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ORIGINAL RESEARCH ARTICLE

A Study on the Lactase Potential of *Kluyveromyces lactis* Grown in Whey

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ABSTRACT

The lactase potential of a non-pathogenic lactose fermenting yeast *Kluyveromyces lactis* isolated from yogurt was tested by growing the strain in whey supplemented with various concentrations of organic nitrogen sources (peptone, tryptone and casein), inorganic nitrogen sources (ammonium sulfate, ammonium nitrate, ammonium chloride), phosphorous sources (sodium di hydrogen phosphate, disodium hydrogen phosphate, trisodium phosphate) and growth factors (beef extract, yeast extract, malt extract) followed by testing the lactose utilization patterns and enzyme activity. The maximum enzyme activity (121.42 ± 0.4 U/mg lactase) was observed with 2.5% yeast extract supplement followed by 1.5% malt extract (111.35 ± 0.4 U/mg lactase), 2% peptone (103.57 ± 0.4 U/mg lactase) and 2% ammonium chloride (101.78 ± 0.3 U/mg lactase). The specific lactase activity was assayed using ONPG.

Key words: β-D-galactosidase, *Kluyveromyces lactis,* Cheese whey,organic and inorganic nitrogen sources.

INTRODUCTION

Lactase (β-D-galactosidase, EC 3.2.1.23) is an enzyme which catalyzes the hydrolysis of lactose to galactose and glucose. β-D-galactosidase has two enzymatic activities: one is responsible for the hydrolysis of lactose and also cleaves cellobiose, cellotriose, cellotetrose and to a certain extent cellulose and the other, splits β-glycosides [1]. The enzyme is continuously synthesized in induced cultures and in batch operations, the maximum yield is obtained at the beginning of the stationary phase of growth, after which the yield decreases [2]. Lactase or β-D-galactosidase enzyme is present in various bacterial species such as *Escherichia coli, Bacillus megaterium, Thermus aquaticus, Streptococcus lactis, Streptococcus thermophilus, Lactobacillus bulgaricus, Lactobacillus helveticus* and lactose fermenting yeasts such as *Kluyveromyces lactis, Kluyveromyces fragilis*, *Candida pseudotropicalis* and fungi such as *Neurospora croussa, Aspergillus foetidus, Aspergillus niger, Aspergillus flavus, Aspergillus oryzae, Aspergillus phoenicis, Mucor pucillus and Mucor meihei* [3]. The most outstanding lactases in terms of technological interest come from yeast of the *Kluyveromyces* genus [4]. *Kluyveromyces lactis* has highest adhesive ability and most attractive for use as a probiotic micro organism [5]. In terms of commercial applications, the yeast *Kluyveromyces lactis* deserves special attention as it is considered by the United States Food and Drug administration as a safe organism for the use and production of lactase aimed at the food and pharmaceutical industry [6].

Cheese whey constitutes a major disposal problem in the dairy industry. The discharge of untreated whey into rivers is being banned in many countries. Lactose content of whey reaches 4.8% and it includes relatively high levels of other nutrients that make it suitable as microbial culture medium [7,8]. Micro organisms capable of using lactose as the sole carbon source are the producers of β-D-galactosidase [9,10]. Lactase deficiency is a wide spread problem occurring in approximately 70% of the world's population. Consumption of milk by lactase deficient individuals results in lactose mal-absorption and often in abdominal pain, diarrhoea and flatulence. Bacterial cultures used in making yogurt produce some of the lactase enzyme required for proper digestion. However, yogurt is well tolerated by lactase deficient individuals and causes significantly less lactose mal-absorption than milk. The improved lactose digestion appears due to auto digestion by microbial β-galactosidase [11]. There is a wide

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spread interest both from nutritionists and commercial producers in reducing the lactose content of some dairy products. Lactose hydrolysis benefits from several advantages of rapid fermentation of glucose, higher sweetness of the liquid in which lactose has been hydrolysed, higher solubility of glucose and galactose, high stability of lactose-hydrolyzed dairy products such as frozen condensed milk, rapid fall of pH in cheese made from lactose-hydrolyzed milk and in consequence rapid development of cheese flavor and texture [12]. Sweet syrup produced by lactose conversion through hydrolysis of whey by β-galactosidase can be used in dairy, confectionary, baking and soft drink industries [13]. Isolation and purification of β-galactosidase is easy because of its large molecular size. Various methods are available for determining β-galactosidase activity. ONPG can be used as a substrate and progress of the reaction can be followed by estimating the chromogen O-nitrophenol [14]. The search continues for organisms yielding large quantities of lactase which can be easily extracted and which possesses characteristics suitable for intended applications. The present study was aimed to investigate the lactase potential of *Kluyveromyces lactis* grown in whey supplemented with various nutritional requirements.

MATERIALS AND METHODS

Sampling

Yogurt sample was purchased in sealed 250 ml plastic containers from retail outlets.

Isolation and identification of yeast cells

5.0ml of yogurt sample was mixed with 5.0ml of 0.1% sterile peptone solution and then serially diluted. 0.2ml volumes of appropriate dilutions were spread on to malt extract agar containing 100µg of filter sterilized oxytetracycline. Plates were incubated at 25ºC for 4 days [15]. Isolated yeast cells were purified by sub-culturing on to malt extract agar slants.

Isolated yeast cells were examined by Gram stain and identified by standard biochemical methods. Acid production from carbohydrates (glucose, maltose, sucrose, lactose, trehalose, galactose, raffinose) in fermentation basal medium was evaluated. Assimilation patterns of various carbohydrates (mannitol, maltose, sucrose, xylose, raffinose, ribose, rhamnose, inositol) in yeast nitrogen base by the organism was tested. Their ability to grow at various temperatures (37^0C and 40^0C) in glucose-peptone-yeast extract broth was examined after 1-2 weeks and their ability to grow in a medium containing 50% glucose [16] was also tested.

Medium for propagation

De proteinized whey was prepared by heating whey at 90ºC for 10 minutes and filtered through Whatmann no.1 filter paper to remove the coagulated protein [17]. pH of the whey was adjusted to 7.0 and it was supplemented with various organic nitrogen sources-peptone, tryptone, casein (0.5-3.0% wt/vol), inorganic nitrogen sources- ammonium sulfate, ammonium nitrate, ammonium chloride (0.5-3.0% wt/vol), phosphorous sources-mono, di and tri basic salts of phosphate (0.5-3.0% wt/vol) and growth factors-beef extract, yeast extract and malt extract (0.5-3.0% wt/vol). Whey containing the nutrients were inoculated with the organism and incubated at 30ºC for 48 hours. After incubation, the cells were harvested by centrifuging at 7000 rpm for 5 minutes at 4ºC. The pelleted cells were washed twice and resuspended in phosphate buffer (0.1M pH 6.6) and subjected to disintegration. The supernatant was tested for lactose utilization.

Lactose utilization

The ability of the organisms to utilize lactose present in whey was studied by determining the amount of lactose present in whey before inoculation and after the incubation period. Quantitative determination of lactose in whey was performed by the method of Teles [18] using Teles' reagent. To 2.5 ml of the supernatent, 0.2ml of 5% Zinc sulphate and 0.2 ml of 4.5% barium hydroxide were added and centrifuged at 2500 rpm for 15-30 seconds. 1 ml of the clear supernatants was transferred to screw cap bottles, 2.5 ml of Teles reagent was added and the bottles were tightly stoppered with a dry rubber stopper. The bottles were immersed in a violently boiling water bath for exactly 6 minutes. They were cooled immediately in tap water and the volume was brought to 12.5 ml with distilled water. The contents were mixed thoroughly and the absorbance was read at 520 nm against a similarly treated reagent blank in which 2.5 ml of distilled water replaces the sample. The results were compared with a standard solution of lactose and the concentration of lactose in samples was determined by using the formula:

$$\text{Lactose (mg / ml)} = \frac{\text{Absorbance of sample x 50}}{\text{Absorbance of standard}}$$

Preparation of cell free extracts

Cell suspensions were shaken in the presence of 0.04% SDS in a shaker at 200 rpm for 1 hour

(30ºC) and centrifuged at 4000 x g for 20 minutes [19]. The supernatant was collected as cell free extract and it was used for the protein estimation and enzyme assayThe concentration of protein in the cell free extract was determined by the method of Lowry *et al*.,1951 [20] .

β-D-Galactosidase enzyme assay

Specific lactase activity of the cell free extracts was assayed using *O*-nitrophenyl -β-D-galactopyranoside (ONPG) as the substrate by the method of Mahoney [10]. Specific activity is a measure of the amount of enzyme present relative to other proteins. To 0.1 ml of cell free extract, 4 ml of 1.25 mm ONPG containing 0.1 M potassium phosphate buffer (pH 7.0) was added and incubated at 37ºC. 1 ml of 0.5 M Na_2Co_3 was added to stop the reaction when a faint yellow colour developed. The reaction time was noted and the absorbance was read at 420nm. The enzyme assay was carried out in triplicates for all cell free extracts. Specific activity was calculated using the formula [21]:

$$\text{Specific Activity [Units / mg]} = \frac{OD_{420} \times 380}{\text{Time (Minutes)} \times \text{Protein (mg)}}$$

RESULTS

Based on the results of standard biochemical tests, the organism isolated from the yogurt was identified as *Kluyveromyces lactis.*

Lactose utilization by Kluyveromyces lactis

Among organic nitrogen supplements 2% peptone showed the highest level (145.45 mg) of stimulation in lactose utilization. When the concentration of peptone was increased, there was a decrease in the lactose utilization. 2.5%, 3% tryptone and 3% casein showed (140.91 mg) of stimulation in lactose utilization.

Among inorganic nitrogen supplements 3% ammonium sulfate and 2% ammonium chloride showed the highest level of stimulation in lactose utilization (145.45 mg). However higher concentrations of ammonium chloride reduced the level of lactose utilization. 2%, 2.5%, 3% ammonium nitrate showed (140.91 mg) of stimulation in lactose utilization.

Among various phosphorous supplements, 2% disodium hydrogen phosphate and 2.5% trisodium phosphate showed the highest level (140.91 mg) of stimulation in lactose utilization. Higher concentrations of it gradually reduced the amount of lactose utilized. The effect of sodium di hydrogen phosphate in lactose utilization was comparatively less. Even 3% sodium di hydrogen phosphate showed only lower level (136.36 mg) of stimulation in lactose utilization.

Among the various growth factor supplements, 2%, 2.5% yeast extract and 1.5% malt extract showed the very high level of stimulation in lactose utilization (150mg) when compared to all other supplements. Higher concentrations of yeast and malt extract was inhibitory. 2% and 2.5% of beef extract showed (140.91mg) of stimulation in lactose utilization.

Estimation of protein

Among organic nitrogen supplements, high concentration of protein was present in 3% tryptone (0.156 mg/ml). In the concentrations of 2% peptone and 3% casein, 0.152mg/ml of protein was present.

Among inorganic nitrogen supplements, high concentration of protein was present in 2% ammonium sulfate, 2.5%, 3% ammonium chloride and 2.5% and 3% ammonium nitrate (0.148 mg/ml).

Among phosphorous supplements, high concentration of protein was present in 2.5% trisodium phosphate (0.156 mg/ml). In disodium hydrogen phosphate 2%, 0.152 mg/ml of protein was present. Among different concentrations of sodium di hydrogen phosphate, 3% contained 0.148 mg/ml of protein.

Among various growth factor supplements, 2% yeast extract, 2% and 2.5% malt extract had the very high concentration of protein (0.164 mg/ml) among various supplements. In 2% and 2.5% beef extract, 0.156 mg/ml of protein was present.

β-D-Galactosidase enzyme assay

Among organic nitrogen supplements, peptone had the greatest stimulatory effect. Addition of 2% peptone increased the lactase yield from 74.50 ± 0.3 U/mg in its absence to 103.57 ± 0.4 U/mg. However, higher concentrations of peptone reduced the yield of lactase. The effect of tryptone was less in lactase yield when compared to peptone. Addition of 2.5% tryptone yielded 89.28 ± 0.2 U/mg lactase. Casein had poor stimulatory effect on lactase production. 1.5 % casein yielded only 82.93 ± 0.3 U/mg lactase. A higher concentration of casein was inhibitory.

Among inorganic nitrogen supplements, 2% ammonium chloride yielded high amount of lactase 101.78 ± 0.3 U/mg. Higher concentrations of it gradually reduced the enzyme yield. Ammonium sulfate 2% yielded 91.69 ± 0.2 U/mg lactase. This was less when compared to ammonium chloride. Higher concentrations of ammonium sulfate also reduced the yield of

lactase enzyme. In 2.5% and 3% Ammonium nitrate, the enzyme yield was little higher 99.03 ± 0.2 U/mg.

The effect of phosphorous supplements in enzyme yield was less comparing to other supplements. Phosphorous supplements had poor stimulatory effect. Among various phosphorous sources, 2% disodium hydrogen phosphate yielded 89.28 ± 0.2 U/mg lactase. Higher concentrations of this salt further reduced the enzyme yield. 3% sodium di hydrogen phosphate yielded 88.03 ± 0.2 U/mg of lactase. 0.5% and 1% trisodium phosphate yielded 87.81 ± 0.3 U/mg lactase. Higher concentrations of it were inhibitory.

Among growth factor supplements, yeast extract had the greatest stimulatory effect among all the other supplements. Addition of 2.5% yeast extract yielded 121.42 ± 0.4 U/mg lactase. Higher concentrations of it were inhibitory. Malt extract also had higher stimulatory effect. 1.5% malt extract yielded 111.35 ± 0.4 U/mg lactase. However, higher concentrations of it were inhibitory. 2% and 2.5 % beef extract yielded 100.91 ± 0.2 U/mg lactase. When the concentration was increased to 3% the enzyme activity was reduced.

Fig 1: Effect of Organic Nitrogen supplements on lactase production by *Kluyveromyces lactis*

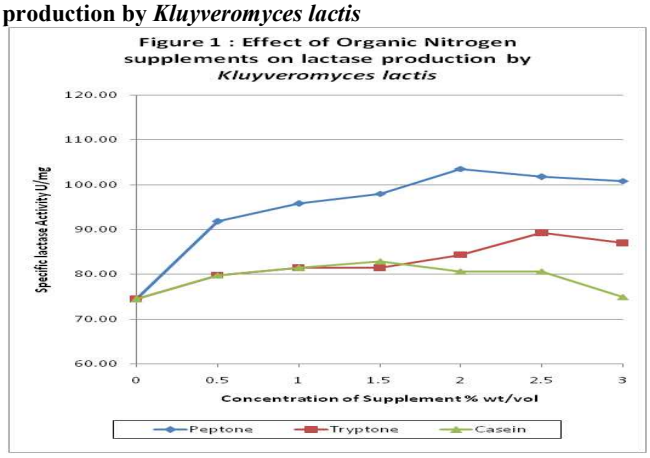


Fig 2: Effect of Inorganic Nitrogen supplements on lactase production by *Kluyveromyces lactis*

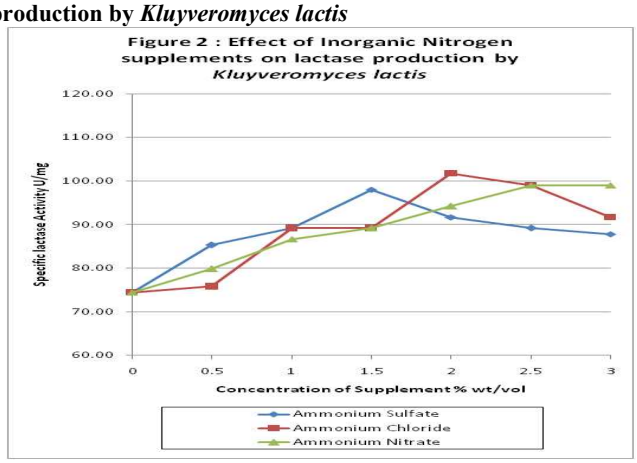


Fig 3: Effect of phosphorus supplements on lactase production by *Kluyveromyces lactis*

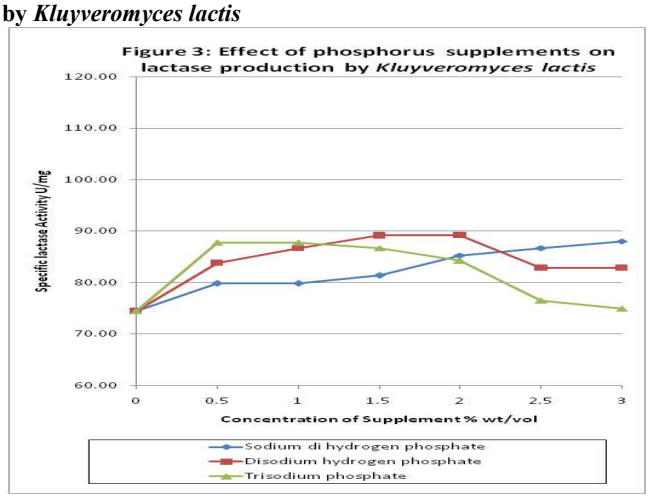
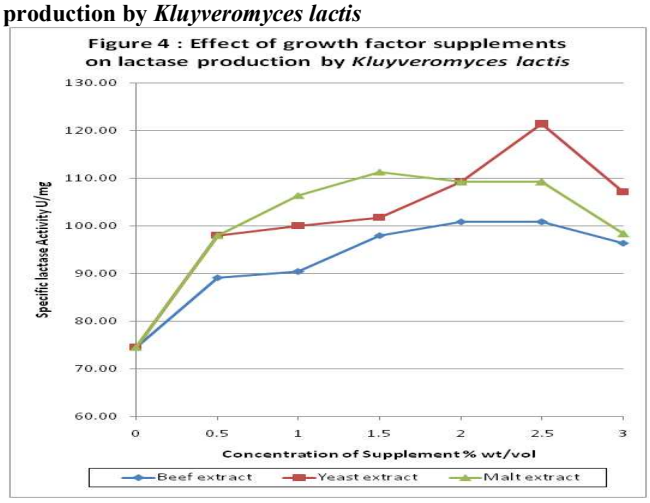


Fig 4: Effect of growth factor supplements on lactase production by *Kluyveromyces lactis*



DISCUSSION

Lactase (β-D-galactosidase, EC 3.2.1.23) is an endo enzyme which catalyzes the hydrolysis of lactose to glucose and galactose. The enzyme is found in plants, animals and micro organisms. Although lactase is found in plants and animals, only the enzyme of microbial origin is of industrial interest. A lactose fermenting yeast *Kluyveromyces lactis* was found to produce highest amount of lactase. It is considered as safe and it was most suitable for use as probiotic micro organism. The lactose fermenting yeast *Kluyveromyces lactis* was isolated from yogurt by serial dilution of yogurt and spread plating on to malt extract agar containing oxytetracycline. After incubation smooth, creamy white, ellipsoidal colonies were formed. Similar type of colonies of *Kluyveromyces lactis* was reported by Savova and Nikolova [22]. In carbohydrate fermentation test, the organism had the ability to ferment glucose, lactose, galactose and raffinose. The organism was unable to ferment maltose and trehalose. In carbohydrate assimilation test, the organism was found to assimilate mannitol, sucrose, xylose and

raffinose for its growth and the organism does not assimilate ribose, rhamnose, inositol and maltose. Similar carbohydrate fermentation and assimilation results were reported by Nahvi and Moeini [16].

When the growth of the organism was tested by inoculating the organism in Glucose-peptone-yeast extract broth and incubating it at37ºC and 40ºC; visible growth was observed in the broth incubated at both 37ºC and 40ºC. Similar results were reported by Nahvi and Moeini [16] and Savova and Nikolova [22]. When the growth of the organism was tested by inoculating the organism in a medium containing 50% glucose; the organism had the ability to grow and produce colonies in 50% glucose medium. Similar results were reported by Nahvi and Moeini [16]. Based on the carbohydrate fermentation pattern, carbohydrate assimilation pattern, growth in 37˚C and 40˚C and 50% glucose; the organism isolated from yogurt was identified as *Kluyveromyces lactis*.

Production of lactase by *Kluyveromyces lactis* grown in whey supplemented with varying concentrations of various nutrients was studied. The organism was propagated in supplemented deproteinized whey, their lactose utilization patterns were studied and cell free extracts were prepared. The protein concentrations of the cell free extracts were determined by the method of Lowry *et al*., [20] and their specific lactase activity was determined using ONPG as the substrate.

In case of *Kluyveromyces lactis*, the highest enzyme activity was observed in 2.5% yeast extract supplement. The specific enzyme activity in 2.5% yeast extract was found to be 121.42 ± 0.4 U/mg. of the various organic nitrogen sources used, peptone (2%) showed the significant stimulatory effect on lactase production yielding 103.57 ± 0.4 U/mg. This was in accordance with the lactose utilization pattern under peptone supplementation (2% peptone stimulated utilization of 145.45 mg of lactose in whey). The protein concentration of 2% peptone was also high (0.152 mg/ml). The similar stimulatory effect of 2% peptone was reported by Rao and Dutta [17] incase of *Streptococcus thermophilus*. In case of tryptone, 2.5% yielded 89.28 ± 0.2 U/mg lactase. This was less when compared to peptone. The lactose utilization pattern of 2.5% and 3% tryptone had the same rate of stimulation in lactose utilization (140.91 mg). The protein concentration in 2.5% tryptone supplement was 0.152 mg/ml, but the protein concentration of 3% tryptone supplement was 0.156 mg/ml. Casein had poor stimulatory effect on lactase production. 1.5% casein yielded only 82.93 ± 0.3 U/mg lactase. However, higher concentrations of it were further inhibitory. Among various inorganic nitrogen sources, 2% ammonium chloride showed significant lactase activity (101.78 ± 0.3 U/mg). The lactose utilization pattern of 2% ammonium chloride was also high (utilization of 145.45 mg of lactose from whey). But the protein concentration was higher in 2.5% ammonium chloride (0.148 mg/ml) when compared to 2% ammonium chloride. Ammonium nitrate 2.5% and 3% yielded 99.03 ± 0.2 U/mg lactase. Similarly lactose utilization was also high (140.91 mg) in 2.5% and 3% ammonium nitrate. Protein concentration of 2.5% and 3% ammonium nitrate was also high (0.148 mg/ml). This was in controversy with the results of Rao and Dutta [17]. According to their result stimulation by inorganic nitrogen sources were poor in case of *Streptococcus thermophilus.* But in present study, inorganic nitrogen sources have some stimulatory effect in case of *Kluyveromyces lactis*. 1.5% ammonium sulfate yielded 98.01 ± 0.3 U/mg lactase. This was in controversy with the results of Barbosa *et al*., [23]. According to their results 0.3% ammonium sulfate increased the enzyme yield in case of *Kluyveromyces fragilis*. There is also a possibility that for *Kluyveromyces lactis* 1.5% ammonium sulfate may be stimulatory.

The stimulatory effects of all the three phosphorous supplements (sodium di hydrogen phosphate, disodium hydrogen phosphate and trisodium phosphate) in lactase yield were similar. Among the three, 2% disodium hydrogen phosphate yielded 89.28 ± 0.2 U/mg lactase. The effects of other phosphorous salts were very less compared to 2% disodium hydrogen phosphate. The supplementation of whey with phosphorous salts had no significant effect on lactose utilization and enzyme yield. Similar results were reported by Bales and Castillo [24].

Of the various growth factor supplements, the greatest enzyme yield was obtained in whey supplemented with 2.5% yeast extract. The specific lactase activity in 2.5% yeast extract was 121.42 ± 0.4 U/mg. This was in accordance with the lactose utilization pattern under yeast extract supplementation (2.5% yeast extract stimulated utilization of 150 mg of lactose in whey). The protein concentration of 2.5% yeast extract was 0.152 mg/ml and the protein concentration of 2% yeast extract was 0.164 mg/ml. Although protein

concentration of 2% yeast extract was high, the enzyme activity was very high in 2.5% yeast extract. The stimulatory effect of yeast extract on lactase production has been reported by several workers (Davies [25]; Mahoney *et al.*,[10] and Shweta et al., [26]). 2% and 2.5% beef extract showed specific lactase activity of 100.91 ± 0.2 U/mg. 1.5% malt extract also produced highest lactase yield 111.35 ± 0.4 U/mg. The lactose utilization was also high in 1.5% malt extract (utilization of 150 mg of lactose in whey). According to Rao and Dutta, [17], malt extract was not stimulatory in case of *Streptococcus thermophilus*. But in case of *Kluyveromyces lactis* 1.5% malt extract was highly stimulatory.

In this present study it was found that *Kluyveromyces lactis* produces high amount of lactase in whey supplemented with 2.5% yeast extract (121.42 ± 0.4 U/mg) followed by 1.5% malt extract (111.35 ± 0.4 U/mg), 2% peptone (103.57 ± 0.4 U/mg) and 2% ammonium chloride (101.78 ± 0.3 U/mg). From the results obtained in the present study it can be inferred that *Kluyveromyces lactis* is a good source for the production of commercial lactase.

CONCLUSION

From the results obtained in the present study it was concluded that production of lactase by *Kluyveromyces lactis* grown in whey would be a viable commercial venture since it was considered as safe to use as a probiotic microorganism and also it gives high enzyme yield. The use of lactase could also reduce the amount of lactose in whey which causes environmental pollution when discharged in large quantities. It also represents as a potential source of lactase for the treatment of milk and sweet whey to reduce their lactose content. The hydrolysis of lactose in dairy products by lactase can help lactose mal-absorbers, alleviate problems and improves processes for dairy products and overcome low solubility and lack of sweetness in concentrated milk products.

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