



Production of β -galactosidase in shake-flask and stirred tank bioreactor cultivations by a newly isolated *Bacillus licheniformis* strain

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ARTICLE INFO

Keywords:

Isolation
Identification
 β -Galactosidase
Production
Bioreactor
Scale-up

ABSTRACT

β -Galactosidase is an industrially important enzyme with many applications in various health, diagnostic and food processing industries. The present work describes the isolation and identification of a new isolate capable of producing β -galactosidase. The work was further extended to optimize the production medium and investigate the kinetics of cell growth and enzyme production during batch cultivation in shake-flask and 5L-stirred tank bioreactor. The isolated strain was identified as *Bacillus licheniformis* using 16S rRNA and BLAST analysis. The final optimized production medium contained (g/L): yeast extract, 14.0; lactose, 8.0; NaCl, 16.0; $(\text{NH}_4)_2\text{HPO}_4$, 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; K_2HPO_4 , 0.1. The optimized medium afforded maximal β -galactosidase production of 116.4 U/mL with a specific yield coefficient ($Y_{P/X}$) of 27.6×10^3 U/g cells. These results corresponds to an increase of about 4.4- and 2.8-folds, respectively, than the initial un-optimized production medium. Further improvement in the production process was achieved by cultivating cells in 5L-stirred tank bioreactor, which increased volumetric enzyme productivity and specific yield coefficient by 8.5- and 6.0-folds than the initial un-optimized production medium, respectively. A new β -galactosidase producing strain was identified and the volumetric as well as specific productivities were greatly enhanced through medium optimization and bioreactor cultivation.

1. Introduction

The global market for industrial enzymes has increased tremendously in the last two decades, driven by the wide and versatile applications of microbial enzymes (Panesar et al., 2018). β -Galactosidase (EC 3.3.1.23), or lactase, is the enzyme responsible for catalyzing the hydrolysis of the disaccharide lactose units into their glucose and galactose mono-sugars (Murugan, 2013). Therefore, it is of great importance in the digestion process of milk sugars, and is now accepted for treating individuals with lactose intolerance problems resulting from lacking the ability to digest dietary lactose (Sumit and Singh, 2014). Lactose-digestion inability is usually associated with experiencing abdominal discomfort, diarrhea, cramps, flatulence, nausea, and vomiting (Gheyntchi et al., 2010). Secondly, β -galactosidases are widely used in food processing industries for the production of ice-creams, condensed milk and frozen dairy products, baking, soft drinks

and sweeteners (Nath et al., 2014). Additionally, β -galactosidases are used in the environmental bioremediation sector to hydrolysis the whey byproduct resulting from cheese industry (Princely et al., 2013). Furthermore, β -galactosidases can perform the *trans*-galactosylation activities to produce the non-digestible galactooligosaccharides (GOSs), which belong to the functional and prebiotic food sector. GOSs are responsible for enhancing growth and activities of symbiotic bacteria in the intestine and modulating the human immune system (Hsu et al., 2006). The annual worldwide production market for GOSs recorded 94.1 kilotons in 2013, and is expected to increase with a compound annual growth rate (CAGR) of 9.3% to reach 175.7 kilotons by 2020 (Panesar et al., 2018). This was largely driven by the increased demand on prebiotic products and infants nutrition products.

β -Galactosidases are widely distributed in microorganisms, plants and animals (Nagy et al., 2001; Flood and Kondo, 2004), but the industrial applications rely on producing them from microorganisms; i.e.

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bacteria, fungi and yeasts (Picard et al., 2005). However, the acceptance of most of these microorganisms for commercial lactase production in food industries is hampered by the fact that the producing microorganism must have the GRAS status (Generally Regarded As Safe), which is a prerequisite for use in food and dairy industries (Vinderola and Reinheimer, 2003). Therefore, continuous research has been conducted to explore the potential of isolating new microorganisms capable of producing β -galactosidase. β -Galactosidase is an inducible enzyme, and its production is largely affected by different nutritional requirements, mainly carbon and nitrogen sources (Al-Jazairi et al., 2015).

In this work, we isolated a bacterial strain, from Egyptian soil sample, capable of producing β -galactosidase. The isolated strain was identified using 16S rRNA technology. Afterwards, the concentrations of different initial medium components were optimized in terms of cell growth as well as enzyme production, in comparison to the initial un-optimized production medium. Furthermore, the production process was transferred from shake-flask level to 5L-stirred tank bioreactor level to investigate the suitability of production process upscaling.

2. Materials and methods

2.1. Sample collection and isolation of the β -galactosidase producing strain

One gram of soil was transferred to 9 mL sterile saline solution (under aseptic conditions), and the tubes were shaken well to get dilution of 10^{-1} . Several serial dilutions were then made to obtain a proper dilution of 10^{-3} . Diluted samples were plated on agar plates (Fiedurek and Ilczuk, 1990) containing 0.5% lactose as the sole carbon source and 0.1% of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal, Takara Bio, Japan). The incubation was carried out at 35°C. After approximately 2 to 3 days, the colonies which appeared blue were collected and streaked onto medium. Once the isolates were purified, the strain producing enzyme of high activity was obtained by fermentation in a shaking flask. The isolated strain was further plated to insure its purity, and then colonies were collected and preserved at -80°C under glycerol.

2.2. Identification of β -galactosidase producing bacteria DNA isolation and PCR amplification

The isolated strain was identified by analyzing its 16S rRNA gene according to Sambrook et al. (1989). The bacterial DNA was isolated and the 16S rRNA was amplified using universal primers (Forward: 5' AGAGTTTGATCMTGGCTCAG3, Reverse: 5'TACGGYTACCTTGTTAC-GAC-TT3'). The sequence similarity was searched within the GenBank database using the Basic Local Alignment Search Tool (BLASTN) according to Hall (1999). The phylogenetic tree was constructed and displayed using the tree view program.

2.3. Inoculum preparation

Inoculum was prepared by inoculating a loop of the isolated bacterial cells in 250 mL Erlenmeyer shake-flasks containing 50 mL of basal lactose broth medium (Kranz et al., 2006). The medium composed of (g/L): lactose, 10.0; yeast extract, 1.0; $(\text{NH}_4)_2\text{HPO}_4$, 0.1; $(\text{NH}_4)_2\text{HPO}_4$, 0.1; MgSO_4 , 0.1; NaCl, 20.0. Flasks were incubated on a rotary shaker (GFL 3033, GFL, Burgwedel, Germany) at 150 rpm at 37°C for 6 h to obtain cells growing in the exponential phase. The optical density of the growing cells was measured at 600 nm with suitable dilutions to inoculate flasks and bioreactor at a concentration of 5% (v/v).

2.4. Shake-flask cultivation

Shake flask cultivations were performed in 250 mL flasks containing 50 mL of lactose broth basal medium (un-optimized). Flasks were inoculated with previously prepared inoculum (5%, v/v), and were then

incubated at 37°C and 200 rpm for 48 h on a rotary shaking incubator (GFL 3033, GFL, Burgwedel, Germany). Before inoculation, the pH of the medium was adjusted to 7.0 before being sterilized by autoclaving at 121°C for 20 min.

2.5. Bioreactor cultivation

Cultivations on bioreactor level were carried out in 5L-stirred tank bioreactor (Bioflo 310, New Brunswick Scientific, New Brunswick, NJ, USA), with a working volume of 3 L. Agitation was performed using a two 6-bladed Rushton turbine impellers ($d_{i(\text{impeller diameter})} = 75 \text{ mm}$; $d_{t(\text{tank diameter})} = 215 \text{ mm}$, $d_i/d_t = 0.35$) at 500 rpm. Filtered sterile air was introduced to the bioreactor at an aeration rate of 1.0 v/v/min. The concentrations of dissolved oxygen were measured using polarographic electrode (Ingold, Switzerland). If applicable, foam was suppressed using silicon antifoam reagent (Fluka, Switzerland). The pH of the bioreactor cultivations was initially adjusted at 7.0 using standard NaOH/HCl solutions, and was kept uncontrolled throughout the bioreactor run. All the experiments were repeated for three times, and the average mean of the data are represented.

2.6. Analysis

2.6.1. Sample preparation and cell dry weight determination

For shake-flask cultivation, samples in the form of three flasks were taken, while for bioreactor cultivations, a sample in the form 20 mL of cultivation broth was withdrawn at different time intervals from the bioreactor vessel through the standard sampling nozzle. Upon sampling, the optical density of the broth was measured at 600 nm after suitable dilution to measure between 0 and 0.5 using spectrophotometer (GeneQuant[®] 1300, Biochrom, Cambridge, UK). Samples were then filtered using pre-weighed filter paper and the biomass was washed twice with distilled water and then dried in an oven at 80°C till a constant dry weight is obtained (Elsayed et al., 2016). Cell dry weight was then determined using previously performed standard curve between OD and cell dry weight. The filtrate was then used to determine enzyme activity.

2.6.2. Determination of β -galactosidase activity

The enzyme activity was determined for the obtained culture filtrates using ONPG as a substrate according to Miller (1998). The enzyme was assayed with 100 μL of enzyme added to 900 μL substrate (*o*-nitrophenyl-D-galactopyranoside, ONPG, 0.4%/0.1 M sodium-phosphate buffer, pH 7.0). The mixture was then incubated at 37°C for 20 min, and the reaction was then terminated with 0.5 mL of 1 M sodium carbonate solution. Absorbance was read at 560 nm using spectrophotometer. The amount of liberated *o*-nitrophenol was calculated from the previously determined standard curve using different concentration of *o*-nitrophenol. Blank tubes contained sodium carbonate solution before enzyme addition. One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 μmol ONPG min^{-1} at 45°C.

2.6.3. Statistical analysis

Data were analysed with the help of SPSS 9.0 and the results were given as mean \pm SD of three separate experiments replicates. The mean comparison between different evaluated parameters was performed using ANOVA one-way analysis of variance. Statistical significance was defined when $p < 0.05$.

3. Results and discussion

3.1. Isolation, identification and molecular characterization of β -galactosidase producing strain

Firstly, the present study aimed at the isolation, molecular

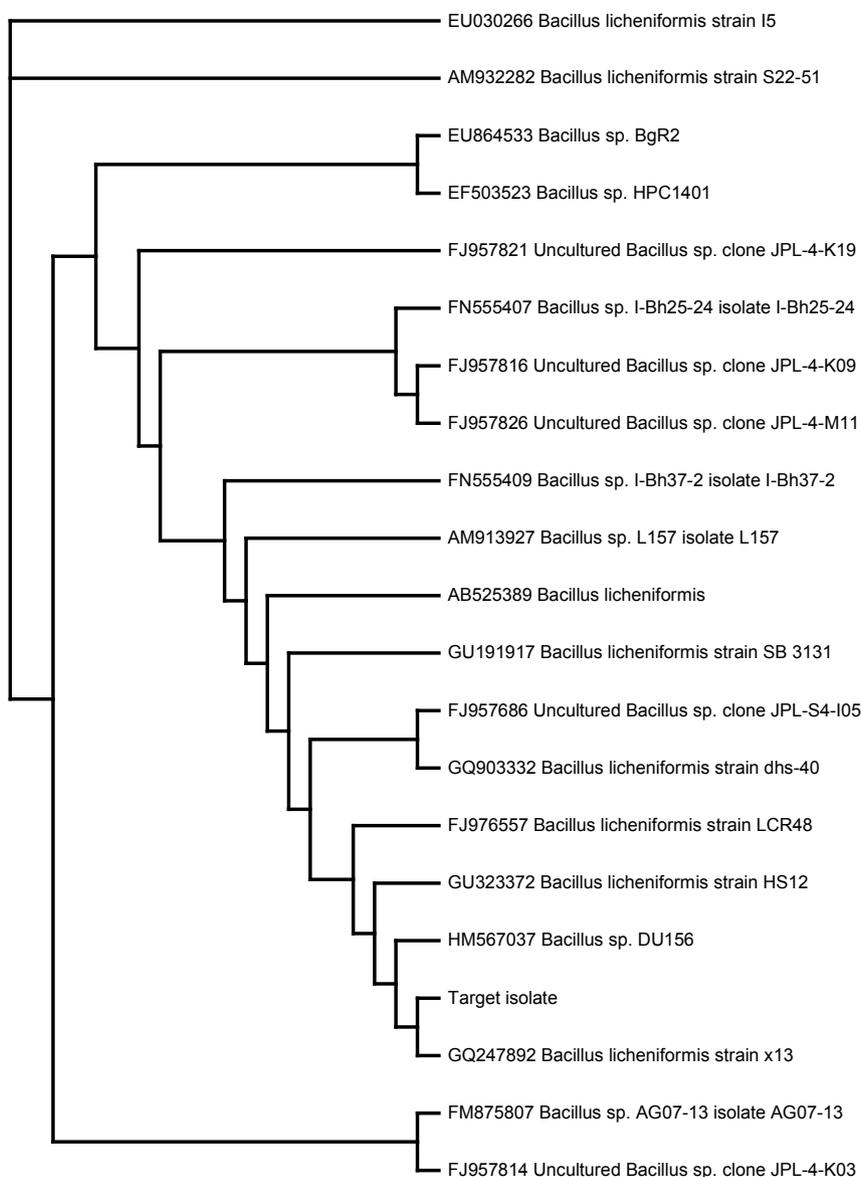


Fig. 1. Neighbor-joining phylogenetic representation of the strains and their closest relatives based on 16S rRNA gene sequences of *B. licheniformis* I5.

characterization and identification of β -galactosidase producing bacterium isolated from soil. During the screening process, 9 different colonies out of 22 isolated strains demonstrated positive β -galactosidase activities. The most active producer among these 9 strains was picked, analysed and further characterized and identified. The isolated strain was gram positive, rod shaped and motile. The alignment of the 16S rRNA gene sequence of the isolated strain with the sequences available in the GenBank database showed that, the isolated organism has 96% identity with various *B. licheniformis* strains. Hence, the strain was confirmed as *B. licheniformis* and the sequence has been deposited in the GenBank database as *B. licheniformis* I5, Accession number EU030266 (Fig. 1). 16S rRNA sequencing was used to identify the isolated producing bacterium as *Bacillus licheniformis* I5 (Bron, 1990; Tran et al., 1998; Osiriphun and Jaturapiree, 2009).

3.2. Growth curve kinetics for β -galactosidase production using un-optimized medium

The vegetative cells were used to inoculate flasks containing the production medium with the initial composition (g/L): yeast extract, 1.0; lactose, 10.0; NaCl, 20; $(\text{NH}_4)_2\text{H}_2\text{PO}_4$, 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1;

K_2HPO_4 , 0.1. Results illustrated (Fig. 2) represent the kinetics of cell growth and β -galactosidase production. It can be clearly observed that cells grew exponentially for the first 6 h of cultivation with an average growth rate of 0.432 g/L/h, reaching 2.97 g/L. Thereafter, cells entered the stationary phase where cell growth rate decreased to 0.013 g/L/h and cell growth slightly increased reaching a maximal cell dry weight of 3.07 g/L at 19 h. After 19 h, cell growth slightly decreased to 2.87 g/L, and remained more or less constant until the end of the cultivation. During cell growth, the pH of the cultivation increased with cell growth, where it reached 8.5 at 48 h. Concerning enzyme production, results showed that after 6 h, β -galactosidase production increased with time during stationary growth phase with an average enzyme production rate of 0.49 U/mL/h. The maximal enzyme production reached 26.4 U/mL after 48 h with a maximal specific yield coefficient ($Y_{P/X}$) of 9.98×10^3 U/g cells.

Generally, it is already accepted that enzyme production is largely influenced by the type of the producing strain as well as nutritional and environmental conditions. Therefore, it is important to optimize such conditions for each isolated strain individually (Li et al., 2018). Results for investigating kinetics of cell growth and β -galactosidase production using the initial un-optimized medium clearly showed that both cellular

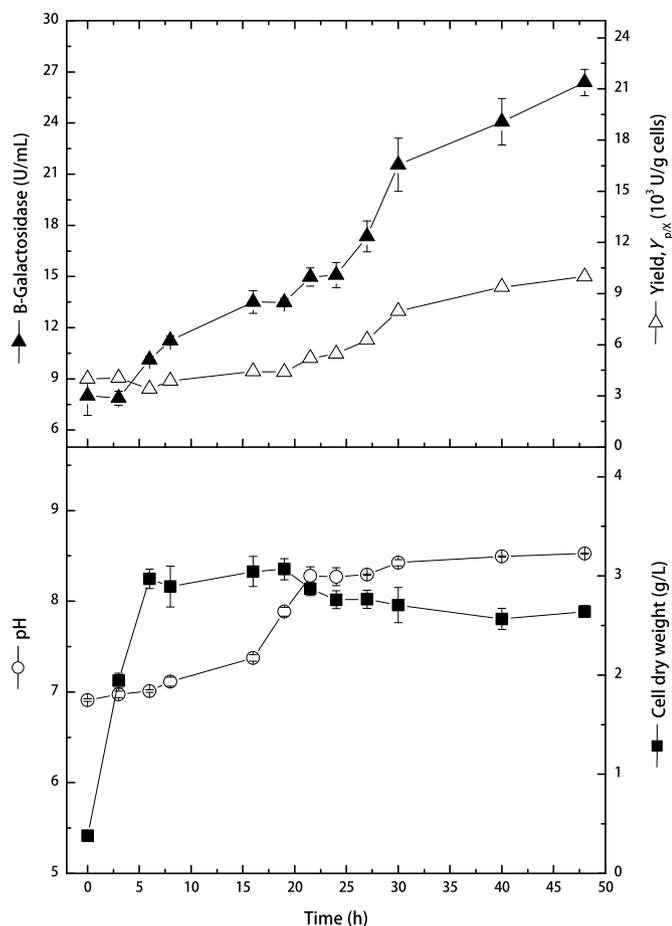


Fig. 2. Growth curve kinetics for cell growth and β -galactosidase production by *B. licheniformis* I5 in shake flask cultivation using un-optimized medium.

growth as well as enzyme production rates increased during the logarithmic growth phase, and then decreased upon entering the stationary phase. These results are in good agreement with those reported by Astapovich and Ryayaba (2006), who investigated β -galactosidase production by different bifidobacterial strains. They found that their highest growth and production rates occurred during the first 3–6 h of cultivation, i.e. logarithmic phase, and then decreased during the stationary phase. They attributed their results to the decline in cell proliferation capacity, which consequently affected enzyme production. However, our results showed that enzyme production continued and reached its maximal by 48 h. This can be explained on the basis that metabolic pathways are highly dependent on the strain and medium composition used. Kumar et al. (2012) produced β -galactosidase from an isolated *Bacillus* sp. MPTK121 and obtained highest enzyme production after 48 h, which declined afterwards. Similar results were reported by Karman et al. (2016), who showed that *Bacillus* strain B-2 produced maximal β -galactosidase at 48 h, and then the production decreased sharply. Furthermore, results showed that enzyme production continued after entering the stationary phase, which can be explained due to continued secretion of enzyme in response to faster autolysis of cell walls (Oyetayo et al., 2011). Accordingly, further optimization experiments were terminated after 48 h.

3.3. Optimization of medium composition

This section of experiments was designed to optimize medium composition for β -galactosidase production. During this section, we evaluated the effect of different concentrations of the factor under optimization, while other components remained as in the initial medium

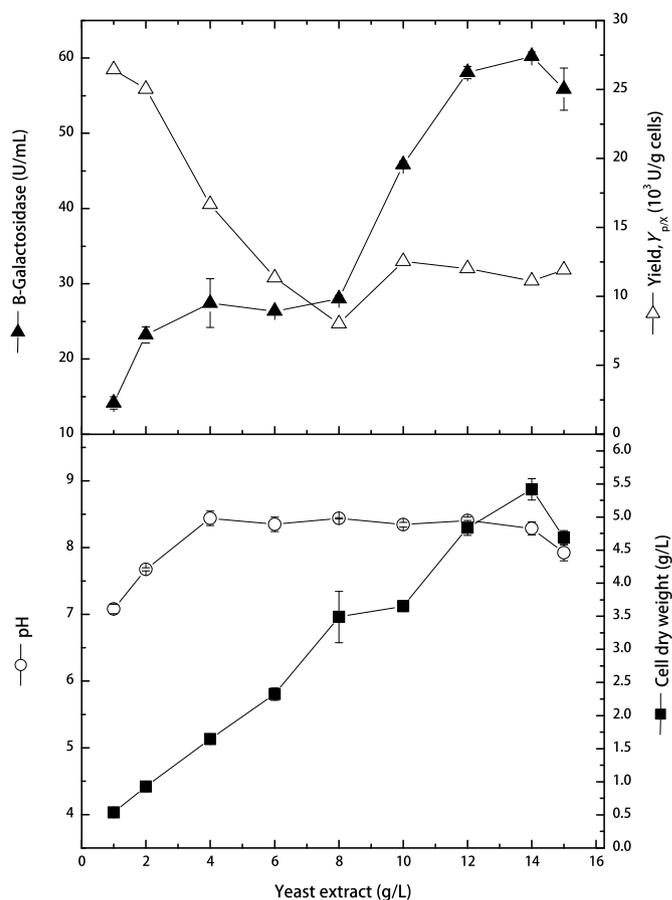


Fig. 3. Effect of different yeast extract concentrations of cell growth and β -galactosidase production by *B. licheniformis* I5.

composition. However, based on our preliminary investigations, ammonium phosphate, magnesium sulphate and sodium phosphate dibasic showed nonsignificant effects on both cell growth and enzyme production (data not shown). Accordingly, their concentrations were not varied and were used as in the initial medium composition.

3.3.1. Effect of different concentrations of yeast extract on cell growth and β -galactosidase production

In this experiment, different yeast concentrations ranging from 1.0 to 15.0 g/L were evaluated for their effect on cell growth and β -galactosidase production. Results (Fig. 3) clearly showed that yeast extract played a significant role in enhancing both cell growth and enzyme production. Increasing concentration of yeast extract from 1.0 to 14.0 g/L proportionally increased cell growth, where a maximal cell growth of 5.42 g/L (76.6% higher than the un-optimized cultivation) was obtained when 14.0 g/L yeast extract were used in the production medium. Further increase in yeast extract concentration above 14.0 g/L decreased cell growth by about 13.4% (4.69 g/L). For enzyme production, 14.0 g/L yeast extract also favored maximal β -galactosidase production, where 60.3 U/mL were produced, which were almost 2.28-folds higher than the maximal enzyme produced in the initial un-optimized medium. Yield coefficient results showed that higher yield values were mainly obtained at lower yeast extract concentrations due to lower cell growth concentrations. On the other hand, using 14.0 g/L yeast extract resulted in a specific production yield ($Y_{P/X}$) of 11.12×10^3 U/g cells, which is slightly higher than the yield obtained in the initial un-optimized medium (9.98×10^3 U/g cells). Accordingly, 14.0 g/L of yeast extract was chosen as the suitable concentration for the following optimization steps.

Microbial growth and consequently enzyme production is

significantly affected by the composition of cultivation medium. Therefore, optimizing medium composition provides growth cells with their optimal nutritional requirements (Dailin et al., 2015, 2016), and accordingly cells grow faster and produce larger amounts of the required enzyme. Similar optimization strategies have been previously adopted to enhance production of industrially valuable microbial enzymes (Boumehira et al., 2016; Elsayed et al., 2016; El-Enshasy and Elsayed, 2017; El-Enshasy et al., 2018). The obtained results showed that increasing yeast extract up to 14.0 g/L promoted maximal cell growth and enzyme production. Our results are in good consistence with those previously reported by Hsu et al. (2005) who investigated the production of β -galactosidase by different Bifidobacterial strains. They found that increasing yeast extract concentration up to 10.0% gradually increased enzyme production, while further increase in yeast extract sharply decreased the production. Yeast extract is generally used as a nitrogen source and a supplement for required growth factors (Dailin et al., 2015). Furthermore, it is worthy to notice that increasing yeast extract concentration greatly increased cell mass produced, which was reflected on the increased enzyme production. During the aforementioned results on un-optimized medium (1.0 g/L yeast extract), it can be seen that cell growth entered stationary phase after 5-8 h of cultivations, and that the depletion of necessary nutrients (mainly carbon source) resulted in cell autolysis. On the other hand, the decrease in both cell growth and enzyme production at higher yeast extract concentrations may be attributed to the increased medium viscosity and hence decreased oxygen availability in the cultivation medium (Karman et al., 2016; Elsayed and El-Enshasy, 2018).

3.3.2. Effect of different concentrations of lactose on cell growth and β -galactosidase production

The effect of different lactose concentrations (4.0-16.0 g/L) on cell growth and enzyme production was evaluated in a medium containing 14.0 g/L yeast extract as the most suitable concentrations. Results (Fig. 4) showed that increasing lactose concentration from 4.0 to 16.0 g/L showed no significant effect on cell growth, where all evaluated concentrations resulted in cell growth concentrations ranging from 4.1 to 4.64 g/L. Similarly, pH behavior was much similar to that of the cell growth behavior (not largely affected). On the other hand, results showed that increasing lactose concentration from 4.0 to 8.0 g/L increased β -galactosidase from 51.02 to 65.9 U/mL (29% increase). Increasing lactose concentration above 8.0 g/L decreased enzyme production by about 7-10% (60.38-60.75 U/mL). The specific production yield coefficient obtained at 8.0 g/L lactose recorded 14.2×10^3 U/g cells. Therefore, the optimized medium chosen for further optimization contained 8.0 g/L lactose, instead of the initial 10.0 g/L concentration.

The obtained results showed that maximal enzyme production and cell specific production are obtained upon using 8.0 g/L lactose in the production medium. These results are in good agreement with those previously reported on β -galactosidase production using lactose (Hsu et al., 2005; Karman et al., 2016). Authors found similar results, where increasing lactose concentration above their obtained optimal concentration resulted in a decrease in β -galactosidase production. When lactose is used at moderately lower concentrations; i.e. up to the optimal concentrations, it acts as a substrate and as an inducer as well for enzyme production resulting in an increase in cell mass and enzyme productivity (Duan et al., 2017). Above its optimal concentration, lactose can lead to increased medium viscosity and therefore, decreased oxygen and nutrient availability due to mixing problems (Rehman et al., 2012). Moreover, increased lactose concentration may increase the concentration of internally produced glucose, which in turn may result in catabolite repression of enzyme biosynthesis (El-Enshasy and Elsayed, 2017). Furthermore, it has been also postulated that higher lactose concentrations may direct the metabolic machinery of the microorganism towards increased precursor polypeptide formation which may lead to protein misfolding (Ishikawa et al., 2015).

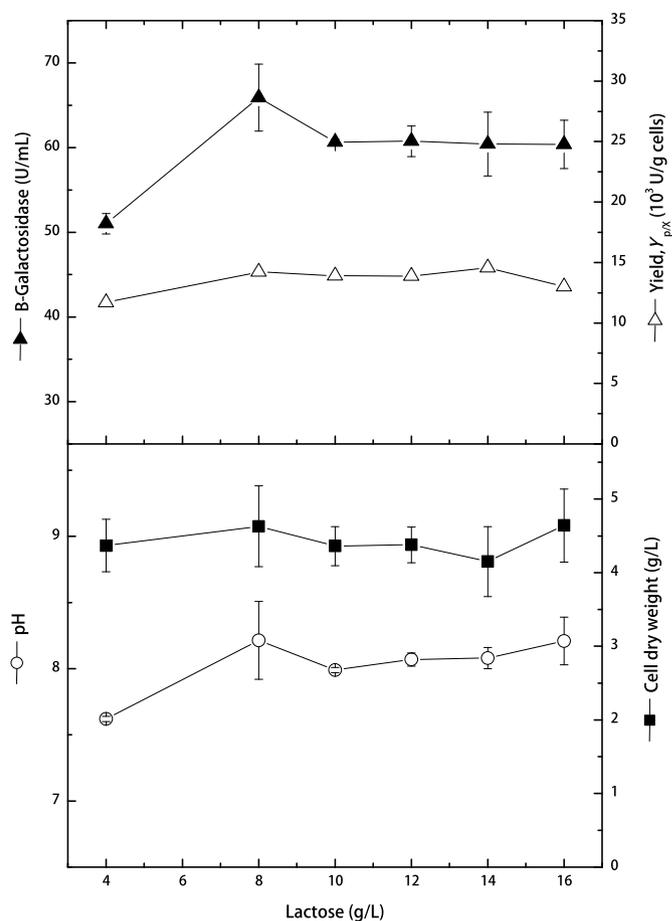


Fig. 4. Effect of different lactose concentrations of cell growth and β -galactosidase production by *B. licheniformis* I5.

3.3.3. Effect of different concentrations of NaCl on cell growth and β -galactosidase production

Sodium chloride plays a crucial role in determining the physiological state of the growing bacterial cells through affecting the pH of the production medium. Since the initial medium composition contained 20.0 g/L NaCl, therefore, different NaCl concentrations of ranging from 4.0 to 25.0 g/L were evaluated for their effects on cell growth as well as β -galactosidase. Results shown in Fig. 5 showed that increasing NaCl concentration from 4.0 to 25.0 g/L had little effect on cell growth where the final cell dry weight concentration ranged from 3.99 to 4.7 g/L. However, varying NaCl greatly influenced the production of β -galactosidase. Increasing NaCl concentration from 4.0 to 16.0 g/L gradually increased enzyme volumetric productivity, where the highest productivity of 62.99 U/mL was obtained upon using 16.0 g/L NaCl. On the other hand, further increase in NaCl concentration showed an inhibitory effect on β -galactosidase production, where the volumetric production decreased gradually till reaching 51.96 U/mL when the highest NaCl concentration (25.0 g/L) was used. Regarding specific production yield coefficient ($Y_{p,x}$), results showed that the highest specific productivity (14.22×10^3 U/g cells) was also obtained when NaCl was used at 16 g/L, which was then decreased upon increased NaCl concentration, mainly due to the decrease in volumetric enzyme productivity. Accordingly, 16 g/L NaCl was used during further medium optimization.

Our results showed that 16.0 g/L NaCl are most suitable for promoting maximal enzyme production, and that further increase in salt concentration adversely affected enzyme production and cell specific production kinetics. Salts have been reported to greatly influence enzymatic activities, either activating or inhibiting, depending on the

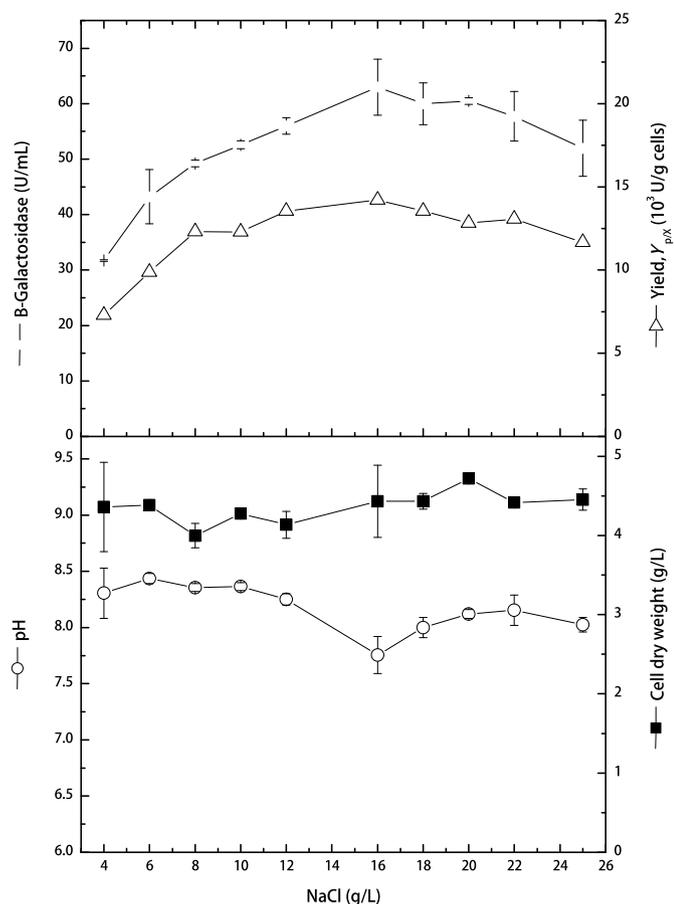


Fig. 5. Effect of different NaCl concentrations of cell growth and β -galactosidase production by *B. licheniformis* 15.

nature of used cations and anions (Lee et al., 2015). Different β -galactosidases have been documented to be either activated or inhibited by mono- and divalent cations (Garman et al., 1996). The increase in enzyme production by increasing NaCl concentration has been reported up to a maximal concentration, above which the enzyme activity decreases sharply. Such effect was attributed to the stabilization effect of the salt on enzyme activity, which was referred to the hydrophobic and electrostatic interactions between enzyme and salt components (Sinha and Datta, 2016). Optimal NaCl concentrations are assumed to increase the ionic interactions between charged substrates and enzyme active sites, leading to stabilization of the active conformational structure of the enzyme and hence, its activity. On the other hand, higher salt concentrations have been found to adversely affect enzyme production and activity, mainly due to disrupting organized structure of water or enzyme molecules (Warren and Cheatum, 1966). Furthermore, it has been postulated that increased salt concentration may directly affect peptide and amide groups through relocating excluded ions at the polar-nonpolar surface, leading to enzyme conformational disruption. Additionally, Malakar et al. (2015) postulated that cellular metabolic machinery reacts towards higher salt concentrations by producing unnecessary proteins required to adapt the microbe towards the new conditions. Such metabolic shift has adverse effects on both cellular growth and enzyme production profiles.

According to the aforementioned results concerning medium optimization, the final optimized medium consisted of (g/L): yeast extract, 14.0; lactose, 8.0; NaCl, 16.0; $(\text{NH}_4)_2\text{HPO}_4$, 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; K_2HPO_4 , 0.1.

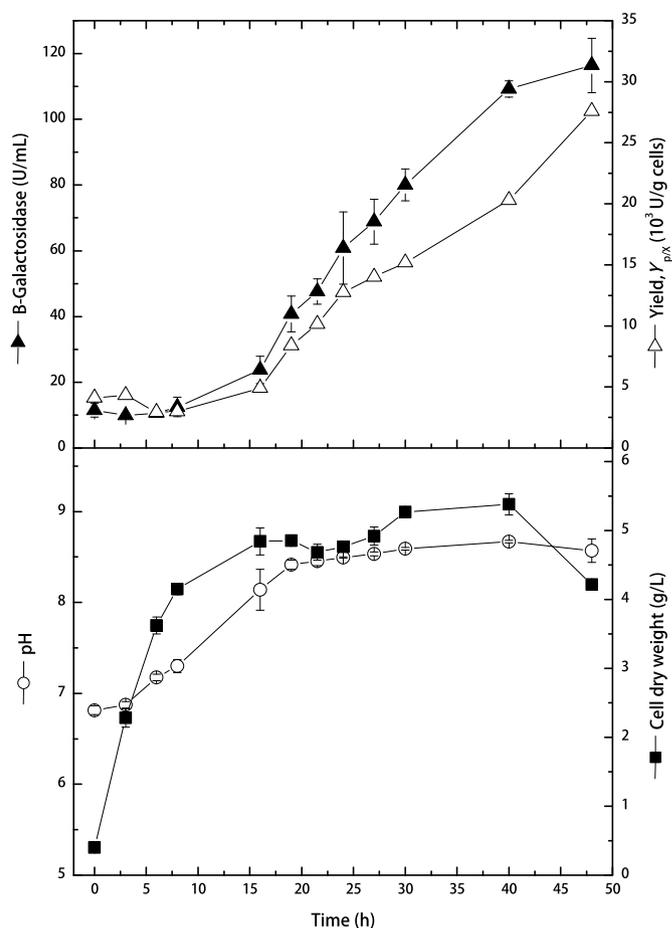


Fig. 6. Growth curve kinetics for cell growth and β -galactosidase production by *B. licheniformis* 15 in shake flask cultivation after medium optimization.

3.4. Growth curve kinetics on shake flask level using optimized medium composition

This experiment was carried out to follow up the kinetic behavior of cell growth and β -galactosidase production in shake flask cultivation after optimization of medium components. Cells were cultivated in 250 mL-Erlenmeyer flasks containing 50 mL of medium, and sample flasks were taken at regular intervals, where necessary analysis were performed. Growth curve kinetic results (Fig. 6) showed that upon inoculation, cells grew exponentially for the first 8 h with an average growth of 0.45 g/L/h, which is similar to the average growth rate obtained when cells were cultivated on the un-optimized medium (0.43 g/L/h). Afterwards, cells entered the stationary phase, where the growth rate decreased to about 0.033 g/L/h. However, the maximal cell growth obtained in this case (4.86 g/L at 19 h) was about 58.3% higher than that obtained in case of un-optimized medium cultivation (3.07 g/L). For β -galactosidase production, similarly to un-optimized medium cultivation, it can be seen that enzyme production started upon entering the stationary phase and continued to the end of the cultivation process. On the other hand, the average enzyme production rate 2.65 U/mL/h was 5.41-folds higher than the average production rate in the un-optimized cultivation (0.49 U/mL/h). This was consequently reflected on the maximal β -galactosidase volumetric productivity, which recorded 116.4 U/mL (about 4.4-folds higher than the maximal enzyme productivity obtained in un-optimized cultivation). Furthermore, maximal specific yield coefficient ($Y_{p/x}$) recorded during cultivation was 27.6×10^3 U/g cells, which was 2.8-folds higher than the maximal yield coefficient obtained in un-optimized cultivation.

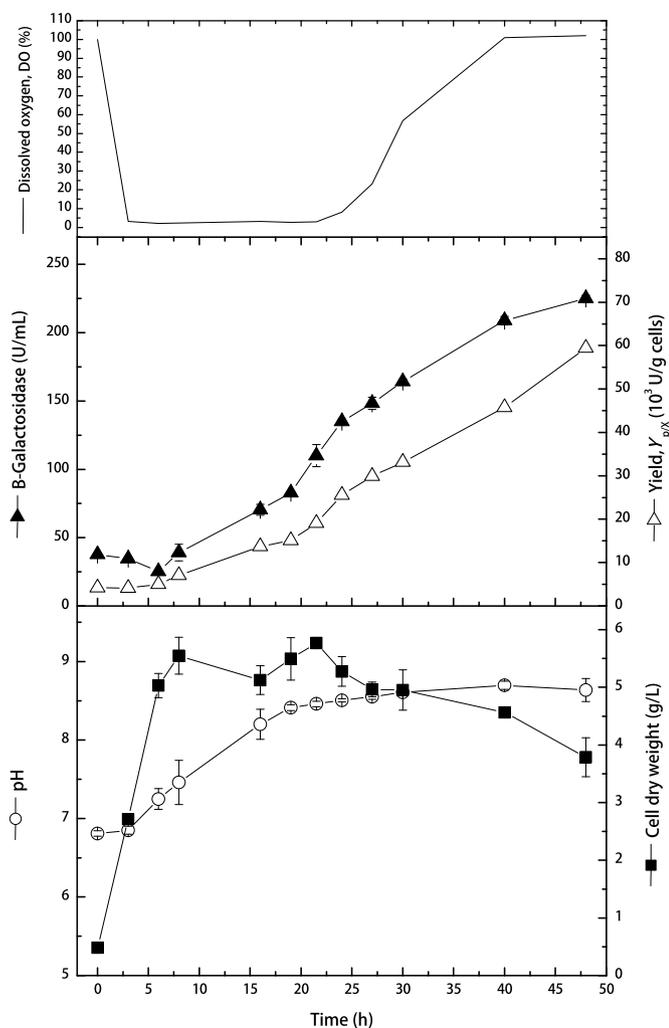


Fig. 7. Growth curve kinetics for cell growth and β -galactosidase production by *B. licheniformis* I5 cultivated in 5L-stirred tank bioreactor.

3.5. Growth curve kinetics on bioreactor level

Generally, microbial production processes are up-scaled in stirred-tank bioreactors due to the better control and optimization environment in bioreactors. Laboratory bioreactor cultivations are considered the first step toward process up-scaling, followed by cultivations in semi-pilot scale and pilot scale bioreactors, and finally production tanks (Connors, 2003). The cultivation environment in bioreactors is homogeneous and more adequate for growing cells due to rapid uniform agitation and mixing and negligible differences in hydrostatic pressures in bioreactor vessel (Dutta et al., 2019). Also, the rheological and viscosity properties of fermentation broth are greatly improved, leading to better cultivation environment (Yegneswaran et al., 1991). Furthermore, oxygen and nutrient uptake rates are superior to their corresponding shake-flask cultivations. Therefore, the production process for β -galactosidase by *B. licheniformis* I5 was transferred to 5L-BioFlo 310 Benchtop bioreactor in order to evaluate process kinetic parameters during scaling up. The bioreactor had a working volume of 3 L, and the cultivation was performed using optimized medium composition obtained from the aforementioned results. Cultivation results (Fig. 7) clearly revealed that bioreactor cultivation showed superior results compared to the shake-flask cultivation in terms of cell growth and production kinetics. During the first 8 h of cultivation, cells grew exponentially with an average growth rate of 0.592 g/L/h, which was higher by about 37.0 and 31.8% than un-optimized and optimized shake flask cultivations, respectively. Maximal cell growth recorded

5.77 g/L at 21.5 h (1.9- and 1.2-folds higher than un-optimized and optimized shake flask cultivations, respectively). Additionally, during stationary phase, the cell growth rate decreased to 0.12 g/L/h. Furthermore, oxygen consumption was at its highest level during the exponential growth phase, where the average oxygen consumption rate recorded 16.3%/h. Upon entering stationary growth phase, cells continued to consume supplied oxygen for about 21.5 h of cultivation, after which, oxygen consumption started to decrease again and the cultivation became oxygen saturated by its end. Regarding enzyme production, it can be seen that cells began to produce β -galactosidase after entering the stationary phase, similarly to previous cultivations. However, β -galactosidase was produced in bioreactor cultivation with an average production rate of 5.78 U/mL/h, which is 11.8- and 2.2-folds higher than that obtained in un-optimized and optimized shake flask cultivations, respectively. Hence, a maximal volumetric production of 225.2 U/mL was obtained in bioreactor cultivation (8.5- and 1.9-folds higher than the maximal enzyme produced in un-optimized and optimized shake flask cultivations, respectively). Concomitantly, β -galactosidase specific yield coefficient ($Y_{p/x}$) reached a maximal of 59.5×10^3 U/g cells, which was 2.2-folds higher than the maximal yield coefficient obtained in shake flask cultivation in optimized medium.

Transferring enzyme production process from shake-flask level to 5L-stirred tank level greatly enhanced the production process parameters in terms of growth and production rates as well as maximal productivity and yield coefficient. Bioreactor cultivation increased cell growth and enzyme production by 1.2- and 1.9-folds, respectively, from the shake-flask cultivation. Our results are in good agreement with those reported earlier, showing that bioreactor cultivations are superior to shake-flask cultivations in terms of higher cell growth and enzyme production rates, which are reflected on the final maximal enzyme productivity (Nadia et al., 2010; Elsayed et al., 2016). Scaling-up production processes from shake-flask to bioreactor level greatly enhances growth as well as production kinetics, mainly due to providing growing cells with better environmental conditions in terms of better oxygenation, agitation and mixing dynamics.

Finally, it can be seen that medium optimization and cultivation up-scaling in stirred tank bioreactor greatly enhanced the volumetric enzyme productivity and specific yield. Under optimized bioreactor conditions, the isolated strain was able to produce 225.2 U/mL, compared to 26.4 and 116.4 U/mL obtained from un-optimized and optimized medium conditions in shake-flask level. Comparing our results with those previously reported in literature, we can see that the isolated *B. licheniformis* I5 can be considered as a potential producer for β -galactosidase production. Astapovich and Ryayaba (2006) investigated growth and β -galactosidase production by 8 *Bifidobacterium* species. However, their maximal production ranged from 0.1 to 0.3 U/mL. On the other hand, Princely et al. (2013) were able to obtain enzyme activities ranging from 6.0 to 16.0 U/mL from their newly isolated *Streptococcus thermophilus* isolated. Furthermore, Hsu et al. (2005) optimized growth conditions for β -galactosidase production using different *B. sp.*, where the enzyme activities increased from the initial 5.0 U/mL up to 18.8 U/mL, with an optimized specific production yield of 12.32×10^3 U/g cells. With this regard, our isolated strain produced higher activities as well as specific yields even in the shake-flask levels. Additionally, Kumar et al. (2012) and Karman et al. (2016) obtained enzyme activities, in shake-flask cultivations, ranging from 25.0 to 70.0 U/mL, respectively. However, they did not report on specific yield data. Finally, Duan et al. (2017) were able to optimize β -galactosidase production using recombinant *B. circulans*, where their enzyme activities increased from 17.2 to 62.5 U/mL, in shake-flask and 3L-bioreactor cultivations, respectively. Upon feeding lactose, authors were able to reach 220.0 U/mL with 2.62×10^3 U/g cells.

Table 1
Growth and production parameters of β -galactosidase production by *B. licheniformis* under different optimization steps.

Parameter	Un-optimized medium	Optimized medium	
		Shake-flask	Bioreactor
X_{max} (g/L)	3.070	4.860	5.770
dx/dt (g/L/h)	0.432	0.450	0.592
P_{max} (U/mL)	26.40	116.4	225.2
Q_p (U/mL/h)	0.490	2.650	5.780
$Y_{P/X}$ (U/g cells)	9.98×10^3	27.6×10^3	59.5×10^3

Abbreviations: X_{max} , maximal cell dry weight; dx/dt , average cell growth rate; P_{max} , maximal volumetric β -galactosidase production; Q_p , average β -galactosidase production rate; $Y_{P/X}$, specific yield coefficient.

4. Conclusion

The current work describes the isolation of a new bacterial isolate capable of producing β -galactosidase from soil. The isolated species was identified using 16S rRNA-PCR technology and was identified as *B. licheniformis* 15. The initial production medium was optimized, followed by comparing cell growth and production patterns in shake-flask and 5L-stirred tank bioreactor levels. Results summarized in Table 1, show that the optimization of medium components increased both maximal cell growth and enzyme production by about 1.58- and 4.4-folds, respectively, from the initial un-optimized medium. Furthermore, scaling-up cultivation process in 5L-stirred tank bioreactor greatly enhanced process kinetics, where maximal cell growth and enzyme production further increased by 1.2- and 1.9-folds, respectively, from the shake-flask cultivation.

Author contributions

EAE and END proposed the research concept, designed the experiments, provided necessary tools for experiments, experimental instructions, analysed and interpreted the data, wrote and revised the manuscript. EAE and END carried out the experimental work. EAE, MAW and HAE analysed and interpreted the data. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgment

Authors are grateful to the Deanship of Scientific Research, King Saud University for funding through Vice Deanship of Scientific Research Chairs.

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