Jurnal Teknologi

Full Paper

SCREENING THE EFFECT OF THE EXPRESSION MEDIUM AND GROWTH CONDITIONS ON THE PERFORMANCE OF ENGINEERED XYLANASE PRODUCED BY IMMOBILIZED RECOMBINANT *E. COLI*

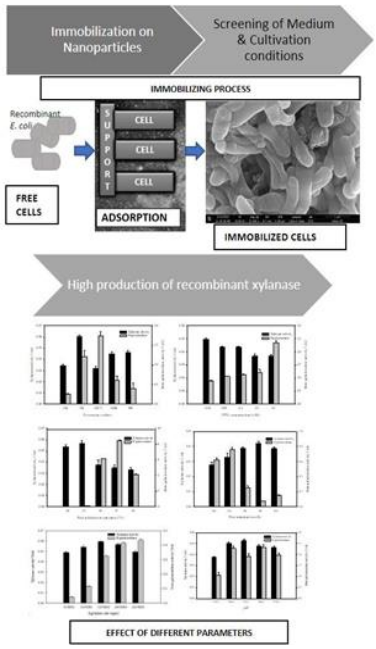
Siti Fatimah Zaharah Mohd Fuzi[a,b*], Nur Atiqah Lyana Nor Ashikin[a], Low Kheng Oon[c]

[a]Faculty of Applied Sciences and Technology, Universiti Tun Hussein Onn Malaysia, 84600, Pagoh, Muar, Johor, Malaysia
[b]Future Food Research Innovation, FAST, UTHM, 84600, Pagoh, Muar, Johor, Malaysia
[c]Malaysia Genome Institute & Vaccine, National Institutes of Biotechnology, Jalan Bangi, 43000 Kajang, Selangor, Malaysia

Article history
Received
26 December 2021
Received in revised form
23 February 2023
Accepted
23 February 2023
Published Online
19 April 2023

*Corresponding author:
fatimahz@uthm.edu.my

Graphical abstract



Abstract

Escherichia coli is the most prevalent host organism for the production of recombinant enzymes. This was feasible due to the possibility of genetic modification and the availability of multiple *E. coli* strains as recombinant systems. The primary disadvantage of using *E. coli* as a host, however, is bacterial cell lysis due to tension build-up in the periplasmic space caused by the overexpression of the recombinant enzyme. Therefore, immobilization is preferable to cytoplasmic excretion for directing the expression of recombinant enzymes into the culture medium. This research investigated the effect of graphene oxide (GO) on the xylanase and β-galactosidase activity of immobilized recombinant *E. coli*. The effect of culture conditions (expression medium, IPTG, post induction temperature, post induction duration, agitation rate, and pH) on xylanase excretion and cell survival of an immobilized cell was studied using the one factor at a time (OFAT) method. After 24 hours of induction, using terrific broth (TB) as a medium increased xylanase excretion to 0.060 U/ml and resulted in decreased β-galactosidase activity (1.218 U/ml). Apart from that, a lower concentration of isopropyl -D-1-thiogalactopyranoside (IPTG) at 0.01 mM, a lower post-induction temperature (25°C), a 5-hour post-induction time, neutral pH, and 150 rpm significantly increased the xylanase excretion of immobilized cells with low β-galactosidase activity. This study established that immobilizing recombinant *E. coli* on GO may be advantageous for the excretion of recombinant proteins with a high cell viability.

Keywords: Immobilized *E. coli*, recombinant xylanase, graphene oxide, induction time, cultural conditions

Abstrak

Escherichia coli ialah sistem perumah yang paling kerap digunakan untuk sintesis enzim rekombinan. Ini boleh dilaksanakan kerana kemungkinan pengubahsuaian genetic dan ketersediaan berbilang strain E. coli sebagai sistem rekombinan. Kelemahan utama menggunakan *E. coli* sebagai perumah, walaubagaimanapun, ialah sel bakteria pecah yang disebabkan oleh pembentukan ketegangan dalam ruang periplasmik yang

disebabkan oleh ekspresi dalam medium kultur. Projek ini mengkaji kesan graphene oksida (GO) terhadap aktiviti xilanase dan β-galactosidase daripada rekombinan *E. coli* yang termobilisasi. Kesan keadaan kultur (medium ekspresi, IPTG, suhu pasca induksi, tempoh pasca induksi, kadar pergolakan, dan pH) ke atas perkumuhan xilanase dan kemandirian sel-sel yang dimobilisasi telah dikaji menggunakan pendekatan satu faktor pada satu masa (OFAT). Selepas 24 jam induksi, menggunakan terrific broth (TB) sebagai medium meningkatkan perkumuhan xilanase kepada 0.060 U/ml dan mengakibatkan penurunan aktiviti β- galactosidase (1.218 U/ml). Selain itu, kepekatan isopropil -D-1-thiogalactopyranoside (IPTG) yang lebih rendah pada 0.01 mM, suhu selepas aruhan yang lebih rendah (25°C), masa selepas aruhan 5 jam, pH neutral dan 150 rpm dengan ketara meningkatkan perkumuhan xilanase sel dimobilisasi dengan aktiviti β-galactosidase yang rendah. Oleh itu, kajian ini membuktikan bahawa memobilisasi rekombinan *E. coli* pada GO mungkin berfaedah untuk perkumuhan protein rekombinan dalam *E. coli* dengan daya maju sel yang tinggi.

Kata kunci: *E. coli* dimobilisasi, xilanase terrekombinasi, graphene oksida, masa aruhan, keadaan kultur

1.0 INTRODUCTION

The development of heterologous proteins using recombinant DNA innovation has generated considerable attention in biotechnology's industrial process and manufacturing level [1]. Despite significant advances in using the *E. coli* cell factory for excretory manufacture of recombinant enzymes, the method of producing expressed protein has hit a specific operational impediment [2]. Protein enzymes are important finding in the industry of bioprocess technology, which is produced by active cells that promote the transformation of chemical species in living systems [3]. The use of enzymes has become a necessary processing strategy with extensive applications in the nanotechnology, animal nutrition, pulp and paper industry, textile, organic synthesis, cellulose and detergent industries, medical, pharmaceutical, biofuels industry, fine chemicals, biomaterials, cosmetic and food industry [4-5].

There are challenges in retrieving appropriately folded proteins from excretion, as overexpression of proteins typically occurs in the inclusion bodies (IB) form, which can only be recovered through a sophisticated and expensive process [6]. Second, the excretion yield of recombinant protein was frequently untraceable or poor; this was mostly due to interactions between the protein's hydrophobic surface areas, which resulted in clumps [2]. Thirdly, cell autolysis as a result of recombinant protein overexpression in the host cell [6].

As a result, various techniques have been developed and applied to address these issues, including lowering the concentration of inducer by controlling the level of expression via various promoters, excretion of recombinant proteins into the culture medium or periplasmic space, and the use of various host strains and specific recombinant proteins to induce chaperone and a class of proteins that aid in protein folding modulation [6-7]. The other frequently used strategy for optimising recombinant protein solubility and folding is to reduce bacterial stress and decrease recombinant protein excretion rates by adjusting the growing temperature. This allows for a more progressive translation and folding process in recombinant proteins [7]. Additionally, decreased yields of a target protein via *E. coli* gene expression are frequently caused by metabolic stress, resulting in decreased cell growth and rerouting of cellular metabolic processes. It has been reported that in some circumstances, it is triggered by the addition of isopropyl -D-1-thiogalactopyranoside (IPTG) inducer [8]. To be precise, once induction is began, it continues until a critical cell mass is reached. This is most likely due to a lack of critical metabolites during the process [9].

On the other hand, cell immobilisation may help to mitigate plasmid instability and cell lysis concerns, hence increasing the productivity of recombinant cells and reducing their susceptibility to pH and temperature as compared to free cells. As a result, the chance of contamination will be reduced, the fermentation time will be reduced, the rate of substrate uptake will be increased, and the predicted concentration of volatile component for the product will increase [10-11]. Meanwhile, researchers continue to focus their efforts on developing a practical way for increasing the production of xylanase enzyme, which has been identified as a high-potential enzyme for a wide variety of industrial applications, including the pulp and paper industry [12-14]. Previously, this work reveals that GO is an advantageous immobilisation matrix for recombinant *E. coli* due to its unique properties, including the ability to increase immobilisation cell efficiency and produce a high level of xylanase expression [14]. Due to its remarkable characteristics such as large specific surface area, strong biological compatibility, superior mechanical property, and long-term stability, as well as its oxygen-containing functional groups, GO has demonstrated

enormous immobilisation potential for enzymes or cells [15-16]. According to other studies, GO-coated carbon composite significantly improved biocompatibility for cell immobilisation and xylitol fermentation [17]. Optimization of cultural conditions for cell immobilisation of recombinant *E. coli* had not been studied till recently.

The high xylanase excretion observed in the immobilized cells of GO was possibly caused by the capability of these cells to be more readily incorporated with the nutrients in culture medium compared to the free cells system by attaining from the adsorbed nutrient at the liquid-solid boundary [10]. Moreover, GO has a high hydrophilicity to enhance the hydrogen (H_2) and oxygen (O_2) penetrability, which made it dissolve in the culture medium or other hydrogen bonding polar solvents [18]. By having this characteristic, it manages to open the way for cell attachment to the matrix [19]. Whereas, the xylanase excretion attained by immobilized cells onto untreated carbon nanotubes (CNTs) and treated CNTs were lower, possibly due to the diffusion restrictions of the matrix due to the high hydrophobic properties of carbon nanotube that may cause deactivation and decrease in kinetic reactions during immobilization [20-21]. Apart from that, immobilized onto CNTs were correlated with 12% lower xylanase excretion compared to GO and 45% higher cell lysis. This is probably due to the hydrophobic properties of CNTs that obstruct their process proficiency. This in turn inhibited their full potential for the process efficiency. Hence, they are usually form unsolvable aggregates [21]. Considering the solubility limitation of CNTs in any solvents, it is also quite hard to separate one carbon nanotube from the other and thus, appropriate amounts of stabilizers are necessary to prevent phase separation and flocculation [21].

The effect of various expression medium on the excretion of xylanase and cell lysis of immobilised recombinant *E. coli* was investigated in this work. *E. coli* was cultured in a variety of different expression mediums (LB, TB, 2xYT, SOB, and M9) under a variety of different conditions, including different concentrations of IPTG as an inducer, post induction temperature, post induction time, agitation rate, and pH, and the activity of xylanase and β-galactosidase was assessed.

2.0 METHODOLOGY

2.1 *E. coli* Strains and Plasmid Selection

The recombinant *E. coli* strain carrying xylanase gene (*xyng*) from *Aspergillus fumigatus* af293 used for this research was constructed by Hakimi *et al*. [22]. In this study, *xyng* with thermostability characteristic at 70°C was cloned into an expression host, *E. coli* BL21 (DE3) containing vector pET21a (+). This vector contains signal peptide M5 that can direct the expressed protein into extracellular space. *E. coli* BL21 (DE3) served as the host for heterologous expression while *E. coli* JM109 was utilized for storage purposes.

2.2 Chemical Reagents

The chemicals and reagents used in this study were all analytical grades which were obtained from several different suppliers including Merck (Merck KGaA, Darmstadt, Germany), Sigma-Aldrich (Merck KGaA, Darmstadt, Germany), Fisher (Thermo Fisher Scientific Inc, US).

2.3 Preparation of Bacterial Glycerol Stock

A single colony was inoculated into 5 ml Luria Bertani (LB) broth (5 g/l yeast extract, 5 g/l NaCl, 10 g/l tryptone) with 100 µg/ml ampicillin concentration and the culture was incubated at 37°C, 200 rpm for overnight. After that, the overnight culture was mixed with 80% of sterile glycerol [final concentration of glycerol is 15% (v/v)] and stored at -80°C. For long-term storage of the reserved stock culture, the culture was kept in bead stock culture system (British Culture Preservation Kit) [22].

2.4 Cell Immobilization

Schematic diagram of immobilized cells is shown in Figure 1.

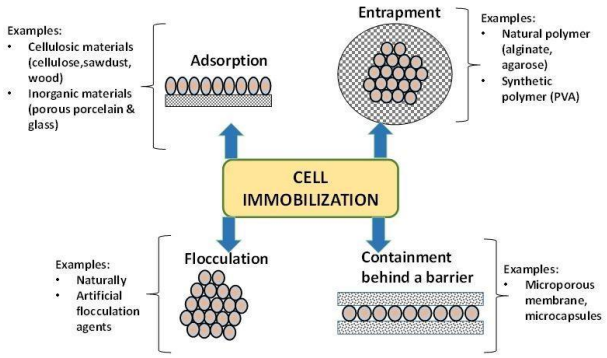


Figure 1 Schematic illustration of cell immobilization types

Immobilization of cells as shown in Figure 2 was performed by adding 10 mg of GO into 50 ml LB broth with 100 µg/ml ampicillin as previously mentioned by Nor Ashikin *et al*. [14].

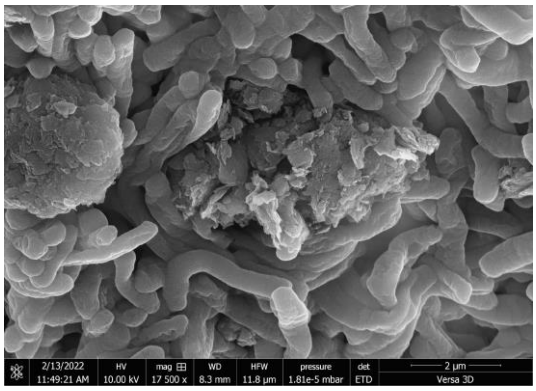


Figure 2 Immobilized recombinant *E. coli* onto GO through adsorption for xylanase production

The optimum weight of GO was gained from the screening process of the optimum matrix's quantity using the OFAT method. The GO was cultivated with recombinant *E. coli* strain from bacterial glycerol stock at 200 rpm and 37 °C. Then, the growth medium was taken out from the flask after the overnight cultivation period (12 hr). The GO was then washed thoroughly with sterilized water to remove the non-immobilized *E. coli*. Meanwhile, the immobilized *E. coli* were transferred to 250-ml flasks consisted of 50 ml TB broth with 100 µg/ml ampicillin concentration for cultivation medium. The free cell suspension (2% v/v) was used as a control in the study and operated under the equal expression and growth conditions as immobilized cell.

2.5 Screening of the Cultural Conditions using One Factor at One Time Method (OFAT)

There were few steps for the screening process as shown in Figure 3.

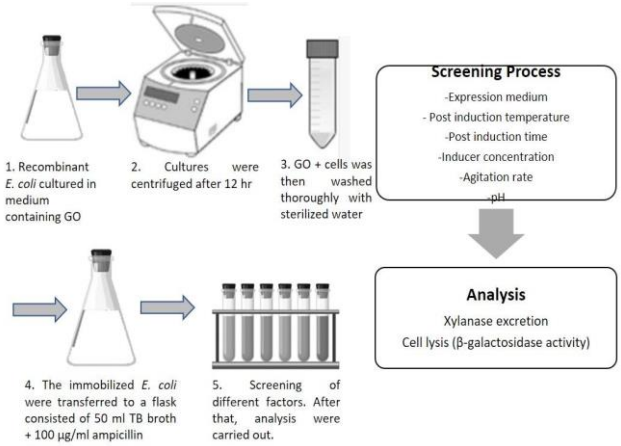


Figure 3 Research overview for production of recombinant xylanase by *E. coli*

The lists of parameters used for OFAT screening are listed in Table 1 and the OFAT cultivation conditions for improvement of xylanase production are listed in Table 2.

Table 1 The list of independent variables and parameters value of OFAT

Variable	Unit	Parameter Value				
		V_1	V_2	V_3	V_4	V_5
Inducer concentration	mM	0.01	0.05	0.1	0.5	1
Post induction temperature	° C	20	25	30	35	40
Post induction time	h	2	3	4	5	6
Agitation rate	rpm	50	100	150	200	250
pH	_	5	6	7	8	9
Expression Medium	The unit and parameter value for expression medium variables are not required					

Remark: the studied factor parameter values (V1, V2, V3, V4, V5) are listed in Table 1

Table 2 The OFAT cultivation condition for improvement of xylanase production

No	Variables	Cultivation condition					
		Medium	Temp. (°C)	pH	Speed (rpm)	Time (h)	IPTG Conc. (mM)
1.	Medium	SF	30	5	200	6	0.01
2.	Temp. (°C)	TB	SF	5	200	6	0.01
3.	pH	TB	25	SF	200	6	0.01
4.	Speed	TB	25	7	SF	6	0.01
5.	Time	TB	25	7	100	SF	0.01
6.	IPTG Conc.	TB	25	7	100	5	SF

*SF-Studied factor

2.5.1 Expression Medium Selection

The impact of the expression medium was examined using five different expression media. The formulations for each medium were listed in Table 3 below. The immobilized cells were expressed onto GO with 0.01mM inducer concentrations at 30°C, 200 rpm, 100 µg/ml ampicillin concentration and pH 5 for 24 hr of post induction time as shown in Table 2.

Table 3 The list of different expression media and formulation

Ingredient	Medium Type				
	LB	OB	TB	2xYT	M9
YE (g/L)	5	5	24	10	-
NaCl (g/L)	5	0.5	-	5	-
Tryptone (g/L)	10	20	12	16	-
KCl (g/L)	-	0.186	-	-	-
MgCl (g/L)	-	2.4	-	-	-
Glycerol (g/L)	-	-	4	-	-
KH_2PO_4 (g/L)	-	-	2.31	-	-
K_2HPO_4 (g/L)	-	-	12.54	-	-
$MgSO_4$ (%)	-	-	-	-	0.024
Glucose (%)	-	-	-	-	0.2
NH_4Cl (%)	-	-	-	-	0.1
Na_2HPO_4 (%)	-	-	-	-	0.6
NaCl (%)	-	-	-	-	0.05
KH_2PO_4 (%)	-	-	-	-	0.3

*YE- Yeast Extract, SOB- Super Optimal Broth, TB- Terrific Broth, 2xYT - 2xYeast-Tryptone and M9- Minimal Medium

2.5.2 Inducer Concentration

The impact of the inducer concentration, isopropyl β-D-1-thiogalactopyranoside (IPTG), was examined by using five different IPTG concentrations of 0.01, 0.05, 0.1, 0.5 and 1 mM as shown in Table 1. The immobilized *E. coli* were expressed onto GO in TB medium with 100 µg/ml ampicillin concentration at 25°C, 100 rpm and pH 7 for 24 hr of post induction time, as shown in Table 2.

2.5.3 Post Induction Temperature

The impact of the post induction temperature was conducted at a certain differential temperature of 20, 25, 30, 35 and 40 °C as shown in Table 1. The immobilized *E. coli* were expressed for 24 hr of post induction time onto the 0.01 g of GO in TB medium with 100 µg/ml ampicillin concentration at 200 rpm, pH 5 and 0.01 mM IPTG concentration as shown in Table 2.

2.5.4 Post Induction Time

The impact of post induction time on the immobilized cell was conducted at various times of 2, 3, 4, 5 and 6 hr as shown in Table 1. The immobilized *E. coli* were expressed onto the 0.01 g of GO in TB medium with 100 µg/ml ampicillin concentration at 25 °C, 100 rpm, pH 7 and 0.01 mM IPTG concentration [24], as shown in Table 2.

2.5.5 Agitation Rate

The effect of shaking rate on the immobilized cell was determined at different speeds of 50, 100, 150, 200 and 250 rpm as shown in Table 1. The immobilized *E. coli* were expressed onto the 0.01 g of GO in TB medium with 100 µg/ml ampicillin concentration at 25 °C, pH 7 and 0.01 mM IPTG concentration for 24 hr of post induction time as shown in Table 2.

2.5.6 pH Medium

The effect of pH medium on immobilized cell was examined by carrying out the cell expression at several different pH conditions of 5, 6, 7, 8 and 9 as shown in Table 1. The types of buffer solutions used for the medium were Sodium-acetate buffer (pH 5), Phosphate-citrate buffer (pH 6), Sodium-phosphate buffer (pH 7 and pH 8) and Tris-HCl buffer (pH 9). The immobilized *E. coli* were expressed onto GO in TB medium with 100 µg/ml ampicillin concentration at 30 °C, 200 rpm and 0.01 mM IPTG concentration for 24 hr of post induction time as shown in Table 2.

2.6 Xylanase Activity Assay

Xylanase excretion was evaluated by incubating appropriately diluted enzyme in sodium acetate buffer (pH 5.0) and temperature of 50˚C for 10 min by using a substrate solution of 1% (w/v) beechwood xylan (Merck). Reducing sugars were assayed by adding 500 µL of DNS (2-hydroxy-3, 5 dinitrosalicylic acid) reagent, boiling for 5 min, followed by cooling and reading the absorbance at 540 nm using UV-vis spectrophotometer. One unit of xylanase excretion was described as the amount of enzyme releasing 1 µmol of reducing sugar (xylose equivalent) per min under the experimental condition [22,23].

2.7 Beta-Galactosidase Activity (Cell Lysis) Assay

Cell lysis was measured by determining the quantity of β-galactosidase in the extracellular medium using 0-nitrophenyl-β-D-galactopyranosid (ONPG). A total of 1 ml of substrate buffer consisting of 4 mg/ml of ONPG in 0.1 M phosphate buffer (pH 7.4) was added with 0.1 ml of sample before it was incubated in 37°C water bath for 10 min. The absorbance at 420 nm was recorded after the reaction was ended by adding 0.5 ml of 1 M sodium carbonate. One unit of xylanase excretion was described as the quantity of enzyme that forms 10-8 moles of ONPG per min under the investigation condition [22].

3.0 RESULTS AND DISCUSSION

3.1 Expression Medium Selection

The results showed that when xylanase was cultivated in LB and 2xYT medium, the activity was lower with 0.035 U/ml and 0.032 U/ml, (Figure 4). Nevertheless, xylanase was slightly higher in SOB and M9 medium with 0.045 U/ml and 0.046 U/ml. Whereas, by using TB as a medium the xylanase excretion was highest with 0.060 U/ml after 24 hr of induction time. The highest xylanase excretion was observed in TB medium may possibly due to the amount of carbon sources including yeast extract (24 g/L) and Tryptone (12 g/L) in the medium as it is the most essential source of

energy available in the medium for microorganisms [25]. The medium composition, specifically the constituent of yeast extract, salts and peptone can potentially improve the recombinant protein concentration [26-27]. Medium selection starts by altering the carbon sources of cultivation media as it is an important energy as well as nutritional factors for xylanase producing microorganisms and it is essential to ensure that the microbial cell manage to utilize carbon sources in the media [25]. Moreover, the addition of complex nitrogen sources such as tryptone present in LB enhanced the stability of plasmids. In fact, a complex medium that contains an abundance of amino acids to support protein expression in *E. coli* [1,22]. Meanwhile, the enhancement of high cell-density culture could be reached in the shake flask fermentation by optimization or development of culture medium.

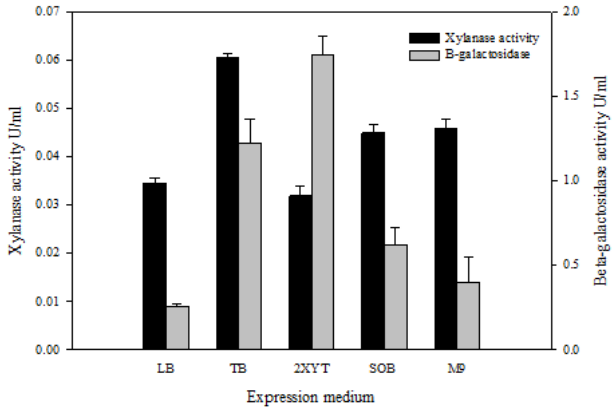


Figure 4 The effect of expression medium on xylanase excretion and β-galactosidase activity of immobilized *E. coli*

As shown in Figure 4, the lowest β-galactosidase activity was demonstrated in LB medium (0.254 U/mL), followed by M9 medium (0.400 U/mL), SOB medium (0.618 U/mL), TB medium (1.218 U/mL) and 2xYT medium (1.746 U/mL). The highest cell lysis demonstrated by 2xYT probably due to the low concentration of NaCl in the media that lead to an alteration in the electrochemical property of the cell wall. Hence, influence the way autolysis interacts with the cell walls which affect the cellular expansion and cause cellular lysis [28]. This was due to the cell wall contraction that led to an increase of electrostatic interaction among the charged of peptidoglycan groups, while the repulsion forces will effect on the swelling of the cell wall [28].

3.2 Effect of Inducer Concentration on Xylanase Excretion and Cell Viability of Immobilized *E. coli*

Figure 5 shows the xylanase excretion and β-galactosidase activity obtained for the different IPTG concentrations. The uppermost excretion of xylanase excretion was perceived from 0.01 mM IPTG concentration (0.050 U/mL), followed by 0.05 mM (0.044 U/mL), 0.1 mM (0.043 U/mL), 0.5 mM (0.037U/mL) and 1.0 mM (0.036 U/mL). Meanwhile, the highest cell lysis activity was observed from 1.0 mM IPTG concentration (2.347 U/mL), followed by 0.5 mM (1.204 U/mL), 0.1 mM (1.118 U/mL), 0.05 mM (1.065 U/mL), and 0.01 mM (0.891 U/mL).

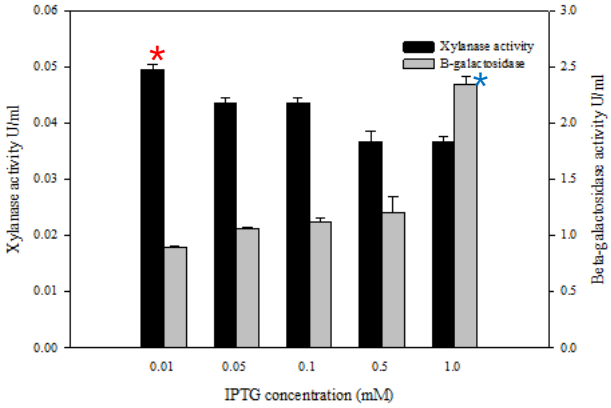


Figure 5 The effect of IPTG concentrations on xylanase excretion and β-galactosidase activity of immobilized *E. coli*

The xylanase excretion was 1.38 fold lower when the concentration of IPTG was increased from 0.01 M to 1.0 mM, indicating that a less inducer concentration is necessary to trigger the lac operon transcription for xylanase expression. The high amount of IPTG could depress the recombinant protein expression, causing low production of the target protein acquired during the process, as the high gene induction could lead to the accumulation of cytoplasmic inclusion bodies. Consequently, impairing the translocation and influence the cell expression ability [24]. In addition, the rise in toxicity level caused by IPTG will consequently effect on the synthesis of protein and altered the system function of the cells, as the optimum inducer concentration is depends on the cell system. Thus, the metabolic change occurs at particular IPTG concentration might occur at different concentrations of IPTG in another system [24,29].
Furthermore, the β-galactosidase activity of the cell was 1.13 fold higher or increased by 13% when the IPTG concentration was increased from 0.01 mM to 1.0 mM due to the higher metabolic stress exposed to the cell when higher IPTG concentration was used to initiate the cell expression. Therefore, the generically applicable approach to increase the recombinant protein productivity is by minimizing the exposed stress on expression cell. Since, by reducing the inducer concentration could enhance the correct folding of recombinant proteins by allowing recombinant protein translation to proceed more slowly and reducing protein synthesis rate.

3.3 Effect of Post Induction Temperature on Xylanase Excretion and Cell Viability of Immobilized *E. coli*

The results demonstrated that when the cells were cultivated at 30 °C, 35 °C and 40 °C of post induction temperature, the xylanase excretion was low with 0.038 U/ml, 0.035 U/ml and 0.033 U/ml, as shown in Figure 6. Nevertheless, xylanase was slightly increased at 20 °C with 0.053 U/ml of unit activities. While at 25 °C, the reaction for xylanase excretion demonstrated the highest activity with 0.057 U/ml after 24 hr induction time.

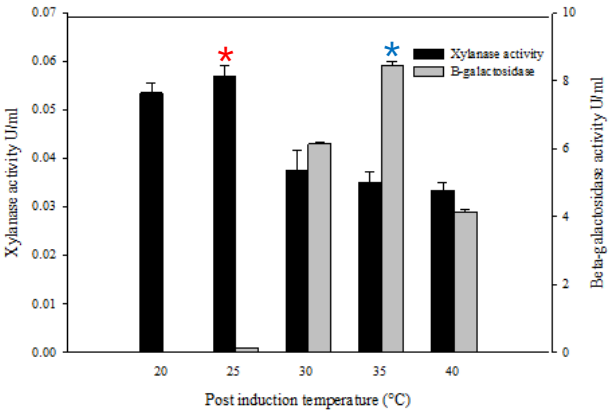


Figure 6 The effect of post induction temperature on xylanase excretion and β-galactosidase activity of immobilized *E. coli*

The optimum temperature to be used for cell expression was supposed to be at temperature 25 °C. Thus, any alteration of cultivation temperature above or below the optimum temperature could lead to alteration in xylanase protein structure and change the three-dimensional shape or xylanase denaturation consequently altering its catalytic activities or its ability to bind substrates [30]. Resulting change in the reaction and lead to reduction in cellular metabolic activities rate, with subsequent inhibition in protein synthesis and cell growth [31].

While, at the temperature of 30 °C, 35 °C, and 40 °C, the xylanase excretion was declined probably due to the denaturation or inactivation of xylanase enzyme that cause the rate of enzyme catalyzed reaction to decrease [32-33]. According to Behnam *et al*. [34] and Patel & Gupte [35], most of the xylanases or proteins are receptive to be defective when being exposed to high temperature. Since such condition may limit the synthesis of essential proteins for microbial growth and other physiological processes which cause the thermal denaturation of protein and enzymes.

The lowest β-galactosidase activity was demonstrated at 20 °C post induction temperature (0.020 U/mL), followed by 25 °C (0.135 U/mL), 40 °C (4.131 U/mL), 30 °C (6.149 U/mL) and 35 °C (8.461 U/mL). According to the Papaneophytou & Kontopidis [26], even though an increase in temperature could stimulate the cells growth, it is adverse for protein excretion, since a faster growth rate could promote the mispartition of a construct vector and increase the plasmid free possibility. Moreover, the higher temperature could cause a metabolic stress on the microorganisms which may lead to increase in β-galactosidase activity compared to the lower post-induction temperature. Besides, the production of inclusion bodies could be inhibited by reducing the post induction temperature to reduce the protein synthesis rate. This approach has been validated practical for certain of difficult recombinant proteins [26].

3.4 Effect of Post-Induction Time on Xylanase Excretion and Cell Viability of Immobilized *E. coli*

Figure 7 shows the xylanase excretion and β-galactosidase activity obtained for five different induction time of cultivated cells. The uppermost excretion of xylanase excretion was obtained at 5 hr of induction time (0.042 U/mL), followed by 6 hr (0.039 U/mL), 4 hr (0.038 U/mL), 3 hr (0.033U/mL) and 2 hr (0.028 U/mL). While, the highest cell lysis activity was observed at 3 hr (0.756 U/mL), followed by 2 hr (0.624 U/mL), 4 hr (0.255 U/mL), 6 hr (0.148 U/mL), and 5 hr (0.075 U/mL). The best induction point was at the mid-log phase, which were equivalent to the 5 hr. During this time, the expression level of the cell was correlated with the highest xylanase excretion with the lowest β-galactosidase activity (cell lysis) compared to the 2, 3, 4 and 6 hr of post induction time.

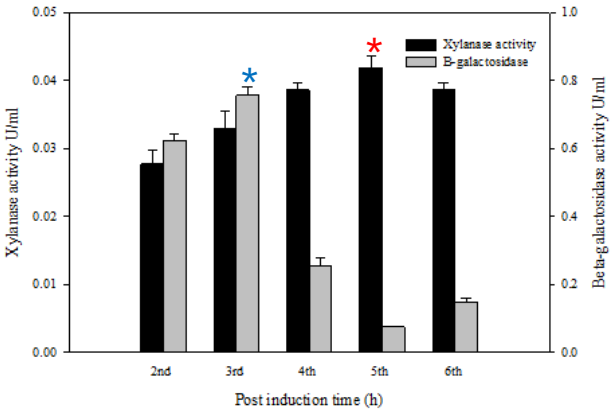


Figure 7 The effect of induction time on xylanase excretion and β-galactosidase activity of immobilized *E. coli*

This is in alignment with the previous studies by Nor *et al*. [36] which mentioned the common optimum point of induction time for recombinant enzyme expression in periplasmic space of *E. coli* is at the middle of log phase. This is because an effective enzyme expression involves an induction of host cell at the mid of log phase when the cell growth is at its maximum rate and both of the cell growth as well as protein translation were rapid [36-38].

Therefore, the recombinant protein production during cultivation process is influenced by the induction point and the length of induction process [36]. Extended induction time could elongate the metabolic load exposed to the cells which lead to an exhaustion of the nutrition sources in the media [36]. Thus, induction point is generally executed at the early mid-point of an exponential phase. However, the induction process can also be performed during the static point and even in the late of exponential phase as it depends on the type of system that was implemented for the process [26].

3.5 Effect of Agitation Rate on Xylanase Excretion and Cell Viability of Immobilized *E. coli*

Figure 8 demonstrates that when xylanase was cultivated at 50 rpm, 100 rpm and 250 rpm of agitation rate, the activity was lower with 0.049 U/ml, 0.054 U/ml and 0.050 U/ml, respectively. Nevertheless, xylanase was slightly increased at 200 rpm with 0.057 U/ml xylanase activities. Meanwhile, at 150 rpm condition, the xylanase excretion was highest with 0.060 U/ml after 24 hr induction time. The xylanase excretion of the immobilized cell at 50 rpm was lower compared to 150 rpm may be due to improper mixing of the medium components that lead to the mass transfer limitations as well as limitation of the dissolved oxygen. Thus, the cells have lower metabolic energy and cellular activity that compulsory for the effectiveness of enzyme excretion. Consequently, it leads to the improper nutrients mixing and poor air dispersion that impacted on the cells development which consequently resulted in low protein excretion [24]. While, at higher agitation rate of 150 rpm, the higher xylanase excretion was generated. This may possibly due to the sufficient oxygen resource in cultivation medium when the cell was cultured at high agitation rate [24, 30].

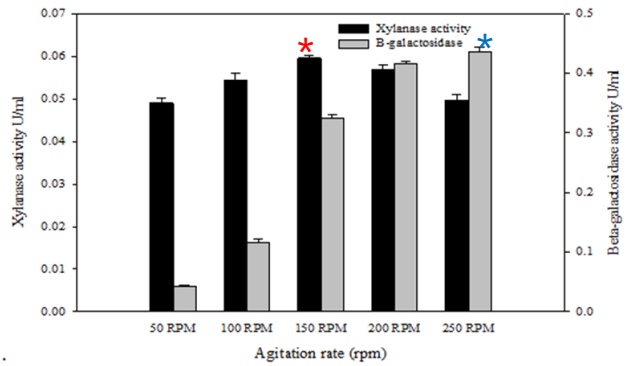


Figure 8 The effect of agitation rate on xylanase excretion and β-galactosidase activity of immobilized *E. coli*

The lowest β-galactosidase activity was demonstrated at 50 rpm agitation rate (0.042 U/mL), followed by 100 rpm (0.116 U/mL), 150 rpm (0.324 U/mL), 200 rpm (0.416 U/mL) and 250 rpm (0.436 U/mL) as shown in the Figure 8. At the high agitation speed of 250 rpm, the cell was correlated with 10.38-fold higher of cell lysis activity compared to the cell cultured at 50 rpm. This was due to the higher shear stress generated at the higher shaking rate that effect negatively on cell development. As the agitation rate increases to 250 rpm, a lower xylanase excretion was gained. Thus, a faster shaking rate would lead to a notable rising in β-galactosidase activity caused by the shear stress produced by higher agitation rate which in turn bring about the negative effects on the strains. For instance, the detachment of cells from the immobilization matrix to the medium and the further metabolic stress on the cells which in turn cause the occurrences of autolysis and cells rupture [24,39].

3.6 Effect of pH Selection on Xylanase Excretion and Cell Viability of Immobilized *E. coli*

The results in Figure 9 demonstrated that when xylanase was cultivated at pH 8.0 and 9.0, the activity was low with 0.048 U/ml and 0.047 U/ml, respectively. Nevertheless, xylanase was slightly stable at pH 7.0 and 6.0 with 0.052 U/ml and 0.049 U/ml respectively. While at pH 5.0 the xylanase excretion was the lowest with 0.037 U/ml.

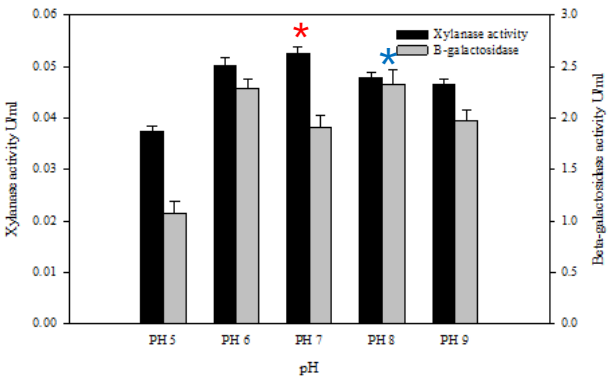


Figure 9 The effect of pH on xylanase excretion and β-galactosidase activity of immobilized *E. coli*

The maximum xylanase excretion demonstrated at pH 7.0 (0.052 U/ml) with soluble xylan as the carbon source and substrate. Hence, it showed that the xylanase enzyme was stable in surroundings where the pH is retained near neutral conditions. In several experimental conducted with lower pH values, the protein excretion was too low [40]. This described that the optimum pH for isolated xylanases from different bacterium are at neutral pH condition and the xylanase isolated from *Aspergillus fumigatus* was active in the pH range from 6.0 to 8.0 [41]. Consequently, any alteration in the pH above or lower its optimum range can possibly change the three-dimensional structure of an enzyme and consequently lead to a drop in enzymatic activity due to the protein instability and inactivation [42].

Meanwhile, the lowest β-galactosidase activity was demonstrated in pH 5 (0.97 U/mL), followed by pH 7 (1.89 U/mL), pH 9 (1.97 U/mL), pH 6 (2.28 U/mL) and pH 8 (2.31 U/mL). Based on Ramírez-Nuñez *et al*. [28], the increased in bacteria cell autolysis under acidic environment and low salt concentration is due to the elastic, flexible and extensible of the cell wall. Peptidoglycan is accountable for bacterial cell wall properties and if it is enzymatically damaged by autolysis, it will become to be water-soluble and unable to work as structural mechanical support to produce cellular lysis. The alterations in the electrochemical property of the bacterial cell wall influence the change of autolysis adhesion which leads to the peptidoglycans hydrolysis [1].

The most encouraging environment for immobilized cells to excrete enzyme is neutral condition that correlated with lower autolysis compared to acidic pH. The higher H+ ions can cause the formation of unstable biofilm and weak attachment due to the cations repulsive forces [24]. Thus, by varying pH medium condition consequently, it can affect autolysis interaction and electrochemical properties in the cell wall, thus causing expansion or contraction of the cell wall as well as decreasing or increasing cellular lysis [28].

4.0 CONCLUSION

The Terrific Broth (TB) provided an optimal expression medium as it is correlated to lower cell lysis along with the better maintenance of plasmid stability and higher xylanase excretion. TB did not only prevent nutrient inadequacy but also prevent protease activity by supplying complete enzyme substrates. Hence, cells grown in TB did not need to synthesize most of the precursor molecules as they were already present in the medium. Moreover, lower proportion of IPTG at 0.01 mM, lower post-induction temperature (25°C), post-induction time of 5 hours, neutral pH, 150 rpm, were significantly improved the xylanase excretion of immobilized cell with high cell stability. The less inducer concentration was necessary to trigger the lac operon transcription for xylanase expression and improved the correct folding of recombinant protein translation by allowing recombinant protein translation to occur slowly and lowering the metabolic burden imposed to the cells during protein synthesis. The lower temperature provided an adequate period for protein to fold properly. Apart from that proper mixing of the medium components will lead to good mass transfer as well as sufficient availability of dissolved oxygen. The neutral pH condition (pH 7) was the most suitable environment for the immobilized cell to obtain the high xylanase production and maintain plasmid stability with less occurrence of cell lysis. It was due to the strong attachment of the cell onto the GO. Too low or too high pH can impose too much stress on metabolic process of the cells.

Conflicts of Interest

The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

Acknowledgement

We are grateful to the Ministry of Higher Education Malaysia for the funding from the research grant Research Acculturation Grant Scheme Vot R048 as well as Universiti Tun Hussein Onn Malaysia (UTHM). Communication of this research is made possible through monetary assistance by Universiti Tun Hussein Onn Malaysia and the UTHM Publisher's Office via Publication Fund E15216.

References

[1] Man, R. C., Ismail, A. F., Ghazali, N. F., Fuzi, S. F. Z. M., & Illias, R. M. 2015. Effects of the Immobilization of Recombinant Escherichia coli on Cyclodextrin Glucanotransferase (CGTase) Excretion and Cell Viability. *Biochemical Engineering Journal*. 98: 91-98. Doi: 10.1016/j.bej.2015.02.013.

[2] Yoon, S. H., Kim, S. K., & Kim, J. F. 2010. Secretory Production of Recombinant Proteins in Escherichia coli. *Recent Patents on Biotechnology*. 4(1): 23-29. Doi: 10.2174/187220810790069550.

[3] Arnau, J., Yaver, D., Hjort, C. M. 2019. Strategies and Challenges for the Development of Industrial Enzymes Using Fungal Cell Factories. *Grand Challenges in Fungal Biotechnology*. 27: 179-210. Doi: 10.1007/978-3-030-29541-7_7.

[4] Chukwuma, O. B., Rafatullah, M., Tajarudin, H. A., Ismail, N. 2022. Lignocellulolytic Enzymes in Biotechnological and Industrial Processes: A Review. *Sustainability*. 12: 7282. Doi: 10.3390/su12187282.

[5] Chandra, P., Enespa, Singh, R., Arora P. K. 2020. Microbial Lipases and Their Industrial Applications: A Comprehensive Review. *Microbial Cell Factories*. 19: 169. Doi: 10.1186/s12934-020-01428-8.

[6] Choi, J. H., & Lee, S. Y. 2004. Secretory and Extracellular Production of Proteins Using Escherichia coli. *Applied Microbiology and Biotechnology*. 64(5): 625-635. Doi: 10.1007/s00253-004-1559-9.

[7] Wyre, C., & Overton., T. W. 2014. Use of a Stress-Minimisation Paradigm in High Cell Density Fed-Batch *Escherichia coli* Fermentations to Optimise Recombinant Protein Production. *Journal of Microbiology Biotechnology*. 41: 1391-1404. Doi: 10.1007/s10295-014-1489-1.

[8] Ramchuran, S. O., Olle, H., & Nordberg, K. 2005. Effect of Post Induction Nutrient Feed Composition and Use of Lactose as Inducer During Production of Thermostable Xylanase in Escherichia Coli Glucose-limited Fed-batch Cultivations. *Journal of Bioscience and Bioengineering*. 99(5): 477-84. Doi: 10.1263/jbb.99.477.

[9] Morowvat, M. H., Babaeipour, V., Memari, H. R., & Vahidi, H. 2015. Optimization of Fermentation Conditions for Recombinant Human Interferon Beta Production by *Escherichia coli* Using the Response Surface Methodology. *Jundishapur Journal of Microbiology*. 8(4): 1-11. Doi: 10.5812/jjm.8(4)2015.16236.

[10] Nor Ashikin, N. A. L., Fuzi, S. F. Z. M., Abdul Manaf, S. A., Abdul Manas, N. H., Md Shaarani@ Md Nawi, S., Md Illias, R. 2022. Optimization and characterization of immobilized *E. coli* for engineered thermostable xylanase excretion and

cell viability. Arabian Journal of Chemistry,15: 6,103803. Doi: 10.1016/j.arabjc.2022.103803.
[11] Ghoshoon, M. B., Raee, M. J., Shabanpoor, M. R., Dehghani, Z., Ebrahimi, N., Berenjian, A., Negahdaripour, M., Hemmati, S., Sadeghian, I., Ghasemi, Y. 2022. Whole cell immobilization of Recombinant E. coli Cells by Calcium Alginate Beads; Evaluation of Plasmid Stability and Production of Extracellular L-asparaginase. *Separation Science and Technology*. 57(17): 2836-2842. Doi: 10.1080/01496395.2021.1962910.
[12] Tripathi, N. K., Shrivastava, A. 2019. Recent Developments in Bioprocessing of Recombinant Proteins: Expression Hosts and Process Development. *Frontiers In Bioengineering & Biotechnology*. 20(7): 420. Doi: 10.3389/fbioe.2019.00420.
[13] Rosano, G. L., Morales, E. S., Ceccarelli, E. A. 2019. New Tools for Recombinant Protein Production in Escherichia coli: A 5-Year Update. *Protein Science*. 28: 1412–1422. 10.1002/pro.3668.
[14] Nor Ashikin, N. A. L., Wahab, M. K. H. A., Illias, R. M., Fuzi, S. F. Z. M. 2017. Comparison of Thermostable Xylanase Production by Escherichia coli Immobilised onto Different Nanoparticles. *Chemical Engineering Transactions*. 56: 1825-1830. Doi: 10.3303/CET1756305.
[15] Gao, Y., Zheng, H., Hu, N., Hao, M., Wu, Z. 2018. Technology of Fermentation Coupling with Foam Separation for Improving the Production of Nisin using a κ-carrageenan with Loofa Sponges Matrix and an Hourglass-shaped Column. *Biochemical Engineering Journal*. 133: 140-148. Doi: 10.1016/j.bej.2018.02.008.
[16] Wang, C., Chen, X., Jiang, X., Li, N., Zhu, P., Xu, H. 2022. Facile and Green Synthesis of Reduced Graphene Oxide/loofah Sponge for Streptomyces Albulus Immobilization and ε-poly-l-lysine Production. *Bioresource Technology*. 349: 126534. Doi: 10.1016/j.biortech.2021.126534.
[17] Wang, L., Yin, Y., Zhang, S., Wu, D., Lv, Y., Hu, Y., Wei, Q, Yuan, Q., Wang, J. 2019. A Rapid Microwave-assisted Phosphoric-Acid Treatment on Carbon Fiber Surface For Enhanced Cell Immobilization In Xylitol Fermentation. *Colloids and Surfaces B: Biointerfaces*. 175: 697-702. Doi: 10.1016/j.colsurfb.2018.12.045.
[18] Zhang, J., Zhang, J., Zhang, F., Yang, H., Huang, X., Liu, H., & Guo, S. 2010. Graphene Oxide as a Matrix for Enzyme Immobilization. *Langmuir*. 26(9): 6083-6085. Doi:10.1021/la904014z.
[19] Ammar, A., Al, A. M., Alali, M., & Karim, A. 2016. Influence of Graphene Oxide on Mechanical, Morphological, Barrier, and Electrical Properties of Polymer Membranes. *Arabian Journal of Chemistry*. 9(2): 274-286. Doi: 10.1016/j.arabjc.2015.07.006.
[20] Zajkoska, P., Martin, R., & Michal, R. 2013. Biocatalysis with Immobilized Escherichia coli. *Applied Microbiology and Biotechnology*. 97: 1441-1455. Doi: 10.1007/s00253-012-4651-6.
[21] Saifuddin, N., Raziah, A. Z., & Junizah, A. R. 2013. Carbon Nanotubes: A Review on Structure and Their Interaction with Proteins. *Journal of Chemistry*. 1-16. Doi: 10.1155/2013/676815.
[22] Hakimi, M. K., Anuar M., & Illias, R. M. 2016. Thermostability Enhancement of Xylanase *Aspergillus fumigatus* RT-1. *Journal of Molecular Catalysis B: Enzymatic*. 134(A): 154-163. Doi.org/10.1016/j.molcatb.2016.09.020.
[23] Fuzi, S. F. Z. M., Farah, D. A. B., Abdul, M. A. M., & Rosli, M. I. 2011. Development and Validation of a Medium for Recombinant Endo- B -1, 4-Xylanase Production by Kluyveromyces lactis. *Annals of Microbiology*. 62(1). Doi: 10.1007/s13213-011-0258-x.
[24] Man, R. C., Ismail, A. F., Fuzi, S. F. Z. M., Ghazali, N. F., Md Illias, R. 2016. Effects of Culture Conditions of Immobilized Recombinant *Escherichia coli* on Cyclodextrin Glucanotransferase (CGTase) Excretion and Cell Stability. *Process Biochemistry*. 98: 91-98. Doi: 10.1016/j.bej.2015.02.013.
[25] Afzali, N., Bahareh, R. Z., & Nahidhagh, N. 2016. Optimization of Trichoderma reesei Medium for Increasing Xylanase Enzyme Production. *Advances in Bioresearch*. 7: 94-99. Doi: 10.15515/abr.0976-4585.7.3.9499.
[26] Papaneophytou, C. P., & Kontopidis, G. 2014. Statistical Approaches to Maximize Recombinant Protein Expression in Escherichia coli: A General Review. *Protein Expression and Purification*. 94: 22-32. Doi.org/10.1016/j.pep.2013.10.016.
[27] Joseph, B. C., Suthakaran P., Sankaranarayanan, S., & Musti, M. 2015. An Overview of the Parameters for Recombinant Protein Expression in *Escherichia coli*. *Cell Science & Therapy*. 6(5): 1-7. Doi: 10.4172/2157-7013.1000221.
[28] Ramírez-Nuñez, J., Romero-Medrano, R., Nevárez-Moorillón, G. V., & Gutiérrez-Méndez, N. 2011. Effect of pH and Salt Gradient on the Autolysis of *Lactococcus lactis* Strains. *Brazilian Journal of Microbiology*. 52: 1495-1499. Doi: 10.1590/S1517-83822011000400036.
[29] Norsyahida, A., Rahmah, N., & Ahmad, R. M. Y. 2009. Effects of Feeding and Induction Strategy on the Production of Bmr1 Antigen in Recombinant E. Coli. *Applied Microbiology*. 49: 544-50. Doi: 10.1111/j.1472-765X.2009.02694.x.
[30] Nkohla, A., Okaiyeto, K., Olaniran, A. & Nwodo, U. 2017. Optimization of Growth Parameters for Cellulase and Xylanase Production by *Bacillus* Species Isolated from Decaying Biomass. *Journal of Biotech Research*. 8: 33-47.
[31] Fatokun, E. N., Uchechukwu, U. N., Ademola, O. O., & Anthony, I. 2017. Optimization of Process Conditions for the Production of Holocellulase by a Bacillus Species Isolated from Nahoon Beach Sediments. *American Journal of Biochemistry and Biotechnology*. 13(2): 70-80. Doi: 10.3844/ajbbsp.2017.70.80.
[32] Khusro, A., Kannan, B., Valan, M. & Agastian, P. 2016. Statistical Optimization of Thermo-Alkali Stable Xylanase Production from Bacillus tequilensis Strain. *Electronic Journal of Biotechnology*. 22: 16-25. Doi: 10.1016/j.ejbt.2016.04.002.
[33] Shang, T., Si, D., Zhang, D. & Liu, X. 2017. Enhancement of Thermoalkaliphilic Xylanase Production by Pichia Pastoris Through Novel Fed-Batch Strategy in High Cell-Density Fermentation. *BMC Biotechnology*. 17(55): 1-10. Doi: 10.1186/s12896-017-0361-6.
[34] Behnam, S., Karimi, K., Khanahmadi, M. & Salimian, Z. 2016. Optimization of Xylanase Production by Mucor indicus, *Mucor hiemalis*, and *Rhizopus oryzae* through Solid State Fermentation. *Biological Journal of Microorganism*. 4(16): 1-10. Doi: 10.1515_tjb-2016-0036.
[35] Patel, H., & Gupte, A., G. 2016. Optimization of Different Culture Conditions for Enhanced Laccase Production and Its Purification from Tricholoma giganteum AGHP. *Bioresourcing and Bioprocessing*. 3(11): 1-10. Doi: 10.1186/s40643-016-0088-6.
[36] Nor, S., Azaman, A., Ramanan, R., & Tan, J. 2010. Screening for the Optimal Induction Parameters for Periplasmic Producing Interferon-A2b in *Escherichia coli*. *African Journal of Biotechnology*. 9(38): 6345-6354. Doi: 10.5897/AJB10.556.
[37] Krause, M., Ukkonen, K., Haataja, T., & Ruottinen, M. 2010. A Novel Fed-batch Based Cultivation Method Provides High Cell-Density and Improves Yield of Soluble Recombinant Proteins in Shaken Cultures. Microbiology Cell Factories, 9(11): 1-11. Doi: 10.1186/1475-2859-9-11.
[38] Jiang, Y. M., Wang, Y. T. & Dong, Z. W. 2007. Effects of Induction Starting Time and Ca2+ on Expression of Active Penicillin G Acylase in Escherichia coli. *Biotechnology Progress*. 23(5): 1031-1037. Doi: 10.1021/bp070100.
[39] Huang, R., Chao, Z., Hui, T., & Ruming, Z. 2013. Effect of Culture Conditions on Production of Xylanase by

Trichoderma reesei. *Advanced Materials Research*. 784: 856-860. Doi: 10.4028/www.scientific.net/AMR.781-784.856.
[40] Silva, L. C. A., Honorato, T. L., Cavalcante, R. S., Franco, T. T., & Rodrigues, S. 2016. Effect of pH and Temperature on Enzyme Activity of Chitosanase Produced under Solid Stated Fermentation by Trichoderma spp. *Indian Journal of Microbiology*. 52(1): 60-65. Doi:10.1007/s12088.
[41] Cavalieri, N., Guimaraes, D. A., Carvalho, S. D., & Nogueira, P. 2013. Effect of Xylanase from *Aspergillus niger* and *Aspergillus flavus* on Pulp Biobleaching and Enzyme Production using Agroindustrial Residues as Substract. *Bioprocess and Biotechnology*. 2-9. Doi: 10.1186/2193.
[42] Andrew, G. M., Keith, F. T. 2022. Parameter Reliability and Understanding Enzyme Function. *Molecules*. 27(1): 263. Doi:10.3390/molecules27010263.