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Isolation, production and optimisation of beta-galactosidase by utilizing yeasts isolated from selected dairy products

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ABSTRACT

β-galactosidase is an enzyme that converts lactose into glucose and galactose. It alleviates the issue associated with intolerance to lactose and pollution caused as a result of milk by-products (whey). This study aimed to isolate and select yeast strains that can produce β-galactosidase from fresh milk, yoghurt and locally made cheese. A total of 115 yeasts were isolated from the samples, 9 yeast isolates had the ability to produce β-galactosidase and three (3) were selected for further analysis. β-galactosidase producers were screened using Ortho-nitrophenyl-β-D-galactopyranoside (ONPG) assay. *Kluyveromyces marxianus* strain SLDY–005 produced the highest β-galactosidase. It showed maximum enzyme activity (277 U/mL) at a temperature of 30ºC, pH 5.5, after an incubation period of 36 h. Glucose decreased β-galactosidase production while yeast extract and urea were considered appropriate nitrogen sources for the best enzyme synthesis. Crude β-galactosidase produced by *Kluyveromyces marxianus* SLDY – 005 was purified. The partially purified enzyme after dialysis showed a specific activity of 165.12 U/mL and had a purification fold of 6.02 and yield of 45.91 %. The purified enzyme had an optimum temperature of 40ºC and a pH 6.0. *Kluyveromyces marxianus* strain isolated from local cheese is a potential candidate for the production of β-galactosidase and could be used to combat the problem of lactose intolerance.

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1. Introduction

The enzyme *βeta*-galactosidase is categorized as the glycoside hydrolase subject to its substrate specificity (1).

The capability of the enzyme to catalyze the hydrolysis of monosaccharides (glucose, galactose) as well as prebiotics (galactooligosaccharides, lactulose) made it have potential applications in pharmaceutical, biotechnology and food industries (2, 3).

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The hydrolysis of lactose also provides alternatives for people who have lactose intolerance (4). The lactose hydrolyzed milk could also be used for the production of dairy products for example, ice creams and condensed milk. It could also help to resolve the environmental problems that are associated with whey disposal (5).

The enzyme, *βeta*-galactosidase could also be obtained from several sources: microorganisms, plants and animals (6).

With the use of microorganisms, higher yields of *β*-galactosidase are obtained compared with plants and animals. Furthermore, seasonal variation does not have any effect on the production of *β*-galactosidase (7). So many microbial sources of β-galactosidase are available but not all of them could be taken or endorsed as safe for food use. Yeasts are also known as a predominant microbial enzyme source for food applications which include lactase. Enzyme activity is predisposed by the type of strains and also the growth medium composition (8). The most commonly used microorganisms for the production of *β*-galactosidase are: *Kluyveromyces marxianus, Lactobacillus bulgaricus, Escherichia coli Bacillus circulans, Aspergillus niger, Kluyveromyces lactis* and *Aspergillus oryzae* (9). *Kluyveromyces marxianus* have more advantages for example: it has good growth yield, which is a vital economic impact in the food industry; it is accepted as a safe microorganism for the production of an enzyme, and it has higher β-galactosidase activity than other yeasts (10). The properties, stability and specificity of the enzyme vary with the source of the enzyme (11).

Yeast has been reported to produce an intracellular enzyme with respect to the fungus that is extracellular in nature (12). Hence, yeast cells have to be disrupted by different disruption techniques such as physical and chemical methods (13) for the extraction of intracellular enzyme Lactase enzyme is essential for lactose intolerant person, and has industrial importance because it has been utilized to avoid crystallization of lactose, increases the solubility of milk products and also resolves the problem created with the usage and disposal of whey which can result in ecological or environmental pollution (14). In addition, the transgalactosylation property of β-galactosidase has prominent therapeutic applications such as: the improvement of digestive supplements and the treatment of disorders. It is also applied in biosensors, bioremediation and diagnosis (15). Therefore, the work aimed at the isolation, production and characterization of βeta-galactosidase from yeast strains isolated from dairy products.

2. Materials and Methods

2.1. Isolation of Yeast

Samples were serially diluted in sterile distilled water and plated out using the pour plate method using Yeast Extract Agar (YEA, LAB M) The media was prepared and sterilized according to the Manufacturer's specification. The media was formulated with glucose to enhance yeast growth and Streptomycin was added to suppress the growth of bacteria respectively.

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The plates were incubated at 30°C for about 5 days. Yeast cells were purified by streaking on fresh plates to obtain a pure colony.

The pure colonies were examined and identified under a microscope. The pure cultures were then stored in Yeast Extract broth and covered with glycerol at 4°C for further use.

2.2. Media Preparation and β-galactosidase production

The composition of the medium in g/L include; Yeast Extract 1 g, Lactose 30 g, K_2HPO_4 2 g, $NH_4H_2PO_4$ 1 g, $(NH_4)_2HPO_4$ 1 g and $MgSO_4.7H_2O$ 0.1 g. The pH was 7.0 (16).

2.2.1. β-galactosidase Assay

The isolates were subjected to screening for their ability to produce βeta-galactosidase. This was done by using o-Nitrophenyl β-D-galactopyranoside (ONPG) according to Miller (17). ONPG broth was prepared and filter sterilized. The composition in g/L include; Casein peptone (7.5 g), Na_2HPO_4 (0.35 g), ONPG (1.5 g). NaCl (3.75 g), pH (7.5 ±0.2), temperature (25°C). The mixture was boiled for complete dissolution. The broth was aseptically dispensed in sterile tubes and inoculated with the yeast isolates. The color change from colorless to yellow was observed every four hours for up to 24 h. The secondary screening was done by subjecting yeast isolates with positive results by repeating the above procedure. At the end of the screening experiment, three yeast isolates were selected for further study.

2.3. Optimization of culture conditions for βeta-galactosidase production

The optimization was performed based on the modification of environmental and nutritional parameters.

The effect of environmental factors was determined by a modification of temperature of the fermenting medium in the range of (25, 30, 35, 40 and 45°C), pH in the range of (4.5, 5.0, 5.5, 6.0 and 6.5), incubation period in the range of (24, 36, 48 and 72 h), different nitrogen sources (malt extract, yeast extract, peptone, urea and ammonium sulphate) and different carbon sources (fructose, lactose, sucrose, glucose, galactose, maltose).

2.3.1. Temperature

The optimum temperature was done at different temperatures. The medium was inoculated with the selected isolates (25, 30, 35, 40 and 45°C) for 48 h. After an incubation period at a certain temperature, the amount of intracellular enzyme produced was carried out.

2.3.2. pH

The pH for the production of β-galactosidase was determined by incubating the medium inoculated with the selected yeast isolates at different pH. The fermentation medium was prepared at pH values ranging from 4.5 to 6.5. The buffer employed was 0.1 M Citrate-Phosphate buffer. After an incubation period with certain pH, the amount of intracellular enzyme produced was carried out.

2.3.3. Carbon Sources

Different carbon sources such as fructose, galactose, glucose, maltose, sucrose, and lactose were employed to determine the suitable carbon source for β-galactosidase production by the selected yeast isolates.

2.3.4. Nitrogen Sources

Nitrogen sources such as malt extract, yeast extract, peptone, urea, and $(NH_4)_2SO_4$ were used to determine the suitable nitrogen source for β-galactosidase production by the selected yeast isolates.

2.3.5. Lactose Concentration

Different lactose concentrations (1% - 5%) were employed to find the optimum concentration of lactose for the highest β-galactosidase production.

2.3.6. Incubation Time

This was done by incubating the production medium for different incubation periods (24, 36, 48, 72 h) at the optimum temperature. After incubating at different time, the amount of intracellular enzyme produced was carried out.

2.4. β-galactosidase Assay

Two (2) mL of fermentation medium was centrifuged at 4000 rpm for 15 min and the supernatant was decanted to obtain the pellet. The pellet was washed two times with sterile distilled water (2 mL) and was suspended again in 2 mL 0.1 M phosphate buffer (pH 7) and vortexed with glass beads for 10 min and the resulting mixture was centrifuged at 4000 rpm for 15 min (18). The obtained supernatant was the crude enzyme and was used to assay for β-galactosidase activity. Temperature, pH, carbon sources, nitrogen sources, substrate concentration, and incubation time were the parameters employed to assay for β-galactosidase activity.

The assay was done by adding 2 mL of the crude enzyme with 200 µL of ONPG (4 mg/mL 0.1 M Phosphate buffer, pH 7) and incubating at 30°C for 10 min. After the sufficient yellow colour was noticed, the reaction was altered by adding 0.5 mL 1 M Na_2CO_3.

The optical density was read at 420 nm by the use of a spectrophotometer for determining the enzyme activity (Miller, 1972). One unit of enzyme is defined as the amount of β-galactosidase that releases 1µmol of o-nitrophenol (ONP) from ONPG per minute under the assay conditions (Miller, 1972)

2.5. Molecular identification of the highest β-galactosidase producer

DNA Extraction Protocol

The extraction of the yeast genomic DNA for molecular analysis was done using the method of Arnold *et al*. (19). One hundred mg of fungal mycelia was taken into a sterile mortal, then 1ml of DNA Extraction Buffer (DEB) containing proteinase K (0.05 mg/mL) was added and macerated with a sterile pestle. The extract was transferred into a 1.5 mL Eppendorf tube. 50 µL of 20% Sodium Dodecyl Sulphate (SDS) was added and incubated in a water bath at 65°C for 30 min. The tubes were allowed to cool to room temperature and 100 µl of 7.5 M Potassium Acetate was added and mixed briefly. The mixture was centrifuged at 13000 rpm for 10 min and the supernatant was then transferred into fresh autoclaved tubes. To the supernatant 2/3 volumes of cold Isopropanol / Isopropyl alcohol were added, and the tubes were inverted (3-5) times gently and incubated at -20°C for 1 h. After incubation, the mixture was again centrifuged at 13000rpm for 10 min and the supernatant was discarded.

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Five hundred µL of 70% ethanol was added and centrifuged for another 5 min at 13000 rpm. The supernatant was carefully discarded with the DNA pellet intact. Traces of ethanol were removed and the DNA pellets dried at 37°C for 10-15 min. DNA pellets were then resuspended in 50 µL of Tris-EDTA (TE) buffer. Aliquot DNA was stored at -20°C for further lab analysis.

2.6. Protein Concentration of partially purified β-galactosidase

Protein content was done using the method of Lowry *et al. (20)* and Bovine Serum Albumin (BSA) was used as standard. Samples were read at 660 nm using a spectrophotometer. The samples were analyzed against respective blank solutions. Protein concentration readings were read in duplicate using average value was used for the calculation.

2.7. Characterization of Partly Purified β-galactosidase

For the determination of optimum temperature, activity was done by carrying out the standard assay at several temperature values. The reaction mixture was incubated for 30 min in the temperature range of 30 – 70°C with an interval of 10°C.

For the determination of the thermal stability of β-galactosidase, the crude enzyme was incubated at different temperatures from 30°C – 70°C (interval of 10°C) for 30 min before the addition of ONPG solution. The residual activity was measured by following the standard assay conditions. Enzyme-buffer mix was stored at optimum temperature (30°C) for 30 min and used as the control.

The effect of pH of β-galactosidase activity was determined by assaying at different pH values (4 to 8) with an interval of 1 in all pH ranges. The buffer used is citrate phosphate buffer. β-galactosidase activity was measured following the standard assay conditions (17).

2.8. Enzyme Purification

Partial Purification of β-galactosidase was done by using the following methods. The steps involved were conducted at room temperature except when specified.

2.8.1. Ammonium Sulphate Precipitation

A 40 ml of crude extract that has βeta-galactosidase activity was precipitated by slowly adding 16 g ammonium sulphate over a period of time and placed on ice with a constant stirring up to a final concentration of 40 % (w/v) (21, 22). The mixture was allowed to stay overnight. The centrifuged precipitate at 10000 rpm, for 10 min, and at a temperature of 4°C was collected with the use of a cold centrifuge (Eppendorf, Centrifuge 5427 R) and used for further purification.

2.8.2. Gel-Filtration Chromatography

A 16 mL of the partly purified enzyme above was eluted in a column (XK 26 Pharmacia) packed with Sephadex G-25 solution (Sephadex G-25 in PBS solution). Samples were eluted with the same buffer at a flow rate of 75 mL/ h and a total of 50 fractions of the eluted enzyme were collected the optical density at 660 nm was read with a spectrophotometer in order to determine the enzyme activity and protein concentration.

The fractions with the highest enzyme activity were pooled and assayed for protein content. The specific activity of the purified enzyme was compared to that of the crude enzyme and fold purification was calculated (23).

2.8.3. Dialysis

A 5 ml of the precipitate obtained with 40% saturation of ammonium sulphate was dialyzed against 0.1 M phosphate buffer saline (pH 7) solution overnight (Basha *et al.,* 2009; Mukesh *et al.,* 2012). The precipitate obtained was decanted into sterile sampling bottles. The optical density of the dialyzed and non-dialyzed enzyme was read at 660nm using a spectrophotometer for the determination of the enzyme activity and protein concentration.

2.9. Statistical Analysis

The β-galactosidase activity experiment was done in triplicate and the standard deviation was determined using SPSS. Analysis of variance (ANOVA) was also used to compare the various treatment groups.

3. Results

3.1. Screening of selected yeast isolates for β-galactosidase production on lactose

Selected yeast isolates (belonging to *Kluyveromyces* spp) were screened for their ability to secrete β-galactosidase (lactase) in the presence of ONPG, a substrate for β-galactosidase production and their ability to produce β-galactosidase is characterized by the formation of yellow colouration which results from the breakdown of ONPG into ONP (a yellow compound) and galactose. *Kluyveromyces marxianus strain SLDY-005* gave the highest enzyme activity of 277 U/mL.

3.2. Effect of incubation temperature on β-galactosidase production

The effect of incubation temperatures (25, 30, 35, 40, and 45^{o}C) on β-galactosidase production by *Kluyveromyces marxianus* SLDY-005 under submerged state fermentation is shown in Fig. 1. A gradual increase in β-galactosidase production was observed with increase in temperature until 30^{o}C when it began to drop. The enzyme activity at this optimal temperature was 155 U/mL.

3.3. Effect of pH on β-galactosidase production

The enzyme activity of β-galactosidase by *Kluyveromyces marxianus* SLDY-005 was done by varying the pH of the fermentation media from 4.5 to 6.5 (Fig. 2). Maximum enzyme activity of β-galactosidase (145 U/mL) was achieved when the pH of the medium was kept at 6.0. An increase in the pH resulted into a drop in the enzyme activity.

3.4. Effect of Incubation period on β-galactosidase production

The fermentation medium in five flasks was incubated at different time duration; 24, 30, 36, 42 and 48 h and β-galactosidase activities were 89 U/mL, 119 U/mL, 126 U/mL, 121 U/mL and 109 U/mL as shown in Fig. 5. Thus, at 36 h of incubation, maximum yield was obtained. The production of enzymes increased with an increase in the fermentation period beyond 36 h, and a decline in enzyme production was observed.

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3.5. Effect of different carbon sources on β-galactosidase production

This was done by varying five carbon sources; fructose, maltose galactose, glucose and sucrose. Lactose is evident to show the highest β-galactosidase production with enzyme activity of 112 U/mL. It was also discovered that the presence of glucose hinders β-galactosidase production with a low activity of 38 U/mL. In this analysis, lactose was used as a control as it's a natural inducer of β-galactosidase (Fig. 4).

3.6. Effect of different nitrogen sources on β-galactosidase production

In this study, five nitrogen sources; malt extract, ammonium sulphate, peptone, yeast extract and urea were added separately to the fermentation medium replacing yeast extract from the mineral salt medium. Among all these nitrogen sources, yeast extract gave the maximum production of β-galactosidase with an activity of 145 U/mL and ammonium sulphate showed the least β-galactosidase production with an activity of 78 U/mL as shown in Fig. 3.

3.7. Molecular Identification

Gene sequences from the isolate characterized showed 90% identity similar to *Kluyveromyces marxianus strain SLDY-005* internal transcribed spacer 1, partial sequence; 5.8 S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene partial sequence.

3.8. Characterization of Partially Purified β-galactosidase

3.8.1. Effect of Temperature on Partially Purified β-galactosidase Activity

This was done by determining the activity at different temperature ranges. The results obtained showed that β-galactosidase activity increased with an increase in temperature but was optimal at 40^{o}C (147 U/mL) and thermally active between 40^{o}C and 50^{o}C (147 – 114 U/mL). A drastic decrease in the enzyme activity (from 114 U/mL to 57.9 U/mL) was observed as the temperature increased above 50^{o}C (Fig. 6).

3.9. Effect of pH on the Enzymatic Activity

This was done by the determination of the activity at various pH ranges. Results obtained indicated that the enzyme activity was optimally active at pH 6.0 (Fig. 7) with an activity of 136 U/mL.

3.10. Protein Content

Table 1 shows the protein content of partially purified β-galactosidase at each of the three purification stages. It was observed that the protein content decreases as the purification steps increases. The protein content is indirectly proportional to the specific activity of the enzyme which increases as the purification step increases.

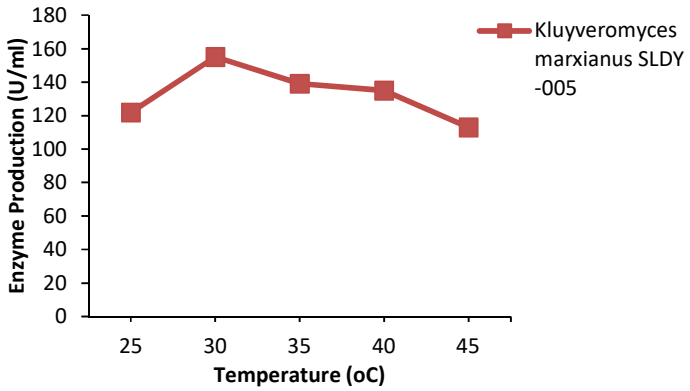

Figure 1. Effect of temperature on β-galactosidase production

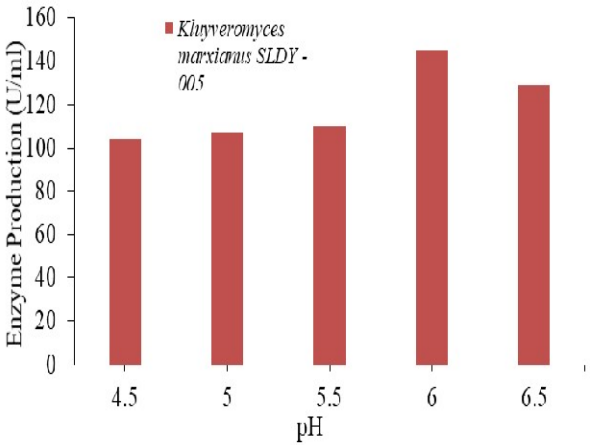

Figure 2. Effect of pH on β-galactosidase production

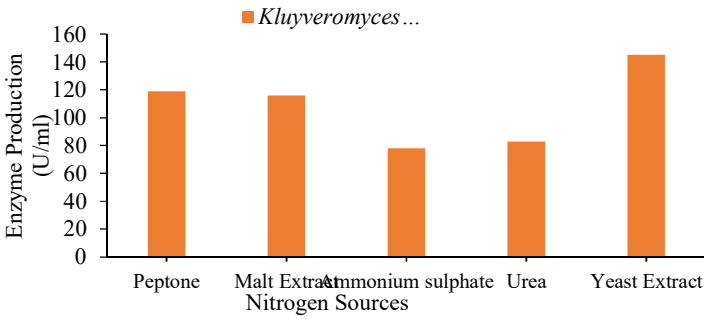

Figure 3. Effect of different nitrogen sources on β-galactosidase production

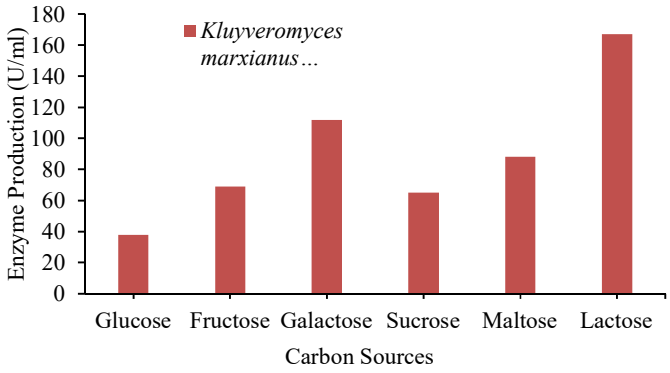

Figure 4. Effect of different carbon sources on β-galactosidase production

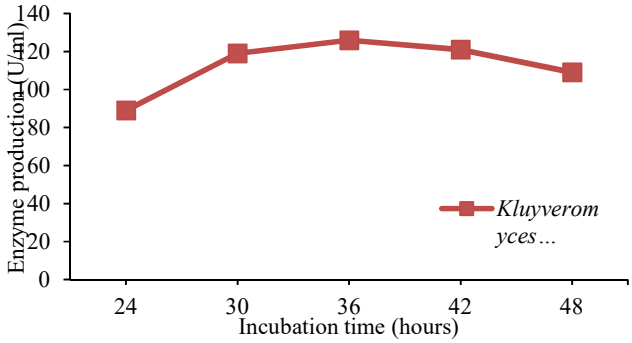

Figure 5. Effect of incubation temperature on partially purified β-galactosidase

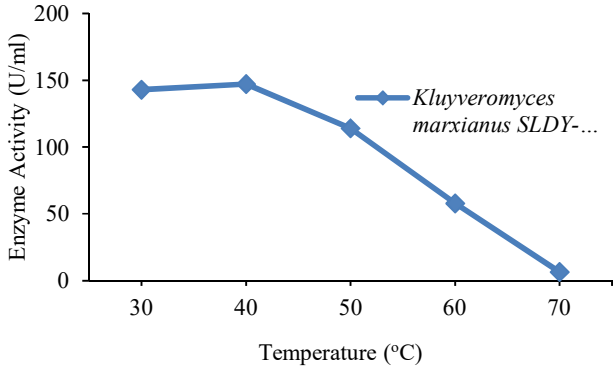

Figure 6. Effect of temperature on partially purified β – galactosidase

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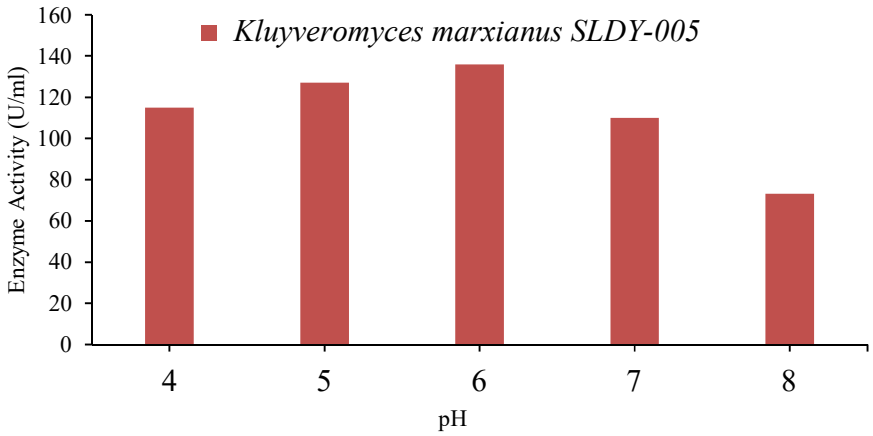

Figure 7. Effect of pH on partially purified β-galactosidase

Table 1. Protein Content of β-galactosidase produced by *Kluyveromyces marxianus* SLDY-005

Purification Step	Total Volume (ml)	Total enzyme activity (U)	Total Protein Concentration (mg)	Specific Activity (U/mL)	Purification Fold	Yield (%)
Crude β-galactosidase	40	428	15.6	27.4	1	100
Ammonium Sulphate Precipitation	40	240	8.6	27.9	1.01	56.07
Gel Filtration Chromatography	16	204.8	2.45	83.59	3.05	47.85
Dialysis	5	196.5	1.19	165.12	6.02	45.91

4.Discussion

This study has demonstrated that the Nigerian local cheese is a rich source for the isolation of yeasts with β-galactosidase producing ability. The biochemical identification and molecular identification of the selected yeast strains indicated that *Kluyveromyces* spp is the dominant group of yeast that is found in dairy products and *Kluyveromyces marxianus* is one of the most frequently occurring yeast in cheese. *Kluyveromyces marxianus* SLDY-005 used in this study has attributes such as, good growth yield, which is important in the food industry; it is accepted as safe yeast for enzyme production, it also has higher β-galactosidase activity when compared with some other yeast (24).

According to Rech and Ayub (19), this yeast has been known for it's efficient ability to utilize high lactose concentration products and convert this sugar into high value-added products in the production of enzymes like βeta-galactosidase (20) and bio-ethanol (21).

Fermentation parameters: pH, carbon and nitrogen, incubation time and temperature sources are significant and important factors that affect β-galactosidase production. In addition, the highest accumulation of intracellular β-galactosidase was observed after 36 h of fermentation. There was a slight reduction in the production of enzymes after 36 h and Rech and Ayub (19) have attributed this to a reduction in oxygen limitation caused by the high oxidative metabolism of yeast. Ramirez-Matthew and Rivas (22) reported an optimal incubation period between 18 and 24 h using *Kluyveromyces marxianus* NCIM 3551 while Gupte and Nair (23) signaled a stationary phase after 30 h of cultivation. There was an increase in β-galactosidase activity of the cells with an increase in incubation time, this may be due to an increase in the cell biomass. There was a further reduction in the β-galactosidase activity which could be a result of inhibition in cell growth at the stationary phase of growth. In this study, it showed that pH, temperature, carbon and nitrogen sources, and incubation time influenced β-galactosidase enzyme production by *Kluyveromyces marxianus* SLDY-005

The optimum temperature was observed at 30°C for maximum β-galactosidase activity with a decrease above this optimum temperature. Rosso in his research stated that (24), when the temperature of the environment is higher or lower than the temperature required, the activity of the microorganism is reduced and this result in a decrease in enzyme activity or cell lysis. The variation of enzyme activity as a function of temperature showed that, there was an increase in βeta-galactosidase activity with an increase in temperature up to 30°C. The results obtained are in accordance with the one obtained by Babu et al., (25) who reported that better β-galactosidase production is observed at 30°C using *Kluyveromyces marxianus* isolated from paneer whey. In another way, this is not in accordance with the result obtained by Kujumdzieva et al., (26) who cited a temperature of 40°C for β-galactosidase production.

The pH is also a factor that affects the cell growth and synthesis of enzymes. In this research work, the best pH for the production of β-galactosidase by the yeast isolates was 6.0 and a pH above this led to a reduction in β-galactosidase production. Results obtained by Gupte and Nair (23) assumed that a pH of 5 is an appropriate pH for good productivity of β-galactosidase by *Kluyveromyces marxianus*. However, the result of this study is not in accordance with the

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above observation. However, similar results which suggested a pH between 5.5 and 6.0 as the optimum pH for β-galactosidase production by *Kluyveromyces marxianus* were found in most of the available literature. A pH of 5.0 was also observed for *Kluyveromyces lactis* by Gupte and Nair (23). Therefore, the results in this work were similar to those obtained by (27-31). Besides, Klovrychev et al. (32) and Gupte and Nair (23) have scientifically established that the pH of the medium has an effect on RNA and protein synthesis. Glucose was an inefficient inducer of β-galactosidase and this agreed with the work of Hsu et al., (33) who described glucose to be a poor inducer of β-galactosidase.

It was observed that all the nitrogen sources used significantly enhanced the production and activity of the enzyme but to variable degrees. This difference may be attributed to their variable composition. The enzyme production increase was noticed with the addition of nitrogen into the media, and this could be due to the important role of nitrogen which serves as a main component of protein and nucleic acids in the growth of microorganisms. Nitrogen sources affect the microbial biosynthesis of β-galactosidase. According to Akcan (34), organic and inorganic forms of nitrogen sources are metabolized by the microbial cells to produce proteins, nucleic acids, amino acids and cell wall compounds. From the study, yeast extract gave the maximum β-galactosidase activity by the isolates and this result is compatible with those described by Manera et al., (35).

Kumari (3) reported urea as the highest source of nitrogen for β-galactosidase by novel yeast isolated from whey. Rao and Dutta (36) signaled that malt extract was highly stimulatory for β-galactosidase production.

5. Conclusion

The genus *Kluyveromyces* has been implicated for their ability to hydrolyze lactose efficiently to synthesize β-galactosidase and galacto-oligosaccharides which has been known for their prebiotic potentials. However, for the yeast strain *Kluyveromyces marxianus* SLDY-005, incubation temperature at 30^{o}C, incubation time of 36 h, and pH of production medium at 6.0, was the most suitable conditions for β-galactosidase production. However, it can be concluded from this study that *Kluyveromyces marxianus* strain isolated from local cheese is a potential candidate for β-galactosidase production. This also adds to existing information by some other researchers that *Kluyveromyces marxianus* is good yeast for β-galactosidase production. All the properties of β-galactosidase suggest its possible use in the reduction of the high lactose content of dairy and its products for people who are lactose intolerant, the remediation of pollution caused as a result of dairy waste and conversion into economically useful materials.

Conflict of interest

The authors declare no conflict of interest.

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