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Producción de α-galactosidasa por Bacillus sp. LX-1 en fermentación en estado sólido para su aplicación como potencial aditivo alimenticio

Produção de α-galactosidase por Bacillus sp. LX-1 em fermentação em estado sólido para o uso como um potencial aditivo na alimentação

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Summary

Background: α-galacto-oligosaccharides, including raffinose and stachyose, are present in soybean meal and used widely as a protein source in poultry diets. These compounds have anti-nutritive effects that ultimately reduce performance and value of birds. Thus, the addition of exogenous α–galactosidase to poultry diets —which can initiate the digestion of these non-digestible sugars— can be an effective strategy to solve the nutritional disorders associated with consumption of these oligosacharides. Solid state fermentation (SSF) has drawn attention for the production of microbial enzymes, due to the possibility of using cheap and abundant agro-industrial residues as substrates. **Objective:** to present information on α–galactosidase production under SSF conditions by an Antarctic bacterial isolate, *Bacillus* LX-1. **Methods:** initially, wheat bran, soybean meal, corn flour and the combinations of these individual substrates with nutritive supplements containing 1% galactose, 0.5% yeast extract, 1% tryptone, and 0.001% $MnSO_4 4H_2O$ were evaluated to select an optimal medium in SSF to produce extracellular α–galactosidase. Certain fermentation parameters involving incubation time, moisture content, and initial pH were investigated separately. Additional studies were conducted to evaluate the influence on enzyme production of different carbon sources (glucose, sucrose, galactose, lactose, and maltose) and nitrogen sources (peptone, tryptone, sodium nitrate and ammonium sulfate). **Results:** a medium containing soybean meal resulted in best α-galactosidase synthesis and was used for further SSF explorations with *Bacillus* sp. LX-1. Maximum enzyme production was observed at a growth period of 72 h, 75% moisture content and pH 8.0. Enzyme activity was enhanced in the presence of galactose or lactose as the carbon source, and tryptone or peptone as the nitrogen source. **Conclusion:** this SSF technique could be potentially used to produce **α-**galactosidase for poultry feed.

Key words: *α-galacto-oligosaccharides, enzyme, poultry feeds, soybean meal.*

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Resumen

Antecedentes: los α-galacto-oligosacáridos, incluyendo rafinosa y estaquiosa, están presentes en la harina de soja, la cual es utilizada ampliamente como fuente de proteína en dietas de aves. Estos compuestos tienen efectos anti-nutricionales que reducen el rendimiento de las aves. La adición de α-galactosidasa exógena a dietas de aves puede iniciar la digestión de esos azúcares, resultando en una estrategia eficaz para resolver los desórdenes nutricionales asociados con el consumo de dichos oligosacáridos. La fermentación en estado sólido (SSF) se puede usar para producir enzimas microbianas, debido a la posibilidad de utilizar residuos agro-industriales abundantes y baratos como sustrato. **Objetivo:** informar sobre la producción de α-galactosidasas por una bacteria antártica (*Bacillus* LX-1) bajo condiciones de SSF. **Métodos:** se evaluaron salvado de trigo, harina de soja, harina de maíz y las combinaciones individuales de estos sustratos con suplementos nutritivos conteniendo 1% de galactosa, 0,5% de extracto de levadura, 1% de triptona y 0,001% de $MnSO_4 4H_2O$ para seleccionar un medio óptimo de SSF para producir α-galactosidasa extracelular. Ciertos parámetros de fermentación incluyendo tiempo de incubación, contenido de humedad, y pH inicial se evaluaron por separado. Se realizaron estudios adicionales para evaluar la influencia de diferentes fuentes de carbono (glucosa, sucrosa, galactosa, lactosa y maltosa) y de nitrógeno (peptona, triptona, nitrato de sodio y sulfato de amonio) sobre la producción enzimatica. **Resultados:** un medio con harina de soja resultó ser el mejor para la síntesis de α-galactosidasa y se utilizó para nuevas exploraciones de SSF con *Bacillus* sp. LX-1. La producción máxima de la enzima se observó en un periodo de crecimiento de 72 h, a 75% de humedad y pH 8,0. La actividad enzimática mejoró en presencia de galactosa o lactosa como fuente de carbono, y triptona o peptona como fuente de nitrógeno. **Conclusión:** la técnica de SSF podría ser utilizada para producir α-galactosidasa destinada a la alimentación de aves de corral.

Palabras clave: *α-galacto-oligosacáridos, alimentación en aves de corral, enzima, harina de soja.*

Resumo

Antecedentes: os α-galacto-oligossacarídeos, incluindo rafinose e estaquiose, estão presentes na farinha de soja, que é amplamente utilizado como uma fonte de proteína em dietas de aves domésticas. Estes compostos têm efeitos anti-nutricionais que reduzem o desempenho das aves. A adição de α-galactosidase exógena em dietas de aves pode começar a digerir estes açúcares, resultando em uma estratégia eficaz para resolver os problemas nutricionais associados com o consumo desses oligossacarídeos. A fermentação em estado sólido (SSF) pode ser usada para produzir enzimas microbianas, devido à possibilidade de utilização de resíduos agroindustriais como substrato abundante e barato. **Objetivo:** relatar a produção de α-galactosidase por uma bactéria da Antártida (Bacillus LX- 1) em SSF. **Métodos:** foram avaliados o farelo de trigo, farelo de soja, farelo de milho e combinações específicas destes substratos com suplementos nutricionais contendo 1% de galactose, 0,5% de extrato de levedura, 1% de triptona e 0,001% de MnSO44H2O para selecionar um meio ideal de SSF para produzir extracelular α-galactosidase. Certos parâmetros de fermentação, incluindo tempo de incubação, teor de umidade e pH inicial foram avaliados separadamente. Estudos adicionais foram conduzidos para avaliar a influência de diferentes fontes de carbono (glucose, sacarose, galactose, lactose e maltose) e de azoto (peptona, triptona, nitrato de sódio e sulfato de amónio) na produção da enzima. **Resultados:** um meio com farelo de soja acabou por ser o melhor para a síntese de α-galactosidase e foi usado para uma maior exploração de SSF com *Bacillus* sp. LX - 1. A produção máxima da enzima foi observada em um período de crescimento de 72 h, 75% de humidade e pH 8,0. Melhoria da atividade enzimática na presença de galactose ou lactose como fonte de carbono e de triptona e peptona como fonte de azoto. **Conclusão:** A técnica da SSF poderia ser usada para produzir α-galactosidase para a alimentação de aves domésticas.

Palavras chave: α*-galacto-olígosacarídeos, ração para aves, enzima, farelo de soja.*

Introduction

Soybean meal (SBM) is widely used as a protein source in poultry diets. However, it contains α-galacto-oligosaccharides, such as raffinose and stachyose, which cannot be digested in the small intestine due to the absence of the endogenous α-galactosidase enzyme (Gitzelmann and Auricchio, 1965). This enzyme catalyzes the hydrolysis of terminal non-reducing α-1,6-linked galactosyl residues from galacto-oligosaccharides and polysaccharides (Naumoff, 2004). The presence of non-digested oligosaccharides

in the intestine leads to fluid retention and increases the flow rate of the digesta, which can hinder the digestion and absorption of other nutrients (Wiggins, 1984), as well as cause flatulence and gastrointestinal distress that reduce feed efficiency (Ulezlo and Zaprometova, 1982). Furthermore, galacto-oligosaccharides are highly hygroscopic. This leads to increased wetness of excreta (Bedford, 1995) favoring bacterial growth and exacerbating footpad lesions and breast-skin discoloration (Chesson, 1993), ultimately reducing bird performance and value (Graham *et al*., 2002). Thus, the addition of exogenous α-galactosidase to poultry diets can be an effective strategy to solve this nutritional disorder (Kidd *et al*., 2001; Graham *et al*., 2002).

Solid state fermentation (SSF) for microbial enzyme production has drawn attention due to the possibility of using cheap and abundant agro-industrial residues as substrates (Shankar and Mulimani, 2007), and because of the interest in processes where the crude fermented product may be used directly as the enzyme source (Pandey *et al*., 1999; Holker and Lenz, 2005; Krishna, 2005). The production of feed-grade enzymes, such as amylases, cellulases, tannases and phytases, by SSF and their application in feed have been extensively examined (Bogar *et al*., 2002; Gautam *et al*., 2002; Sabu *et al*., 2002; Singhania *et al*., 2009). Even the use of SSF feed produced by microbial strains can improve piglet performance and benefit gastrointestinal microflora (Van Winsen *et al*., 2001; Feng *et al*., 2007).

Until now, most SSF studies concerning α–galactosidase production have been conducted by using filamentous fungi (Liu *et al*., 2007a; Rajoka *et al*., 2009; Kapnoor and Mulimani, 2010) because of their abilities to survive on a complete solid substrate and to produce a variety of extracellular enzymes (Krishna, 2005). However, little information on α–galactosidase production in SSF using bacteria is available in the scientific literature, except for the filamentous actinobacterium *Streptomyces griseoloalbus* (Anisha *et al*., 2008a and 2008b; Anisha *et al*., 2010). The objective of this work was to explore the production of α–galactosidase under SSF conditions by an Antarctic bacterial isolate, *Bacillus* LX-1.

Materials and methods

Reagents

p-Nitrophenyl-α-D-galactopyranoside (*p*NPG) substrate for the α-galactosidase assay was purchased from Sigma-Aldrich (St. Louis, MO, USA). Peptone, tryptone, yeast extract, tryptic soy broth and bacto agar were purchased from BD Biosciences (San Jose, CA, USA). All other chemicals used in this study were of analytical grade and also purchased from Sigma-Aldrich.

Microorganism and inoculum

The bacterial strain *Bacillus* sp. LX-1 has been already described (Lee *et al*., 2012). The strain was used for α-galactosidase production. It was grown for inoculum preparation in tryptic soy agar consisting of tryptic soy broth and 1.5% bacto agar at 37 ºC for 24 h. A single fresh colony was retrieved and added to a 50 mL Falcon conical tube (BD Biosciences) containing 5 mL of tryptic soy broth at 37 ºC for 12 h on a rotary shaker (220 rpm). A 1% volume of the resulting 12 h culture was transferred to a 250 mL Erlenmeyer flask containing 50 mL of tryptic soy broth and incubated at 37 ºC for 24 h. This pre-culture was used as the inoculum for SSF.

SSF

Commercial quality wheat bran, soybean meal, and corn flour obtained from local suppliers were initially evaluated to select the appropriate solid substrate for the fermentation. Briefly, 10 g of each substrate was placed alone in 250 mL Erlenmeyer flasks or together with nutritive supplements (galactose: 10 g, yeast extract: 5 g, tryptone: 10 g and $MnSO_4 4H_2O$: 0.01 g per liter). Initial pH and moisture content was 5.5 to 6.0 and 50%, respectively. After autoclaving at 121 ºC for 15 min, the flasks were inoculated with a 4% inoculum and incubated at 28 ºC with vigorous shaking (220 rpm). Unless otherwise stated, SSF was obtained using 10 g soybean meal (75% moisture content) in 250 mL Erlenmeyer flasks with a 4% inoculum, followed by incubation at 28 ºC for 72 h. The effects of various physicochemical parameters including moisture content (33.3 to 80%) of the substrate, incubation time (0 to 168 h) and pH

(5 to 9) were investigated for the optimum production of α-galactosidase by *Bacillus* sp. LX-1. Additional studies were conducted to evaluate the influence of carbon sources (glucose, sucrose, galactose, lactose and maltose; final concentration of each: 1%) and nitrogen sources (peptone, tryptone, sodium nitrate and ammonium sulfate; final concentration of each: 1%) on enzyme production.

Enzyme extraction

Crude enzymes were extracted by mixing a weighed quantity of the fermented matter with 40 mL cold distilled water and shaking the mixture on a rotary shaker (220 rpm) at 28 ºC for 1 h. The suspension was spun down by centrifugation (4 ºC, 10 min and 5,000 rpm), and the supernatant was used for the α-galactosidase assay.

Enzyme assay

Unless otherwise stated, α-galactosidase activity was assayed at 40 ºC for 15 min in a reaction mixture consisting of 0.8 mL 50 mM sodium phosphate (pH 7.0), 0.1 mL 10 mM *p*NPG, 0.08 mL distilled water and 0.02 mL of the crude enzyme. The reaction was stopped by adding 1 mL of 1 M Na_2CO_3, and color development was measured at a wavelength of 405 nm ($OD_{405\ nm}$). One unit (U) of enzyme activity was defined as the amount of enzyme required to produce 1 nmol of *p*-nitrophenol per second under the given assay conditions and expressed as U/g of dry substrate in SSF.

Statistical analysis

Data were processed by analysis of variance using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC, USA). When *F*-tests were significant, means were separated using Duncan's multiple range test (Duncan, 1965). The statistical significance was set at an alpha level of 0.05. The experimental units for the calculation of mean values were the crude enzymes of the supernatant samples extracted from the fermented matter under SSF conditions.

Results

Substrate selection for α–galactosidase production in SSF

As shown in Table 1, soybean meal was the most effective substrate for α–galactosidase production by *Bacillus* sp. LX-1 in the absence or presence of nutritive supplements ($p<0.05$). Meanwhile, combinations of individual substrate with nutritive supplements containing 1% galactose, 0.5% yeast extract, 1% tryptone and 0.001% $MnSO_4 4H_2O$ showed no effect on enzyme synthesis, except for wheat bran. Therefore, soybean meal was the only substrate used for further studying SSF with *Bacillus* sp. LX-1.

Table 1. α-Galactosidase synthesis by *Bacillus* sp. LX-1 using different solid substrates.

Solid substrate	[2]Enzyme activity (U/g)
wheat bran	843[c]
soybean meal	31,759[a]
corn flour	737[c]
wheat bran + [1]nutritive supplements	13,058[b]
soybean meal + [1]nutritive supplements	29,939[a]
corn flour + [1]nutritive supplements	809[c]
Standard error of the mean	1,132
p-value	<0.001

[1]Nutritive supplements consisted of 10 g/L galactose, 5 g/L yeast extract, 10 g/L tryptone, and 0.01 g/L $MnSO_4 4H_2O$.

[2]Enzyme activity was assayed at 30 ºC by the method described in the text. Each least square of mean represents three experiments. Different superscript letters indicate significant difference ($p<0.05$).

Enzyme production at different fermentation periods

α–Galactosidase production was followed for 168 h. Maximum enzyme activity was observed at 72 h of fermentation. Thereafter the enzyme production level did not improve (Figure 1).

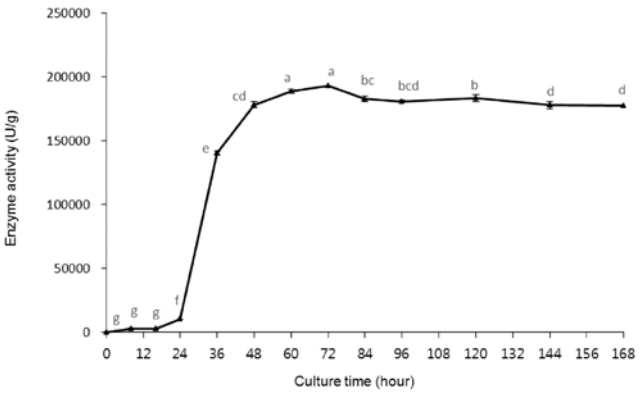

Figure 1. Time course of LX-1 α-galactosidase production in SSF (28 ºC, 75% moisture) using soybean meal. Data were expressed as mean±standard error from three experiments. [a-g] means without a common letter differ (p<0.05).

Effect of initial moisture content on enzyme production

The highest α–galactosidase production (228,294 U/g, mean of enzyme activity) was obtained with 75% moisture content (p<0.05; Figure 2). However, about 30% (72,884 U/g, mean of enzyme activity) of the maximal enzyme production was maintained at 50% moisture content (Figure 2).

Effect of initial pH of the medium on enzyme production

α–Galactosidase synthesis occurred in a relatively wide pH range (5 to 9) and optimal enzyme yield was observed at pH 8 (p<0.05; Figure 3).

Effect of additional carbon sources on α-galactosidase production

Galactose and lactose enhanced the enzyme activity (p<0.05), even if other carbon sources had no positive impact on the enzyme synthesis (Figure 4). On the other hand, glucose considerably repressed production of the enzyme (p<0.05).

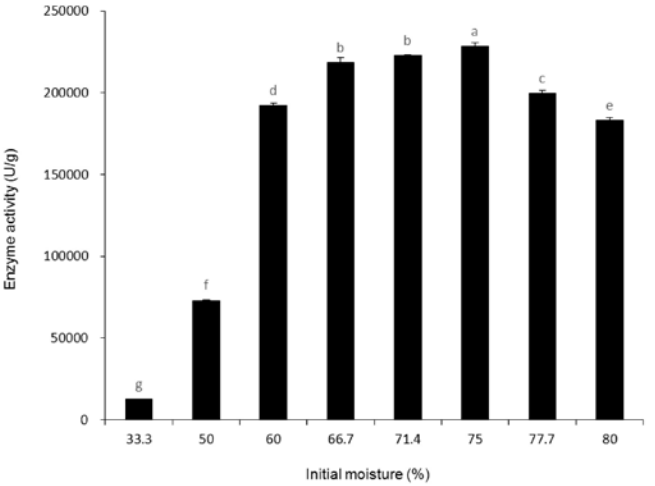

Figure 2. Effect of initial moisture content on LX-1 α-galactosidase production in SSF (28 ºC, 96 h cultivation). Data were expressed as mean ± standard error from three experiments. [a-g] means without a common letter differ (p<0.05).

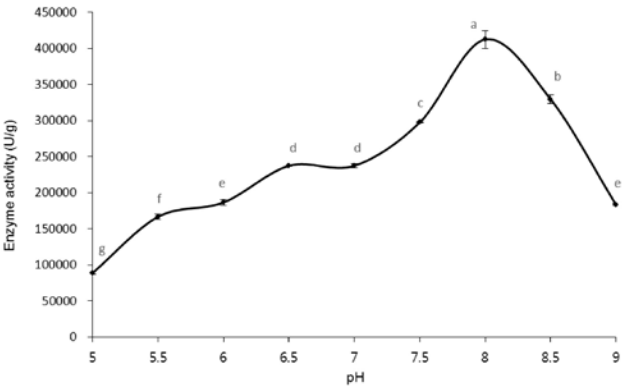

Figure 3. Effect of initial pH of the fermentation medium on LX-1 α-galactosidase production in SSF (75% moisture, 28 ºC, 72 h cultivation). Data were expressed as mean ± standard error from three experiments. [a-g] means without a common letter differ (p<0.05).

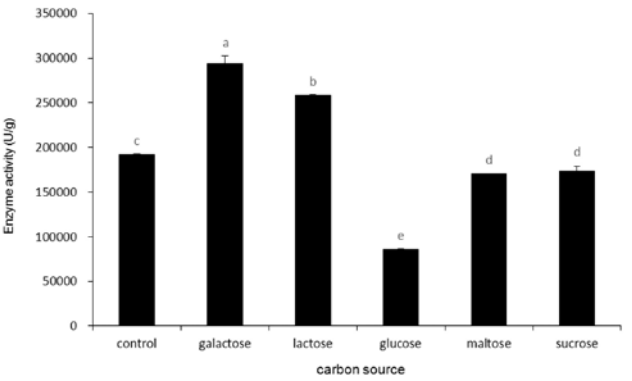

Figure 4. Effect of different carbon sources on LX-1 α-galactosidase production in SSF (75% moisture, 28 ºC, 72 h cultivation). Fermentation medium without any carbon supplementation was used as control. Data were expressed as mean ± standard error from three experiments. [a-e] means without a common letter differ (p<0.05).

Effect of additional nitrogen sources on α-galactosidase production

No major effects on enzyme activity were observed in the presence of ammonium sulfate and sodium nitrate. However, addition of tryptone and peptone remarkably enhanced enzyme synthesis ($p<0.05$; Figure 5).

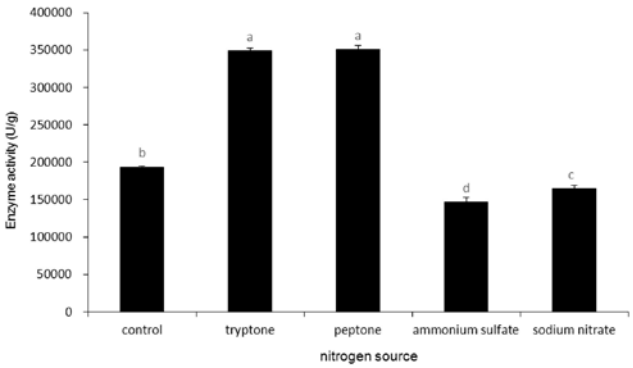

Figure 5. Effect of nitrogen source on LX-1 α-galactosidase production in SSF (75% moisture, 28 ºC, 72 h cultivation). Fermentation medium without nitrogen supplementation was used as control. Data were expressed as mean ± standard error from three experiments. [a-d] means without a common letter differ ($p<0.05$).

Discussion

This study presents information on the production of α–galactosidase in SSF by *Bacillus* LX-1. In general, *Bacillus* sp. has been known as a good source for the production of feed enzymes (Park and Cho, 2011). To the best of our knowledge, this information is novel, although other hydrolytic enzymes involved in digestion of nutrients such as amylase, protease and xylanase have been described in SSF by some *Bacillus* strains (Rajagopalan and Krishnan, 2010; Garg *et al*., 2011; Madhuri *et al*., 2012).

Bacillus LX-1 exhibited the highest α–galactosidase activity when grown under SSF conditions with SBM serving as a solid substrate in the absence or presence of nutritive supplements ($p<0.05$; Table 1). A high enzyme yield obtained with SBM may be associated with the presence of galacto-oligosaccharides metabolic intermediates (Wang *et al*., 2004). Abundant galacto-oligosaccharides in SBM or soy flour induced the highest α–galactosidase yield in thermophilic fungus *Humicola* sp. and actinobacterium *Streptomyces griseoloalbus* under SSF conditions (Kotwal *et al*., 1998; Anisha *et al*., 2008b), although the mechanisms remain unclear.

α–Galactosidase production by *Bacillus* LX-1 in SSF increased steeply in the exponential phase from 24 to 72 h, followed by a stationary phase, though there were no statistical differences between 60 and 72 h (Figure 1). A decrease in the enzyme activity was observed after 72 h of fermentation, which could be due to the depletion of nutrients for enzyme synthesis and proteolytic degradation of already synthesized enzymes (Anisha *et al*., 2008b).

Initial moisture content critically affects microbial growth and enzyme production in SSF. Thus, optimum moisture level should be maintained for appropriate growth and enzyme production (Shankar and Mulimani, 2007). Just as most filamentous microorganisms grow best when the substrate moisture content is between 50 and 75% (Pandey *et al*., 2001), a relatively high moisture content of 75% also supported the highest α–galactosidase production in SSF by *Bacillus* LX-1 ($p<0.05$; Figure 2). Meanwhile, optimum moisture level for maximal α–galactosidase production from *Aspergillus oryzae* and *Streptomyces griseoloalbus* was reported to be only 35 and 40%, respectively (Annunziato *et al*., 1986; Anisha *et al*., 2008b). It is accepted that low moisture tends to reduce stability and substrate swelling, and excessively high moisture levels lead to particle agglomeration, gas transfer limitation, and competition among bacteria (Gowthaman *et al*., 2001).

pH is an important factor affecting cell growth and enzyme production during SSF (Kunamneni *et al*., 2005; Mahanta *et al*., 2008). The optimum pH of medium for α–galactosidase production under SSF conditions by microorganisms ranges from 5 to 7 (Wang *et al*., 2004; Shankar and Mulimani, 2007; Liu *et al*., 2007b; Anisha *et al*., 2008b). In our study, *Bacillus* sp. LX-1 comparatively showed a better enzyme yield in the alkaline range (pH 8.0 to 8.5) ($p<0.05$; Figure 3), which helps reducing the risk of contamination (Gessesse and Mamo, 1999).

The carbon source used for microbial enzyme production is an important factor (Liu *et al*., 2007a). Galactose and lactose had inducible effects on

α–galactosidase synthesis by *Bacillus* sp. LX-1 ($p<0.05$; Figure 4), as the former also acts as an inducer for the enzyme production in *Streptomyces griseoloalbus* (Anisha *et al*., 2008a) and *Aspergillus oryzae* (Shankar *et al*., 2006). Usually, the substrates of an enzyme or its structural analogs can serve as inducers (Anisha *et al*., 2008a). Furthermore, the hydrolysis rate of a substrate or its binding with α–galactosidase depends upon the substrate having a pyranoid ring structure, and the configuration of carbon atoms 1-4 must be similar to that in D-galactose (Dey and Pridham, 1972). In contrast, the lowest α–galactosidase activity was found in SSF by *Aspergillus foetidus* ZU-G1 when lactose was used (Liu *et al*., 2007a).

Addition of organic nitrogen sources such as tryptone and peptone showed significantly positive effects on α–galactosidase production by *Bacillus* sp. LX-1 under SSF conditions ($p<0.05$; Figure 5). This may have occurred due to the presence of surplus nutrients such as vitamins, minerals and amino acids in the organic nitrogen sources that support active cellular function (Awan *et al*., 2009). Conversely, inorganic sources including ammonium sulfate and sodium nitrate did not have appreciable effects, consistent with previous results obtained in *Aspergillus niger* and *Aspergillus foetidus* ZU-G1 (Liu *et al*., 2007a; Awan *et al*., 2009).

In conclusion, this SSF technique can potentially be employed as a cost-effective manner to produce α–galactosidase for utilizing indigestible α–galacto-oligosaccharides such as raffinose and stachyose in the feed industry. Scale-up experiments for the industrial development of the enzyme production are in progress.

Acknowledgement

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