biochemical techniques for isolation of strains of Lactobacillus sp. from the dairy effleunt, screening of the isolate using standard substrates of o-NPG and p-NPG respectively for production of β-galactosidase and molecular identification of the Lactobacillus producing β-galactosidase.

2. Material and Methods

Materials

All the reagents, chemicals, equipments used in the present study were products of BDh, Bristol, Sigma Aldrich and are all of analytical grade. The equipments are in good working conditions.

Sample Collection

Dairy industrial waste water was collected from effluent drainage surge tank of Dairy industry located at Rumuekini front terminal, Portharcourt, Rivers state, Nigeria. The collected waste was taken to the laboratory in a clean sterile sample bottle for further analysis.

Waste Water Analysis

Waste water sample from the industry reservoir site prior to microbial isolations were subjected to various physicochemical water profiling analysis as described in Journal of America Toxicology Substances and Disease Registry (ATSDR) (2010). Physicochemical properties include: pH, conductivity, dissolved oxygen, biochemical oxygen demand, total solids, total dissolved solid, total suspended solid, total oxidizable carbon, total organic matter content and temperature of the waste water were analysed.

Isolation and Identification of Strains of *Lactobacilli* sp. from the Dairy Industry Waste Water.

Strains of the bacteria (*Lactobacilli* sp.) was isolated from the dairy waste water using standard microbiology (culturing/coliform counting, gram staining and microscopy mounting) and biochemical (sugar fermentations, nitrogen digestions) technique as described by Ezeonu *et al.*(2013).

Colony forming units counts (CFU/ml) was calculated using the formular: TOTAL HETEROTROPHIC COUNTS X RECIPROCAL OF VOL.OF INOCULUM X RECIPROCAL OF DILUTION FACTOR

Screening of *Lactobacilli* sp. for β-galactosidase Production

Identified *Lactobacilli* sp. standardized using the Macfarland solution ($BaCl_2/H2SO_4$) at wavelength of 610 nm and then screened for β-galactosidase producing ability using Demanragoshie sharpie broth supplemented with 2mM p-NPG as described by Gheytanchi *et al.*(2010). The inoculated culture broth was incubated at 37°C for 3 days.

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).

Table 1 Conditions for Amplification of the Bacteria Genome using RT- PCR

TREATMENT	TEMPRETURE (°C)	TIME (Min)
Pre-denaturation	95	5
Denaturation	94	1
Annealing	52	1
Elongation	71	7
Final elongation	72	7

Assay protocol for the Enzyme Activity

β-galactosidase was assayed according to the method described by Chilaka *et al*. (2002) using p-NPG as the substrate. Assay mixture contained 0.5 ml of enzyme solution, 0.1 ml of 2mM of p-NPG and 0.5 ml of phosphate buffer solution (pH 6.5). The mixture is incubated at 50°C for 30 min. thereafter, the reaction was stopped using 4ml of 0.1M NaOH. Absorbance was taken at 400 nm.

Protein Determination

The total protein content of the enzyme was estimated as described by Lowry *et al*. (1951) using bovine serum albumin (BSA) as the standard protein. Absorbance was taken at 750 nm.

Optimization of Microbial Growth Rate

Inoculum sizes ranged from 0.5 to 2% v/v used for production of β-galactosidase was incubated for 48 hours at pH 6.0 and room temperature (37°C). Aliquot of the inoculum were collected every 12 hours, viable counts of *Lactobacillus acidophilus* was observed till 48 hours of incubation and standardized using the Macfarland solution.

Enzyme Production.

Submerged fermentation technique was used for β-galactosidase production as described by Allam *et al*. (2016). Production parameters such as: Incubation period, pH, and basic macro nutrients (carbon and nitrogen) were optimized during the production process.

Optimized Incubation Period

Conical flasks (250 ml) containing 100 ml of liquid media optimized for β-galactosidase production contained: 1% $(NH_4)S0_4$, 0.4% K_2HPO_4, 1% Lactose, 0.2ml tween 80, 0.01% sodium acetate, 01% di-ammonium citrate, 0.05% $MgSO_4.7H2O$,0.2% $MnSO_4.4H_2O$ were incubated at pH 6.0 and at room temperature (37°C) for eighteen (18) days. The whole setups were sterilized at 121°C/ 15psi for 20 minutes using the electronic autoclave. Samples were drawn at every 24 hours till 18 days, each drawn sample was filtrated using the muslin cloth of pore size 2mM and the filtrate (crude extracts) was used to assay for β-galactosidase activity.

Effect of Physiologic pH

As described above, each conical flask containing 100 ml of the liquid production media were incubated at pH of 4.0-8.5 in the range of 0.5 units. Initial pH of the liquid broth was adjusted using 2% HCl v/v and NaOH w/v. They were incubated till the optimal day of β-galactosidase production.

The effect of incubation period was determined by incubating production medium at different time intervals (18 days) at optimal production conditions.

3. Results

3.1 Physicochemical Properties of the Dairy Waste Water

The table (2) showed a lower pH of 5.52 and higher conductivity of 857 (Ω^{-1} Cm-1) when compared with the control samples. Total solids, suspended solid and total dissolved solids of the waste water were: 618.84, 82.65 and 536.19 mg/ml, respectively. BOD_5 quotient and the temperature of the waste water were observed at 5.22mg/ml and 37.67°C, respectively. Total oxidizable carbon and organic matter contents of the waste water are 72.6 and 89.3 mg/ml, respectively.

Table 2. Physicochemical properties of the dairy waste water.

Physical Factors	Control	Dairy waste water
pH	7.2	5.52
Conductance (Ω^{-1} Cm-1)	423	857.00
TDS (mg/ml)	258.70	536.19
TS (mg/ml)	307.46	618.84
TSS (mg/ml)	48.76	82.65
BOD_5 (mg/ml)	2.26	5.52
TOC (mg/ml)	42.67	72.60
TOM (mg/ml)	89.37	89.30
Temperature (°C)	34.55	37.67

N=3

3.2 Microbial Isolation

Pure colonies of *Lactobacilli* sp. were obtained using differential DeManRagoshie sharpie culture medium. From the culture plate, distinct colonies of the organisms were observed with total heterotrophic number of 2.7 x 10^8 CFU/ml (Plate 1).

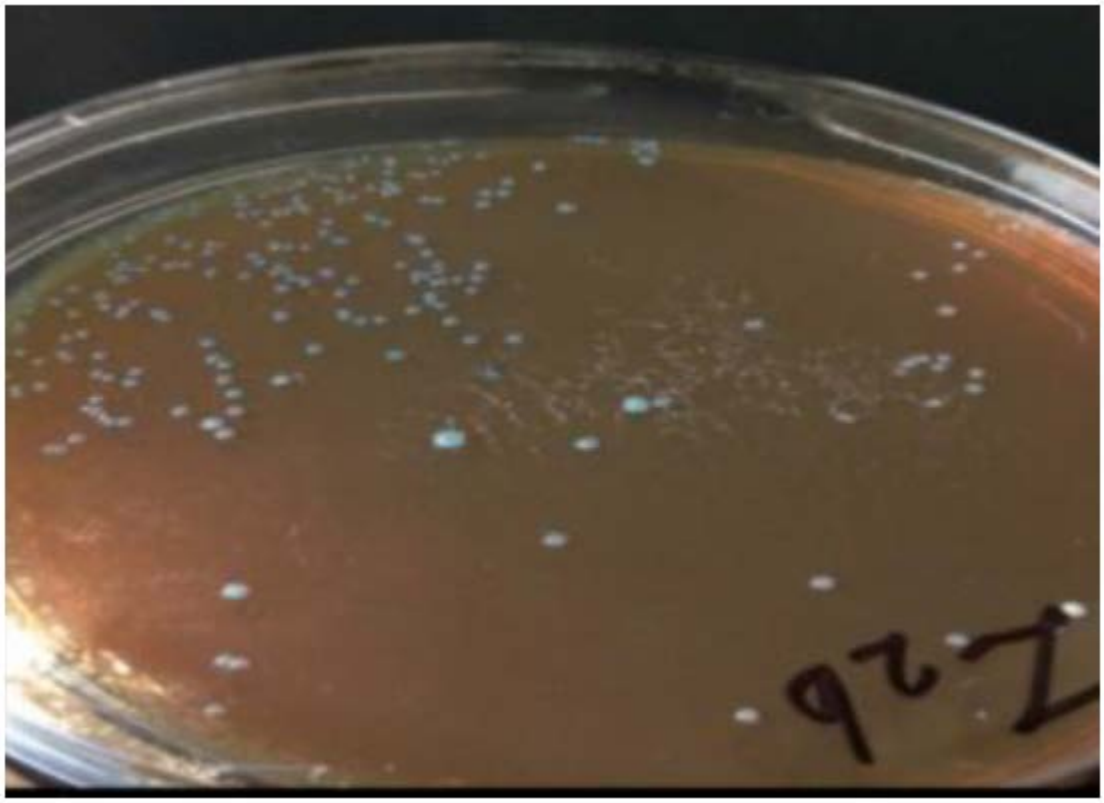

Plate 1. Strains of Lactobacillus sp. on MRS media.

The micrograph of the bacteria suspension under an objective magnification of x100 showed clusters of rod shaped bacteria cells in a red stained background using safranine dye (Plate 2).

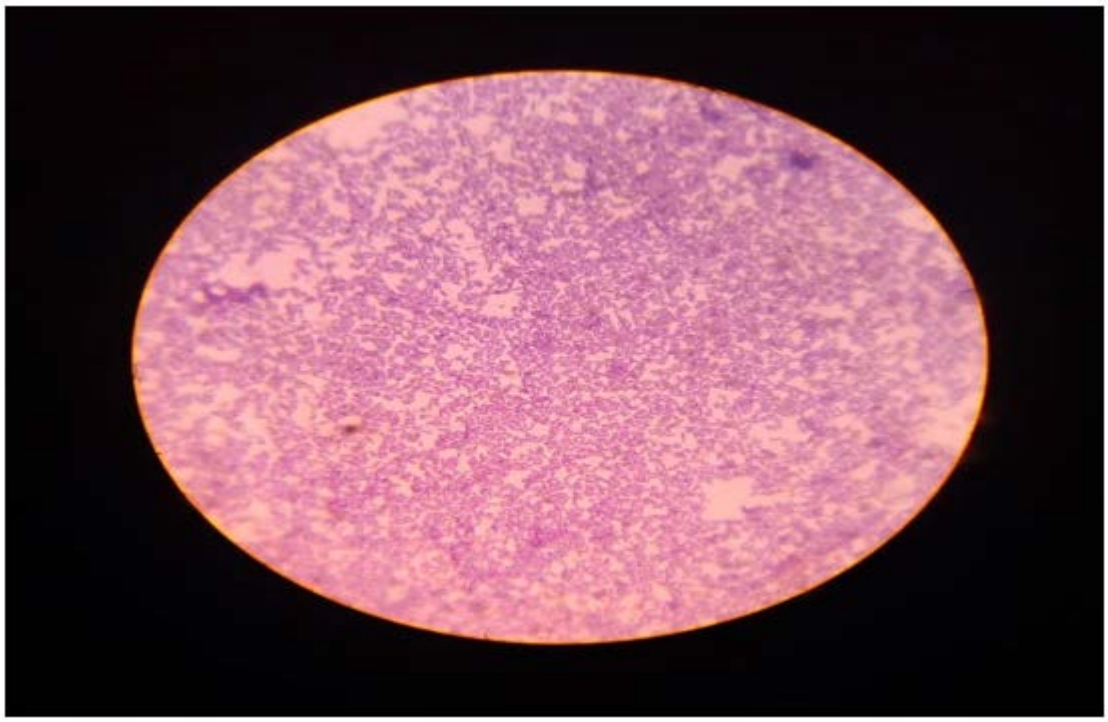

Plate 2. Micrograph of stained strains of Lactobacillus sp. under light microscope (x100).

The basic morphology and biochemical features of strains of *Lactobacilli* sp. isolated from dairy waste water (Table 3). The basic morphological features of the bacteria showed that the isolate are: rod shaped (*Bacilli*), non sporulating and non motile bacteria; biochemically, they are obligate gram-positive, starch hydrolyzing and lactic acid forming organisms with optimum growth at temperature of 25-40^{o}C.

Table 3. Morphology and biochemical characterization of the bacteria isolate (Lactobacilli sp.)

Morphology	Biochemical Tests
Rod shape	Gram positive
Non-sporulating	Starch hydrolysis (+)
Smooth and raised colony	Heamolysis (-)
Cream colour	Catalase (-)
Non-motile	Lactic acid formation (+)
	Temperature range (25-40 **^{o}C)**
	Urea hydrolysis (-)
	Glucose fermentation (+)
	Hydrogen sulphide (-)
	Indole utilization (-)
	Gelatin hydrolysis (+)

3.3 Screening of the Isolates for β-galactosidase Production

Studies on screening of the bacteria isolates for β-galactosidase production showed that p-NPG and o-NPG used in the nutrient broth showed positive test for β-galactosidase production with yellow colouration after three days of incubation, meanwhile the colouration was found intense in broth infused with p-NPG substrate (Figure 1).

Fig. 1 Chromogenic screening of the bacteria isolates using o-NPG and p-NPG.

3.4 Molecular Characterization

Electrophoretogram of the amplified genome of *Lactobacilli* showed a typical band at 750 bp as shown in the Figure 2.

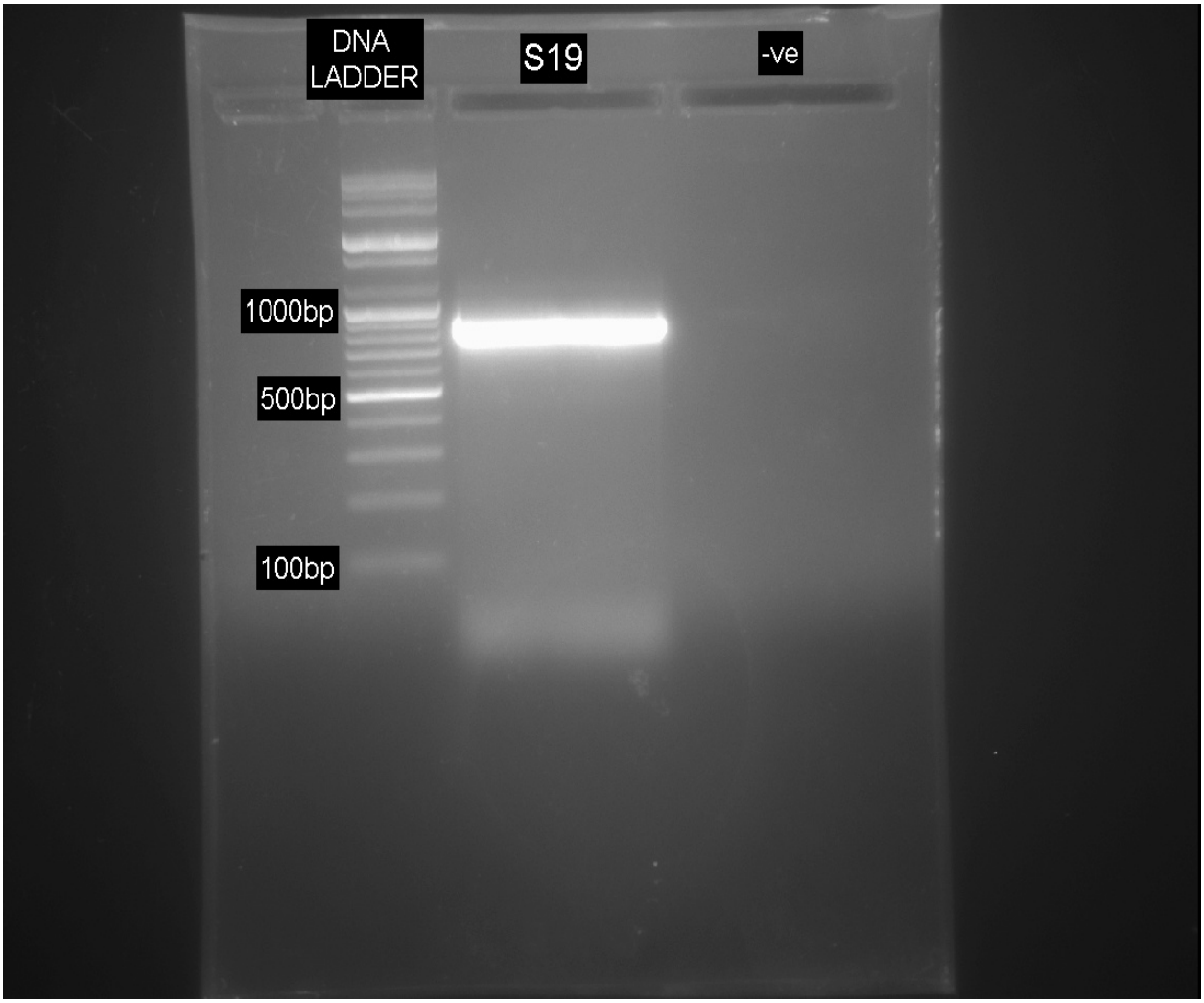

Fig. 2 Electrophoretogram of the amplified genomic DNA viewed on a UV trans-illuminator

Lactobacillus acidophilus was identified after the genomic sequencing with ascribed NCBI accession number of JX255677 as shown in table 4 and Figure 3.

Table 4. Molecular characteristics of the identified strain of Lactobacilli sp.

Sample	NCBI Identification Specie Name	Query cover	Total score	E value	Similarity	Ascension number
Lac.M	*Lactobacilli acidophilus*	97	2728	0.00	99.54	JX255677

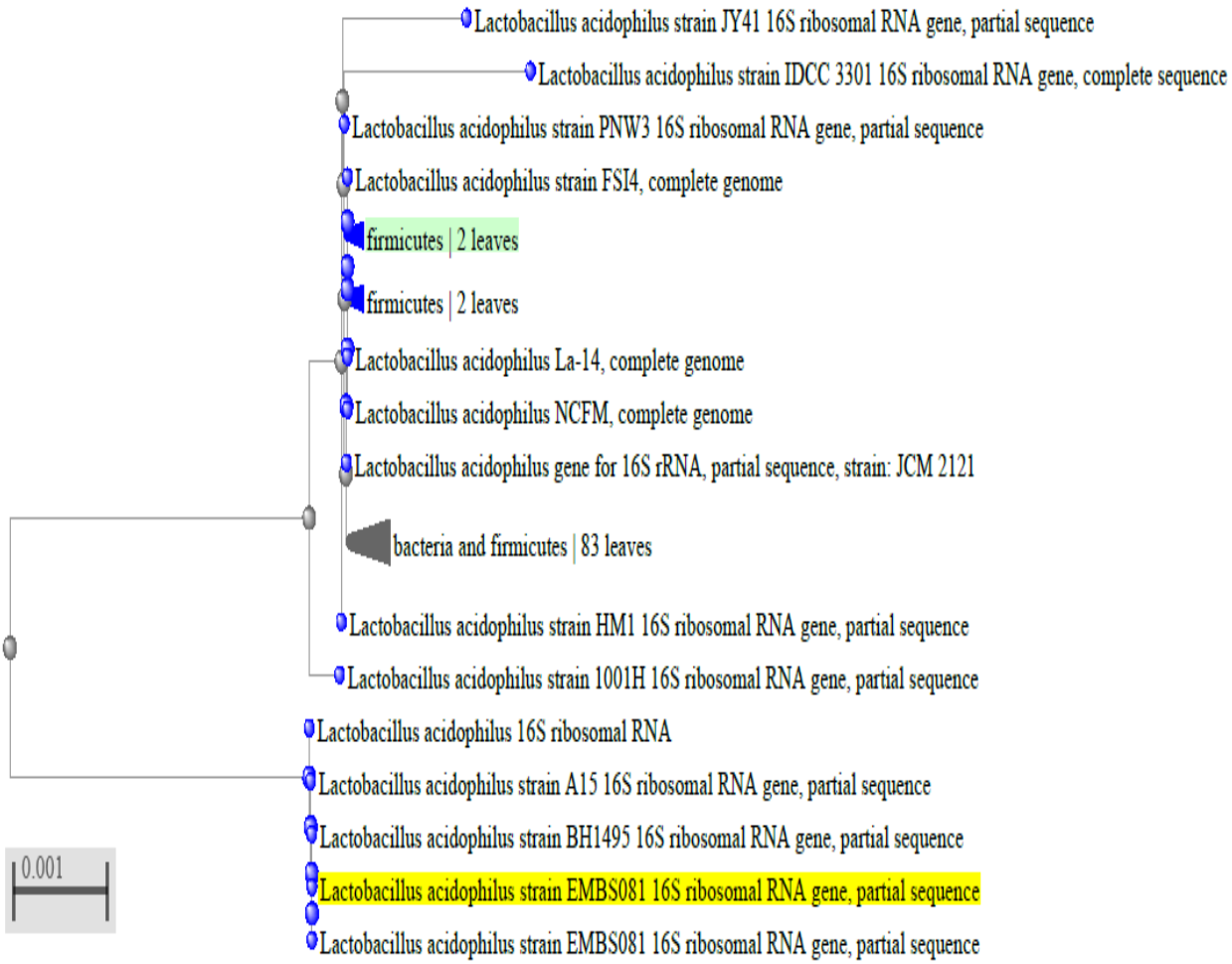

Fig 3. Phylogenic evolutionary relatedness of strains of Lactobacilli acidophilus obtained using NCBI BLAST tools

3.5 β-Galactosidase Production

Effect of Incubation Time on Microbial Growth Rates

Studies on effect of incubation time on the inoculum growth rates at various volumes (0.5 ml, 1.0 ml, 1.5 ml and 2 ml) of the inoculum incubated showed optimum microbial growth at 36th hour of incubation with 2ml of the microbial suspension containing the highest heterotrophic counts of the organism (5.4 x 10^8 CFU/ml) (Figure 4).

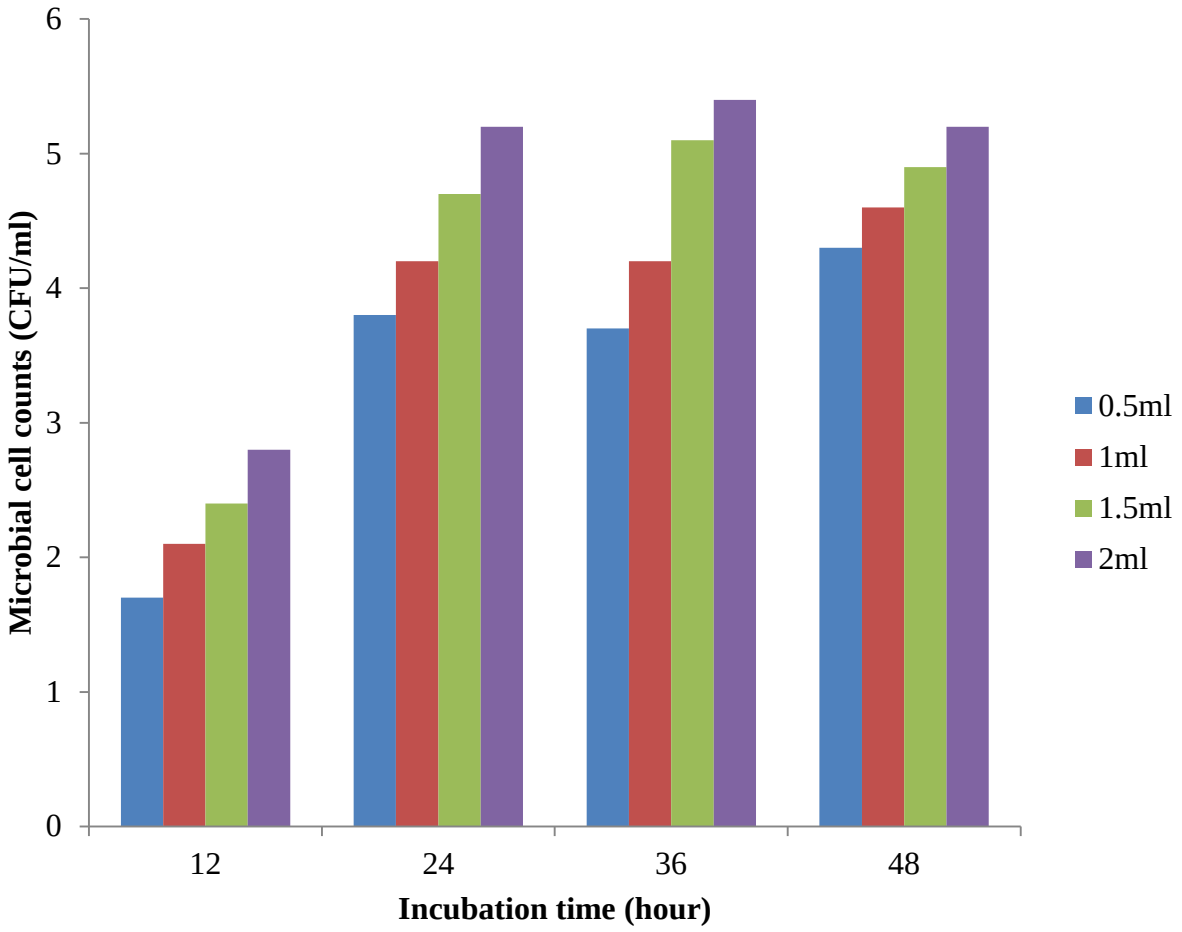

Fig 4. Effect of incubation time on the microbial cell growth rates (CFU/ml) at various inoculum sizes (ml).

Effect of Incubation Time on β- Galactosidase Production

Studies on the effect of incubation time on the production of β-galactosidase from *Lactobacilli acidophilus* in a submerged fermentation system showed that the highest activity and protein concentration were obtained on day 12 (126µmol/min) and day 13 (0.72 mg/ml), respectively of incubation (Figure 5).

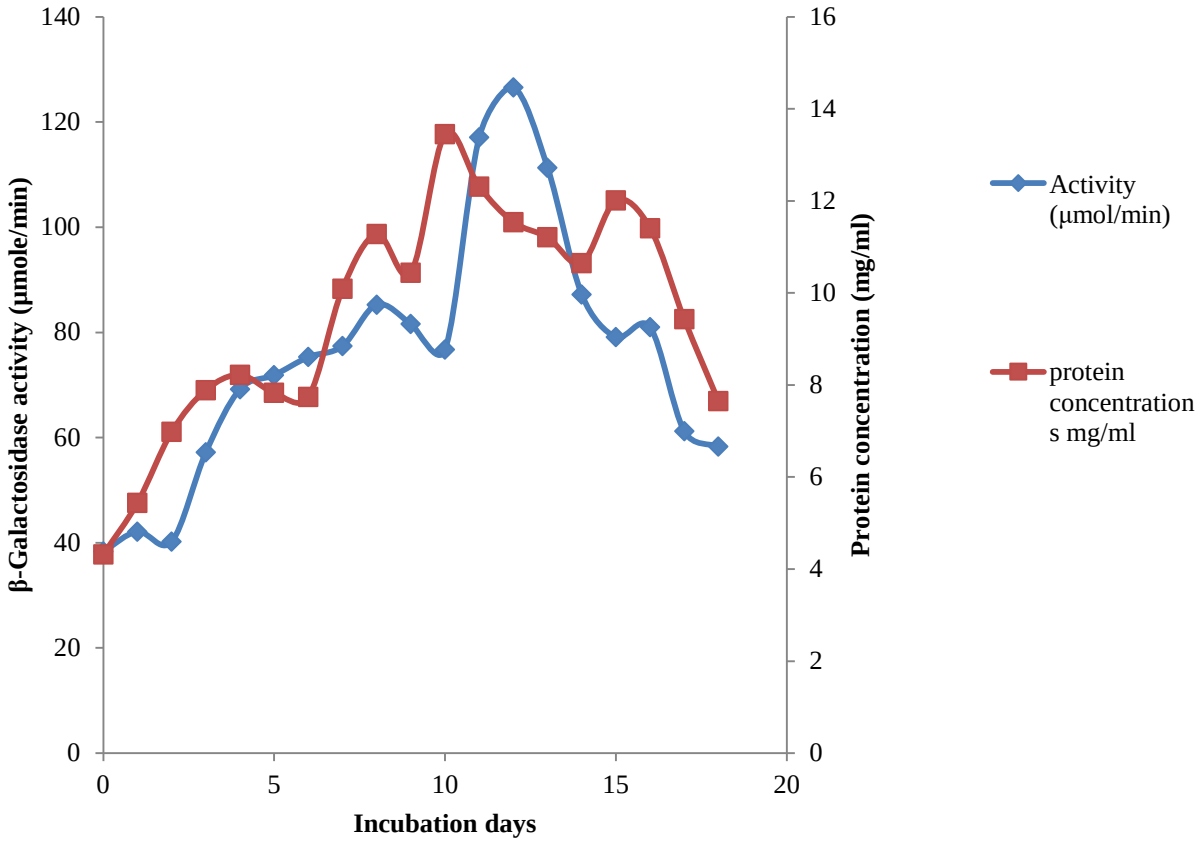

Fig. 5. Effect of incubation days on β- galactosidase production from Lactobacilli acidophilus in a submerged fermentation system.

Effect of pH on Production of β-Galactosidase

β-galactosidase optimum production was found at pH 6.0 with activity of 121.71 µmol/min after 12 days of incubation (Figure 6).

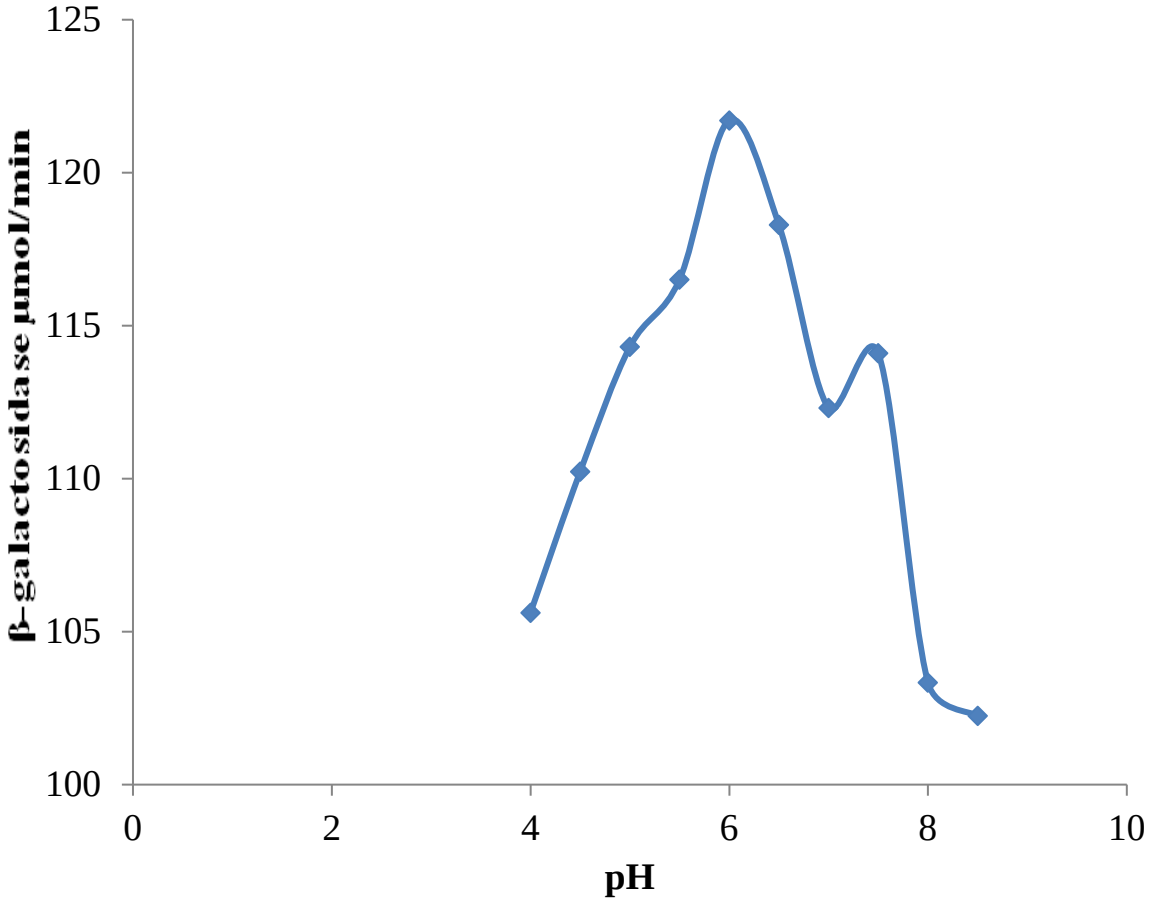

Fig 6. Effect of physiologic pH on production of β–galactosidase from Lactobacilli acidophilus incubated for twelve days (12 days) in a submerged fermentation system using lactose as the sole carbon source.

4. Discussion

Effluent surge tank of the dairy industry located at Rumuekini, Rivers state showed high heterotrophic microbial diversity and activity. In this present study, *Lactobacilli acidophilus* used as starter culture for production of β-galactosidase was isolated from a dairy effluent site located at Rumuekini, Portharcourt, Rivers state. Prior to the organismal isolations and characterizations; physicochemical properties of the dairy waste water was carried out. These properties of samples revealed certain parameters such as the physical and chemical composition of both biological and non biological entities (Valerro, 2010). Physiochemical properties observed from the dairy waste water sample include: Dissolved oxygen concentration (DO) which was at 6.24 mg/ml from which the biochemical oxygen demand (BOD_5) needed for oxidation of carbonaceous organic matter in the water was extrapolated after five days of incubation. The BOD_5 value of the dairy waste water was taken after five days of waste water incubation and was observed at 5.27 mg/ml. This observation varied significantly from the control sample (unpolluted sample) which showed a BOD_5 of 2.26 mg/ml depicting much available dissolved oxygen for biochemical activities of inhabitants of the aquatic environment. Valerro (2010) reported that exposure of water bodies to effluents of biological matter leads to a “BLOOM” in organismal composition of the aquatic

environment at the first phase; however he went further to say that after the initial increase in eutrophication of algal content of the water receiving such effluent of organic matter compositions, organisms biochemical activities increases and such creating a hypoxic condition in the surrounding polluted water. pH and conductivity of the dairy waste water was observed at 5.52 and 879 $\Omega^{-1}cm^{-1}$, these were relatively acidic and high resistivity when compared with the control which showed pH of 7.2 and conductivity of 423 $\Omega^{-1}cm^{-1}$. The low pH range and high conductivity of the dairy wastewater can be attributed to the nature of the recalcitrant in the dairy waste water body such as: whey sugars, additives and preservatives from the dairy industries which are relatively acidic in nature. These components through bio chemodynamic principles integrate itself into the surrounding environment such as water and increase the hydrogen ion concentration of the system (Gheytanchi *et al*. (2010).

Chikere *et al.* (2006) in their study on physicochemical properties of petrochemical effluent from Eleme petrochemical jetty port site reported an acidic pH of 5.02 and effluent conductivity of 1024 $\Omega^{-1}cm^{-1}$ in the contaminated Eleme port effluent. Their finding corroborates with that of the present research. Total dissolved solid (TDS), total suspended solids (TSS) and total solids (TS) of the water were 536.19, 82.65 and 618 mg/ml, respectively. These significant varied from the control experiment which gave 258.70, 48.76 and 307.46 mg/ml for TDS, TSS and TS, respectively. This as reported in the proceedings of ASTDR, 2003 that every exposed water body are characterized by the presence of solid particles which may be suspended within the coastal water axis or dissolved in the olefiers of the water bed. The proceedings went further to state that these solid particles constituents of the water can be as a result of rock weathering, human activities such as querrying, volcanic eruption in the water bed and water bodies eutrophications. Total oxidizable carbon and organic matter content of the diary waste were 72.6 and 89.30 mg/ml, respectively. Temperature of the dairy waste water was observed at 37.67°C which showed no significant variation from the control water sample (34.55°C).

Among the microbes isolated from the dairy waste water, bacteria kingdom was optimal in diversity. Total heterotrophic counts of 2.8 x 10^8 CFU/ml (10^{-2} dilution factor) and 1.65 x 10^5 CFU/ml (10^{-2} dilution factor) of the bacteria isolate were identified on nutrient and differential DeManRagoshie Sharpie (MRS) media plates respectively. This finding showed a relatively higher correlation with that of Corral *et al.* (2006) on ecological relevance in whey surge polluted water. They reported a heterotrophic microbial population of 4.5 x 10^7 CFU/g bacteria on nutrient media plate and that of 2.65 x 10^3 CFU/g of differential media of MRS. Basic morphological and biochemical screening were used to identify the isolate as a *Lactobacilli* genre organism. Basic morphological features of the bacteria showed that strains of *Lactobacilli* are rod shaped, non sporulating and non motile bacteria; biochemically, they are obligate gram positive, starch hydrolyzing and lactic acid forming organisms with optimum growth at 25-40^{o}C. These findings corroborate with that of basic manual for organisms isolations and identifications written by Ezeonu *et al*. (2013)

Organisms are equated as vessels carrying proteins of biochemical relevance (Okpokwasili and Amanchukwu, 1988). Strains of *Lactobacilli acidophilus* isolated from the dairy effluent showed the ability to produce β-galactosidase when screened with standard chromogenic substrates (p-NPG and o-NPG).

Formation of yellow colouration in the nutrient broth containing the organism after 48 hours of incubation at 37^{o}C suggests a positive test for β-galactosidase production. However, the yellow coloration varies in intensity in the infused broth. Intense yellow colouration was seen in the broth infused with p-NPG than that with o-NPG substrate. Para substituted benzene side chains are said to be of less stearic hindrance during chemical reactions especially chemical conformations and cleavages of benzene side chains that any other benzene susbstituted side chains (meta and ortho

positions) (Corral *et al*., 2006). p-NPG however served as the better substrate for the enzyme. Gheytanchi *et al*. (2010) reported similar observation, working with *Lactobacillus* from milk and cheese using o-NPG as their standard screening substrate.

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. *Lactobacillus acidophilus was* identified after the genomic sequencing with ascribed NCBI accession number of JX255677.

Studies on the effect of incubation period on microbial growth rate at different inoculum sizes showed maximum microbial growth of exponential 10^8 CFU/ml in all the inoculum sizes after 36 hours of incubation. Increase in the inoculum size was accompanied proportionately with increase in microbial growth. Decline in microbial growth was observed after the 36^{th} hour of incubation in all the inoculum volume. Ezeonu *et al*., 2013 reported that microbial population largely depend on the inoculum volume, they went further to say that organismal proliferation is categorized in four distinct stages: Lag phase, exponential phase, stationary phase and death phase.

Studies on the effect of incubation period on the production of β-galactosidase from *Lactobacilli acidophilus* showed that the highest β-galactosidase activity and protein concentration were obtained on the 12^{th} and 13^{th} day of fermentation, respectively. Extracellular protein production at these observed days (12 and 13) respectively is evident of catabolite inducement of the substrate present in the fermentation media to the organisms for higher protein production (Allam *et al*., 2013). *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).

pH which is the negative logarithm of hydrogen ion concentration in a medium is an important physiologic factor to be considered in enzyme production. It helps to understand the tolerant nature of the organism to medium's hydrogen ion (H^+). The optimum pH for β-galactosidase production was at 6.0. *Lactobacilli acidophilus* are moderate acidophiles and are tolerant to low pH in production system. Gheytanchi *et al*. (2010) reported pH optimum of 6.5 and 5.5 for β–galactosidase production from milk and cheese respectively.

5. Conclusion

β-galactosidase is a multi-purpose enzyme that has found its way in many production industries including those of foods, clinical and environmental based research industries. The present study has shown the basic enzymology and kinetic properties of β-galactosidase produced from *Lactobacillus acidophilus* isolated from an effluent site. From the results, optimal production of β-galactosidase from cheap available and renewable raw materials was possible.

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Author's Contributions

Oparaji Emeka Henry: Conceived and designed the experiments, performed the experiment and processed the data, analyzed the data and wrote the manuscript.

Ethics

Authors declared no ethical issues that may arise after the publication of this manuscript.

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