D-XYLONIC ACID FROM RECOMBINANT *E. coli* BL21 (DE3): COMPARISON BETWEEN SHAKE FLASK AND BENCHTOP BIOREACTOR FERMENTATION

N A M Rodzri [1], W S W M Zain [1], R M A Hanapiah [1], R A Samah [1] and R M Illias [2]

[1] Faculty of Chemical & Process Engineering Technology, Universiti Malaysia Pahang, 26300 Gambang, Pahang, Malaysia
[2] Faculty of Chemical & Energy Engineering, Universiti Teknologi Malaysia, 81310 Skudai, Johor, Malaysia

salwanis@ump.edu.my

Abstract. Production D-xylonic acid from recombinant *E. coli* BL21 (DE3) at a small scale has been successfully done several times. In an attempt to scale up the production process to a bigger scale, fermentation in bioreactor was conducted. The comparison on growth of recombinant *E. coli* BL21 (DE3) and production of D-xylonic acid in both shake flask and bioreactor was investigated. Higher specific growth rate of recombinant *E. coli* BL21 (DE3) and concentration of D-xylonic acid however, was obtained by fermentation in shake flask which is 0.273 h^{-1} and 9.06 gL^{-1} D-xylonic acid respectively. The result suggests several mistakes done during fermentation in bioreactor that causes low concentration of D-xylonic acid in bioreactor fermentation. Therefore, this calls up for improvement and better scale-up strategies in future work.

Keywords: D-xylonic acid, recombinant *E. coli* BL21 (DE3), scale-up

1. Introduction

In these recent years, production of bio-based materials such as D-xylonic acid that has the potential of replacing fossil-based products has become the forefront of biotechnological field. Having similar properties as gluconic acid and as another derivatives of xylose other than xylitol, D-xylonic acid has its own unique traits that makes itself listed as the top 30 most valuable platform chemicals [1,2]. D-xylonic acid proves to be a cheaper alternative than gluconic acid as it is derived from the hemicellulose sugar xylose that is a non-food carbohydrate [3]. Reported application of D-xylonic extends to pharmaceutical field, in construction field as a complexing agent for concrete dispersal, in agriculture field and also used in polishes and paint [3,4,5].

Despite the advantages of D-xylonic acid, the production of D-xylonic acid at a larger scale is still yet to exist. Many researchers attempt to produce D-xylonic acid form various strains of bacteria such as recombinant *E. coli*, *G. oxydans, P. fragi* [2,3,6] and yeast such as *K. lactis* and *S. cerevisiae* [7,8]. From all the reported literature on D-xylonic acid production, recombinant *E. coli* shows the most promising yield of D-xylonic acid. This is not at all surprising considering that genetic engineering of microorganism is a robust method used to improve or create new pathways in recombinant microbes, therefore improving product yield [19]. Production of D-xylonic acid from recombinant *E. coli* W3110

at small scale and in 5L bioreactor was done using two carbon source, xylose and glucose by Liu et. al. Maximum concentration of 39.2 gL^{-1} D-xylonic acid was obtained from 40 gL^{-1} xylose with average productivity as high as 1.09 $gL^{-1}h^{-1}$ [2]. Another recombinant *E. coli* strain used for D-xylonic acid production was recombinant *E. coli* BL21 (DE3). Under fed-batch fermentation in 5L bioreactor, 27.3 gL^{-1} D-xylonic acid was obtained from 30 gL^{-1} xylose which makes up 88% of theoretical yield. This fermentation however uses sole carbon source which is xylose [9]. High concentration production of D-xylonic acid was also successfully done using recombinant *E. coli* BL21(DE3) at a small scale in previous work by investigating various effect of process parameters such as temperature, agitation rate and pH on D-xylonic acid production (unpublished work). D-xylonic acid concentration as high as 9.95 gL^{-1} was obtained from 10 gL^{-1} with yield of 0.91 g/g xylose when fermentation was conducted at 37°C, pH 7 and 200 rpm. Seeing the potential of D-xylonic acid production at a larger scale, a scale-up process production in a benchtop bioreactor was carried out.

Process scale-up is a critical process that allows fermentation process achieved in research and development stage to operate at a pilot scale for industrial manufacturing [10]. When scaling up a fermentation process, many parameters should be paid attention to in order to minimize the scale-up risk. Common problems that arises during scale-up process are microbial contamination, equipment failure, varies raw materials quality and poor fermentation performances [16, 17]. Therefore, all fermentation parameters that could cause these risks should not be overlooked. In this paper, fermentation of recombinant *E. coli* BL21 (DE3) in shake flask and 2 L bioreactor using batch mode was conducted. Comparison on growth of recombinant *E. coli* BL21 (DE3) and production of D-xylonic acid was then determined and analyzed.

2. Materials & Methods

2.1. Microorganism

Recombinant *E. coli* BL21 (DE3) strain used in this study was acquired from Universiti Teknologi Malaysia's culture collection. The native xylose and xylonic acid catabolism pathway in this recombinant *E. coli* were blocked and two new genes were expressed in the recombinant *E. coli* to allow accumulation of D-xylonic acid in the culture broth. The two genes were xylose dehydrogenase and xylonolactonase genes from *Ralstonia pickettii*, which was cloned into pACYC-Duet plasmid from Novagen and later expressed in the recombinant *E. coli* BL21 (DE3). The strain was maintained as a refrigerated 10% (v/v) glycerol stock.

2.2. Media preparation and cultural conditions

50 mL of sterilized Luria-bertani broth (LB) which consist of 10 gL^{-1} tryptone, 5 gL^{-1} yeast extract and 10 gL^{-1} sodium chloride, together with 34 µg/mL chloramphenicol was used as a media for seed culture. A loopful of recombinant E. coli BL21 (DE3) was transferred into the media and the media was then incubated at 37°C and 200 rpm agitation rate for 18 hours in an incubator shaker. Fermentation in shake flask was conducted in 500 mL Erlenmeyer flask meanwhile for benchtop bioreactor, a 2 L stirred tank bioreactor was used. The media used for both fermentations was the super optimal broth (SOB) containing 20 gL^{-1} tryptone, 5 gL^{-1} yeast extract, 0.5 gL^{-1} NaCl, 0.186 gL^{-1} KCl and 2.4 gL^{-1} MgSO4.

2.3. Experimental setup

For fermentation in shake flasks with working volume of 150 mL, 100 mL of SOB together with 10 gL^{-1} xylose, 34 µg/mL chloramphenicol and 10% (v/v) seed culture was incubated at 37°C and 200 rpm agitation rate for 24 hour. When the OD_{600} of the cells in the culture reach about 0.6, 0.5 mM of IPTG was added into the culture to induce the protein expression. Fermentation in bioreactor with 1.5 L working volume was carried out with the same cultural condition. The bioreactor used was a 2 L Biostat® A (jacketed-single wall glass vessel, Rushton turbine impeller (4 blades, 47 mm diameter, 9 mm blade height)) with automated DO and pH control.

SOB medium was transferred to the bioreactor and sterilized at 121°C for 20 minutes. Separately autoclaved 10 gL^{-1} xylose, filter-sterilized 34 µg/mL chloramphenicol and 150 mL seed culture were also added into the bioreactor prior to the fermentation. The batch fermentation was carried out with the controlled setting of 37°C, stirring speed 200 rpm, airflow of 2 L/min and 100% dissolved oxygen (DO) for 24 hour. During the fermentation in bioreactor, the pH of the media was maintained at pH 7 via automated addition of 0.5 M HCl and 1 M NaOH. Throughout both fermentations, samples were collected at different intervals and the supernatants were used for xylonic acid and xylose determination. Figure 1 below illustrates the schematic diagram of the stirred tank benchtop bioreactor that was used in this study.

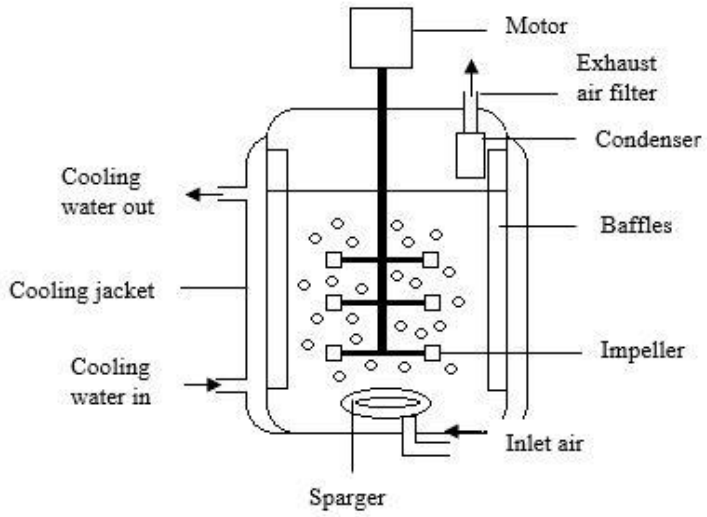

Figure 1. Schematic diagram of a stirred tank benchtop bioreactor

2.4. D-xylonic acid

D-xylonic acid concentration was quantify based on the method described by Liu [2] with slight modification. Samples from fermentation broth was first centrifuge and then were diluted in 0.7 M HCl. The samples were then boiled for 15 minutes at 100°C to convert xylonic acid to xylono-γ-lactone. 500 µL of the diluted samples were then transferred into 1 mL hydroxylamine reagent (2M hydroxylamine HCl in 2 M NaoH). 650 µL of 3.2 M HCl was added to the samples in hydroxylamine reagent followed by 500 µL of 100 g L^{-1} FeCl3 (FeCl3 was prepared in 0.1 M HCl). The absorbance of the solution was quickly measured at 550 nm wavelength to determine xylonic acid concentration using UV-VIS spectrophotometer (Varian Cary® 50).

2.5. Xylose

Xylose concentration was quantified by using Dinitrosalicylic acid (DNS) method. Distilled water was used to diluted samples at ratio of 1:9. 1 mL of DNS reagent was then added to 1 mL of the diluted samples. Then, samples in tubes were covered and were boiled for 10 minutes. The solution was then allowed to cool down before 0.33 mL of potassium sodium tartrate was added into the solution. The absorbance of the solution was quickly measured using UV-Vis spectrophotometer (Varian Cary ® 50) at 575 nm wavelength.

3. Results and Discussions

3.1. Comparison of recombinant E. coli BL21 (DE3) growth profile

Comparison of recombinant *E. coli* BL21 (DE3) growth profile was done by developing the growth curve from fermentation in both shake flask and 2 L benchtop bioreactor. Fermentation in both vessels were conducted under the same parameters which is 37°C temperature, initial pH of medium pH 7 and agitation rate of 200 rpm. Standard curve for cell dry weight (gL^{-1}) was first developed in order to obtain the regression line that correlates the absorbance of cell and cell dry weight. This standard curve is later used to generate the growth profile. It can be observed in Figure 2 that the growth of recombinant *E. coli* BL21 (DE3) increased exponentially after experiencing lag phase for the first hour. The *E. coli*'s exponential growth for both shake flask and bioreactor then reached its maximum value after 4 hours.

As clearly depicted in Figure 2, there is a significant difference in growth profile of recombinant *E. coli* BL21 (DE3) that was grown in shake flask and 2 L bioreactor. The growth profile of *E. coli* in shake flask was unexpectedly higher than in bioreactor, with specific growth rate, μ of 0.273 h^{-1} and doubling time, τd of 2.54 h. The μ and τd of fermentation in bioreactor on the other hand was 0.266 h^{-1} and 2.60 h respectively. The growth profile of bacteria in the bioreactor was supposedly higher than in shake flask, however the result was contrary. There are several aspects that should not be overlooked when scaling-up a fermentation process. These aspects include oxygen transfer, heat transfer of surface-to-volume ratio, quality of mixing, superficial air velocity, time of inoculum transfer, time to set the fermentation and age and stability of the culture [15]. It is believed however, that this result occurs mainly due to the lack of similar k_La value when conducting the fermentation in bioreactor.

Heat transfer of surface-to-volume ratio usually affected fermenters that is 10 000 L and bigger in which it needed a cooling coils, so this factor was ruled out [15]. Several studies have also reported that poor mixing quality affected the process yield. Mixing problems however, is usually caused by cooling coils and therefore commonly happens only in bigger fermenter [18]. A scale-up culture in large fermenters may causes stress on the strain because of non-homogenous mixing, pH changes and temperature. This condition increases the possibility of unwanted strain mutation that could compromise strain stability [20]. This factor might be one the cause of low growth rate of recombinant *E. coli* BL21 (DE3) in a 2L bioreactor.

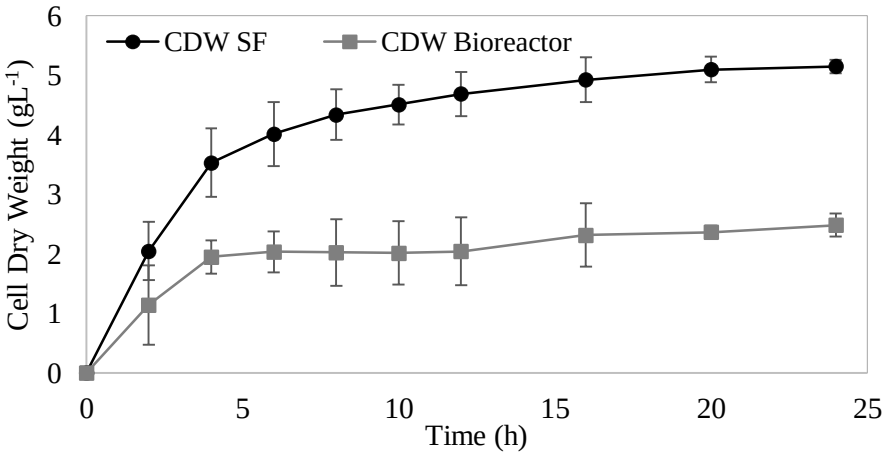

Figure 2. Difference between growth profile of recombinant *E. coli* BL21 (DE3) in shake flask and 2L bioreactor fermentation

Cells that undergoes fermentation or biological batch culture especially in bioreactor takes up oxygen in a non-compound, free forms oxygen called dissolve oxygen (DO). Throughout the

fermentation, DO is supplied (aeration process) and the oxygen dissolves in the cell culture through convection. The oxygen transfers through cells (oxygen transfer rate, OTR) and cells takes up these oxygens (oxygens uptake rate, OUR) with the help of agitation [11]. Oxygen mass transfer coefficient, k_La is the coefficient that correlates the OTR and OUR in cells. The measurement of k_La gives vital information about a bioprocess. OTR, through K_La describes a theoretical maximum cell density that could be achieved in a cell cuture [12]. During lag phase of a culture, OTR or OUR is typically low as the cells focuses more on self-synthesizing and there is a slight gain to cell density. Cells density increases when the cells focuses on growing which is during exponential phase and this is where OUR is continuously increasing until OTR becomes a limiting rate, which is determined by the k_La [13].

Mass transfer rate of oxygen, k_La to the culture media must be equal or exceed the rate of growing cells takes up oxygen in order for the cells to grow effectively [13]. When scaling up fermentation from shake flasks to bioreactor, the k_La value in the shake flasks must be determine and the same value should be provided when carrying out fermentation in bioreactor. A similar aeration condition is provided in both fermentation by matching the k_La value in shake flask and bioreactor [14]. For this study, the agitation rate and stirring speed were both set at 200 rpm and when conducting the fermentation in bioreactor, the k_La value was not taken into consideration. Matching up the k_La value in both fermentations could ensure adequate oxygen supply is available during the fermentation for effective growth of cells. Therefore, it is strongly believed that the lack of similar k_La value is one of the factors affecting the low growth rate of *E. coli* BL21 (DE3) in bioreactor.

3.2. Comparison of D-xylonic acid production in shake flask and bioreactor

D-xylonic acid started to accumulate when the recombinant cells entered exponential phase at 2 hours of fermentation in both shake flask and bioreactor, and it continued to accumulate until 24 hours of fermentation. Concentration of D-xylonic acid in shake flask was higher since recombinant *E. coli* BL21 (DE3) grows better in shake flask fermentation. The maximum concentration of D-xylonic acid in shake flask was 9.06 g/L at 20 hours of fermentation with D-xylonic acid yield ($Y_{p/s}$) of 1.10 g/g xylose. On the other hand, the maximum concentration of D-xylonic acid obtained from bioreactor fermentation was only 6.89 g/L at 24 hours of fermentation with yield of 0.89 g/g xylose. The significant difference of D-xylonic acid obtained from both fermentations is depicted in Figure 3.

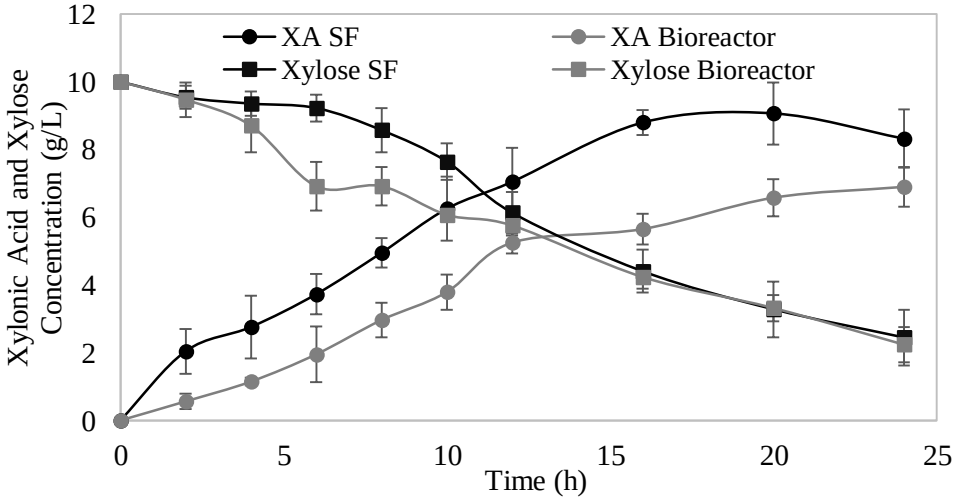

Figure 3. Profile of D-xylonic acid (XA) concentration and xylose consumption in shake flask (SF) and bioreactor

Theoretically, the production of D-xylonic acid in bioreactor fermentation should be higher compared to shake flask fermentation. The result is however vice versa, which is proportional to the growth of recombinant *E. coli* in shake flask and bioreactor. This probably happen due to negligence of k_La value as mentioned previously. In both fermentations, the concentration of D-xylonic acid reached maximum when the carbon source (xylose) is almost exhausted. Xylose is also utilised faster in bioreactor fermentation and this might occur because of the higher oxygen availability in the bioreactor compared to shake flask. After achieving maximum concentration of D-xylonic acid at hour 20 in shake flask fermentation, D-xylonic acid concentration started to drop.

One possible reason for this condition to occur was the assimilation of D-xylonic acid as a result of xylose depletion. D-xylonic acid production notably speeds up during exponential phase, which indicates that D-xylonic acid follows the production pattern of growth-associated product. Considering that this is a batch culture for both fermentations, fermentation in bioreactor was also limit to 24 hours. Extending the fermentation time would cause further xylose exhaustion in the culture media and therefore decreasing D-xylonic acid productivity. The summary of fermentation for both batch processes are tabulated in Table 1.

Table 1. Summary of fermentation for batch processes (shake flask and 2L bioreactor)

Parameter	Shake Flask	Bioreactor
Specific growth rate, μ (h^{-1})	0.273	0.266
Doubling time of recombinant *E. coli* BL21(DE3), τd (h)	2.54	2.60
Maximum D-xylonic acid concentration (gL^{-1})	9.06	6.89
D-xylonic acid yield ($Y_{P/S}$)	1.10	0.89
Maximum D-xylonic acid productivity, P_{max} ($gL^{-1}h^{-1}$)	0.45	0.11
Biomass yield ($Y_{X/S}$)	0.68	0.32
Time to enter stationary growth phase (h)	6	6
Time for maximum D-xylonic acid concentration (h)	20	24

4. Conclusion

The comparison on growth of recombinant *E. coli* BL21 (DE3) and production of D-xylonic acid from the recombinant *E. coli* BL21 (DE3) from fermentation conducted in shake flask and bioreactor was studied in this paper. Specific growth rate, μ of 0.273 h^{-1} and maximum D-xylonic acid concentration of 9.06 gL^{-1} was obtained from fermentation in shake flask. Meanwhile for fermentation in bioreactor, the μ was 0.266 h^{-1} and maximum D-xylonic acid concentration was 6.89 gL^{-1}. It was expected that the growth rate and production of D-xylonic acid would be higher in bioreactor fermentation however the result showed vice versa. It is believed that lack of similar k_La value between the two fermentation plays an important role for the result obtained. Production of D-xylonic acid from recombinant *E. coli* BL21 (DE3) is nonetheless a promising prospect for industrial application provided with a better scale-up strategy.

Acknowledgement

The authors are thankful for the financial support from the Ministry of Education Malaysia for Research Acculturation Grant Scheme (Grant No. RDU150104). Authors are also grateful to Universiti Malaysia Pahang for the Postgraduate Research Scheme (PGRS 180338) and to Faculty of Chemical & Process Engineering Technology, UMP for the research facilities provided.

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