CHAPTER IV

A Simple and Effective Isocratic HPLC Method for Fast Identification and Quantification of Glucose in Complex Fermentation Broth

4.1. Introduction

Bioprocess industry especially related to fermentation activities has identified the advantages of doing specific parameters measurements as close to the fermentation process as possible (Danielsson, 1991). In order to make use of increasing knowledge about the processes control, new analytical methods have to be invented and applied to meet the intend tendency. Fermentation monitoring covers a vast area, encompassing numerous analytical fields (Danielsson, 1991) because of the effective control of fermentation processes demands the measurement of as many significant parameters as possible as frequently as possible (Bradley *et al.,* 1991). Therefore, it is important to have an accurate and consistent set of approaches for measurement for each parameter considered in fermentation process to meet with measurement process. On-line and off-line measurements are frequently used as the main source of information about the condition state of the fermentation process with combination of model-based calculations to have estimations for fermentation condition for monitoring purposes (Dondo, 2001).

Carbohydrates (glucose, fructose, lactose, sucrose, etc) are essential sources for cell growth and product synthesis, it was consumed in biomass growth for biosurfactant production and in biomass maintenance (Casas *et al.,* 1997). Glucose is assimilated during bacterial growth until another essential source depleted (Cooper *et al.,* 1984). This carbon sources and metabolic by-products of fermentation processes can impact the yield and quality of the desired products. Hence, it is desirable to characterize the culture and fermentation broth ingredients to optimize media formulation development, manufacturing process performance with nutrient supplementation, and endpoint definition (Hanko *et al.,* 2000). Therefore, the measurement of glucose

substrate which is often the mainly carbon sources in fermentation works especially for biosurfactant is particular interest (Isa *et al.,* 2008).

The qualitative and quantitative composition of a fermentation broth is very often difficult to determine accurately because the medium and substrate itself contain a complex component of nutrient, cell debris, cells, waste products, and interested products (Hanko *et al.,* 2000). Besides the chemical and physical properties of the broth are continuously changing with time (Buttler *et al.,* 1996). Biosensor commonly used to analyse fermentation broths, however this method cannot simultaneously determine multiple compounds in fermentation broth (Schugerl *et al.,* 1993). Hence, for any method that need to be develop for detection of glucose must satisfies these condition; (a) allow measurement over the range of interest, (b) respond rapidly to changes in analyte concentration; and (c) their signals must be easily interpretable and either continuously or frequently emitted (Bradley *et al.,* 1991).

One of the techniques used for glucose detection is HPLC equipped with various detector such as refractive index (RI) (Rodrigues *et al.,* 2006) and light scattering detector (LSD) (Garcı´a-Ochoa *et al.,* 1999). HPLC are commonly used technique in off-line analysis for determination of changes in glucose concentration in fermentation process especially for biosurfactant works (Rodrigues *et al.,* 2006; Garcı´a-Ochoa *et al.,* 1999; de Oliveira *et al.,* 2013). This HPLC protocol bringing them closer to understanding the fermentation process and fermentation kinetic itself. The obvious advantage of HPLC is its capability for multicomponent analysis in complex media. However, all the previous method that used HPLC procedures was reported used RI (Rodrigues *et al.,* 2006; de Oliveira *et al.,* 2013) and light-scattering detector as detector (LSD) (Garcı´a-Ochoa and Casas, 1999). HPLC is always couple with RI (de Oliveira *et al.,* 2013) as detector for detection of sugar in biosurfactant works, but is limited by poor sensitivity and specificity (Buttler *et al.,* 1993). Besides, the RI detection only reliably be used when the concentration of the analytes is high and the influence of co-eluting compounds is small (Buttler *et al.,* 1996) which is not suit with the samples used in this study.

A reliable technique for glucose analysis would undoubtedly improve greatly the efficiency of fermentation monitoring and allow the information for development of new feedback control strategies. This work was used variable wavelength detector (VWD) as the detector without any changes in current HPLC system. This works describes the determination of glucose concentration in *B. subtilis* ATCC 21332 cell culture and their final fermentation broths. This culture medium was complex and contains mineral salts thus provide a great challenge for separation and detection techniques. In the methods outlined in this paper, the columns Chromolith® NH_2 (Merck, Germany) was used for the determination of glucose concentration in fermentation broths using high performance liquid chromatography (HPLC) equipped with variable wavelength detector (VWD). Detection limits, linearity, and precision are reported for the proposed column suggested. The method was used to determine the changes concentrations of glucose during culture incubation period and took less than 5 minutes per samples analyses.

4.2. Materials and Methods

4.2.1. Apparatus

An Agilent 1200 series HPLC system consisting of a vacuum degasser, quaternary pump, autosampler, thermostatted column compartment, and variable wavelength detector was used. Data acquisition was performed using Chemstation software (Agilent Corporation).

4.3.2. Glucose Standard and Chemicals

All chemical used were analytical grade unless stated. D-(+)-Glucose Anhydrous ()J. Kollins) and Trifluroacetic acid (TFA) solution of HPLC grade (Merck) was used. Deionised water was obtained from the Microbiology Laboratory of Universiti Sains Islam Malaysia (USIM). Deionised water was prepared using a system equipped with 0.2µm filter (Elga).

4.2.3. Chromatographic Conditions

HPLC system equipped with a Chromolith® high performance NH_2 (100 mm x 4.6 mm, 5 µm) column was operated and maintained at 30° C. A mobile phase mixture consisting of 3.8mM TFA solution (100%) were pumped in an isocratic mode with a flow rate of 0.5 mL/min. The injection volume was set at 10µL and was detected through a VWD detector at 195 nm. Each analysis was completed within 5 min.

4.2.4. Preparation of Glucose Stock and Standard Solutions

Glucose stock solution was prepared at 80 g/l. Later, a series of glucose solution range of 0.1 g/L to 80 g/L were prepared by dilution of the stock solution with deionized water and were then stored at 4 °C prior to use.

4.2.5. Preparation of The Mobile Phase Solution

A 3.8 mM TFA solution was prepared in a 1 L volumetric flask filled with deionised water and stirred until complete dissolution. Later, the TFA solution was filtered through 0.45 µm nylon filters and degassed prior to use.

4.2.6. Method Validation

The HPLC assay of the glucose was validated in terms of linearity, repeatability, intermediate precision, sensitivity and recovery with slight modification and approach based on methodology proposed by previous research (Snydeir *et al.,* 1994; Li *et al.,* 2012).

4.2.6.1. Linearity

Ten standard glucose solutions were prepared by dilution of the stock solution with the distilled water as solvent. The calibration curve were constructed by using the method proposed in section 3.2.6.1. The obtained chromatograms were analysed using Chemstation software.

4.2.6.2. Repeatability

Instrumental precision (system precision) and intra-assay precision was determined by using the method proposed in section 3.2.6.2.

4.2.6.3. Intermediate Precision

Intra-day repeatability was obtained by using the method proposed in section 3.2.6.3.

4.2.6.4. Sensitivity

Sensitivity of the HPLC method was determined by the estimation of limit of detection (LOD) and limit of quantification (LOQ) based on the method proposed in section 3.2.6.4.

4.2.7. Sample Preparation

The strain used in the fermentation was *B. subtilis* ATCC 21332 provided by Microbiology Laboratory, Faculty of Science and Technology, Universiti Sains Islam Malaysia(USIM). Inocula prepare in two stages. Firstly the microorganism recover from the strain bank and grown in the nutrient agar. Then a loopful of cells from this culture add to 25ml of nutrient broth containing 40g/L of glucose, which will incubate for 24 h on an orbital shaker for 200rpm at 30 °C. The culture broth will be used to inoculate five conical flasks, each one containing 45 ml of Cooper's medium (Cooper *et al.,* 1981) by using 5mL of inoculum per flask (10%). Later, these flasks incubate under the same conditions as the previous culture. The five conical flasks will be used to inoculate a 5 L containing Cooper's medium consist 5% inoculum (Isa *et al.,* 2007)

The agitation system of the 5L bioreactor (Sartorious stedim) contain two impellers on a single drive shaft connect to a motor which speed control by a fermentation control unit. Dissolved oxygen and pH probes connect to a fermentation control unit and pH maintain constantly by the automatic addition of either 1 M NaOH or 1 M HCl, according to the signal received from the pH electrode. The fermentation conduct at 30 °C, pH 7, for 55 h, under depleted oxygen conditions accomplish by using a low air

flow rate (1 vvm^{-1}) and low stirrer speed (100 rpm). Culture broth samples of approximately 40 mL were taken during the course of the fermentation at regular intervals for determination of glucose concentration. The final fermentation broths obtain by harvesting, clarifying by centrifugation for 10 min at 8000 rpm at room temperature and finally frozen for further studies (Isa *et al.*, 2007).

4.3 Results and Discussions

4.3.1 Determination of Suitable Glucose Separation Condition

Figure 6: HPLC Chromatogram for glucose concentration at 80 g/l.

The total elution time and separation of glucose peaks was highly dependent upon the percentage of TFA presence in deionized water. The optimum separation of glucose peak was achieved with a mobile phase consist of 3.8 mM TFA as shown in the chromatogram in Figure 6. The presence of TFA in water as mobile phase could suppress the dissociation of glucose in fermentation broth. The retention times for glucose were recorded at 3.60 minutes, as shown in Figure 6. The HPLC was run for 5 minutes, which resulted in both satisfactory resolutions of glucose peaks.

4.3.2 Precision

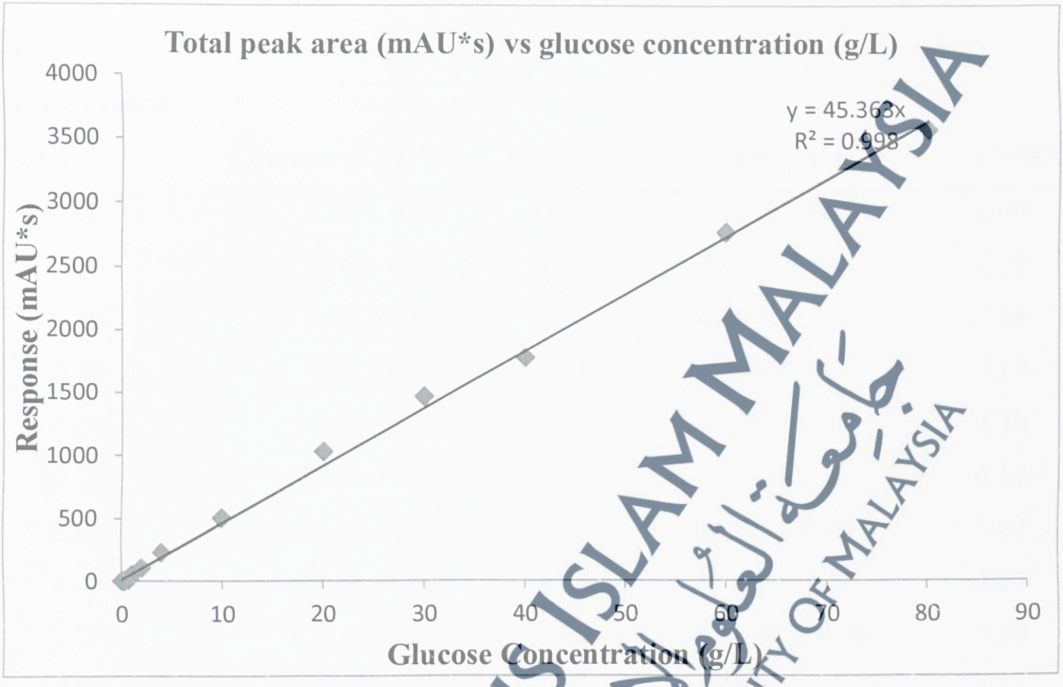
Table 6: Instrumental precision and intra-assay precision (means ± SD)

Glucose Concentration (g/l)	Instrumental Precision[a]		Intra-assay Precision[b]	
	Peak area (mAU*s)	RSD (%)	Peak area (mAU*s)	RSD (%)
0.0	0.00 ± 0.00	0.00	0.00 ±0.00	0.00
0.2	3.74 ±0.07	1.85	3.51 ± 0.14	3.89
0.3	3.48 ± 0.09	2.49	3.85 ± 0.10	2.63
0.4	5.83 ± 0.13	2.18	6.57 ± 0.24	3.72
0.6	11.07 ± 0.38	3.42	11.07 ±0.10	0.86
0.7	15.96 ± 0.97	3.09	15.65 ± 0.44	2.84
0.8	19.60 ± 0.68	3.44	17.77 ± 0.44	2.23
1.0	52.22 ± 0.97	1.86	54.14 ± 0.81	1.49
2.0	109.88 ± 2.69	2.45	101.98 ± 1.32	1.29
4.0	230.47 ± 3.87	1.68	230.34 ±6.46	2.81
10.0	503.53 ± 4.53	0.90	480.39 ± 4.17	0.87
20.0	1024.34 ± 7.25	0.71	948.76 ± 11.26	1.19
30.0	1455.57± 17.32	1.19	1346.43 ± 26.05	1.93
40.0	1766.01 ± 11.94	0.68	1744.18 ± 49.75	2.85
60.0	2755.14 ± 18.39	0.67	2558.25 ± 21.24	0.83
80.0	3554.85 ± 12.42	0.35	3689.13 ± 78.98	2.14

[a] Five injection of each sample (n=5)

[b] Five injection of each sample (n=5)

Figure 7: Plot of TPA versus various concentrationof glucose.



Precision of the proposed method was assessed by repeated injection of a glucose standard solution of various concentrations. The relative standard deviation (RSD) used to assess instrumental precision and intra-assay precision shown in Table 6. The instrumental precision was measured by repeated injection of the same homogeneous sample while Intra-assay precision was determined by measuring independent glucose solutions of varying concentration (Pursell *et al.,* 2004). This was repeated for all of the glucose solutions. A correlation between the analyte peak area and glucose at various concentrations was observed with $r^2 = 0.9980$ and $r^2 = 0.9979$ for standard curves for instrumental precision and intra-assay precision data as shown in Table 6 respectively, with $r^2 \approx 1$ for both measurement. Meanwhile, the RSD value of both instrumental and intra-assay precision of the glucose solution were below 4.0%, indicating both HPLC and the proposed method were highly precise, accurate, and reproducible for quantitave analysis of glucose (Li *et al.,* 2012).

4.3.3. Intermediate Precision

Table 7: Intra-day and inter-day repeatability of the assay (means ± SD, n =5)

Glucose concentration (g/L)	Intra-day repeatability		Inter-day repeatability	
	Average(mAU*s)	RSD(%)	Average(mAU*s)	RSD(%)
0.00	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00
0.20	3.76 ± 0.07	1.85	3.94 ± 0.09	2.27
0.30	3.48 ± 0.09	2.49	4.34 ± 0.12	2.84
0.40	7.03 ±0.13	1.81	7.29 ± 0.23	3.18
0.60	13.47 ± 0.38	2.81	14.65 ± 0.63	4.30
0.70	17.94 ± 0.97	5.42	21.14 ± 0.87	4.12
0.80	23.40 ± 0.68	2.89	26.60 ± 0.98	3.67
1.00	62.02 ± 0.97	1.56	71.42 ± 2.31	3.23
2.00	123.88 ± 2.69	2.17	125.08 ± 4.38	3.50
4.00	251.87 ± 3.87	1.53	275.81 ± 11.33	4.11
10.00	525.73 ± 4.53	0.86	545.39 ± 3.78	0.69
20.00	1097.54 ± 7.25	0.66	1139.44 ± 45.12	3.96
30.00	1515.57 ± 17.32	1.14	1502.67 ± 9.62	0.64
40.00	1850.01 ± 11.94	0.65	1886.69 ± 11.06	0.59
60.00	2835.14 ± 18.39	0.65	2952.49 ± 35.70	1.21
80.00	3758.85 ± 12.42	0.33	3918.70 ± 62.80	1.60

Intermediate precision of the proposed method was determined by assessing intra-day and inter-day reproducibility. The results of intra- and inter-day reproducibility are listed in Table 7. The RSD values for all tested groups were approximately or less than 5.5%, which can be considered an acceptable value for quantitative analysis of glucose.

4.3.4. Sensitivity

The sensitivity of this HPLC method was determined by LOD and the limit of quantification (LOQ). LOD is the lowest analyte concentration detectable by HPLC

4.3.3. Intermediate Precision

Table 7: Intra-day and inter-day repeatability of the assay (means ± SD, n =5)

Glucose concentration (g/L)	Intra-day repeatability		Inter-day repeatability	
	Average(mAU*s)	RSD(%)	Average(mAU*s)	RSD(%)
0.00	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00
0.20	3.76 ± 0.07	1.85	3.94 ± 0.09	2.27
0.30	3.48 ± 0.09	2.49	4.34 ± 0.12	2.84
0.40	7.03 ±0.13	1.81	7.29 ± 0.23	3.18
0.60	13.47 ± 0.38	2.81	14.65 ± 0.63	4.30
0.70	17.94 ± 0.97	5.42	21.14 ± 0.87	4.12
0.80	23.40 ± 0.68	2.89	26.60 ± 0.98	3.67
1.00	62.02 ± 0.97	1.56	71.42 ± 2.31	3.23
2.00	123.88 ± 2.69	2.17	125.08 ± 4.38	3.50
4.00	251.87 ± 3.87	1.53	275.81 ± 11.33	4.11
10.00	525.73 ± 4.53	0.86	545.39 ± 3.78	0.69
20.00	1097.54 ± 7.25	0.66	1139.44 ± 45.12	3.96
30.00	1515.57 ± 17.32	1.14	1502.67 ± 9.62	0.64
40.00	1850.01 ± 11.94	0.65	1886.69 ± 11.06	0.59
60.00	2835.14 ± 18.39	0.65	2952.49 ± 35.70	1.21
80.00	3758.85 ± 1242	0.33	3918.70 ± 62.80	1.60

Intermediate precision of the proposed method was determined by assessing intra-day and inter-day reproducibility. The results of intra- and inter-day reproducibility are listed in Table 7. The RSD values for all tested groups were approximately or less than 5.5%, which can be considered an acceptable value for quantitative analysis of glucose.

4.3.4. Sensitivity

The sensitivity of this HPLC method was determined by LOD and the limit of quantification (LOQ). LOD is the lowest analyte concentration detectable by HPLC

4.3.3. Intermediate Precision

Table 7: Intra-day and inter-day repeatability of the assay (means ± SD, n =5)

Glucose concentration (g/L)	Intra-day repeatability		Inter-day repeatability	
	Average(mAU*s)	RSD(%)	Average(mAU*s)	RSD(%)
0.00	0.00 ± 0.00	0.00	0.00 ± 0.00	0.00
0.20	3.76 ± 0.07	1.85	3.94 ± 0.09	2.27
0.30	3.48 ± 0.09	2.49	4.34 ± 0.12	2.84
0.40	7.03 ±0.13	1.81	7.29 ± 0.23	3.18
0.60	13.47 ± 0.38	2.81	14.65 ± 0.63	4.30
0.70	17.94 ± 0.97	5.42	21.14 ± 0.87	4.12
0.80	23.40 ± 0.68	2.89	26.60 ± 0.98	3.67
1.00	62.02 ± 0.97	1.56	71.42 ± 2.31	3.23
2.00	123.88 ± 2.69	2.17	125.08 ± 4.38	3.50
4.00	251.87 ± 3.87	1.53	275.81 ± 11.33	4.11
10.00	525.73 ± 4.53	0.86	545.39 ± 3.78	0.69
20.00	1097.54 ± 7.25	0.66	1139.44 ± 45.12	3.96
30.00	1515.57 ± 17.32	1.14	1502.67 ± 9.62	0.64
40.00	1850.01 ± 11.94	0.65	1886.69 ± 11.06	0.59
60.00	2835.14 ± 18.39	0.65	2952.49 ± 35.70	1.21
80.00	3758.85 ± 1242	0.33	3918.70 ± 62.80	1.60

Intermediate precision of the proposed method was determined by assessing intra-day and inter-day reproducibility. The results of intra- and inter-day reproducibility are listed in Table 7. The RSD values for all tested groups were approximately or less than 5.5%, which can be considered an acceptable value for quantitative analysis of glucose.

4.3.4. Sensitivity

The sensitivity of this HPLC method was determined by LOD and the limit of quantification (LOQ). LOD is the lowest analyte concentration detectable by HPLC

for the proposed method. On the other hand, the LOQ is the lowest concentration that can be quantified accurately by the proposed method. The LOD and LOQ were 1 g/l and 2 g/l, respectively. Compared to other reported HPLC methods, the sensitivity of the described method was considerably improved.

4.3.5. Recovery

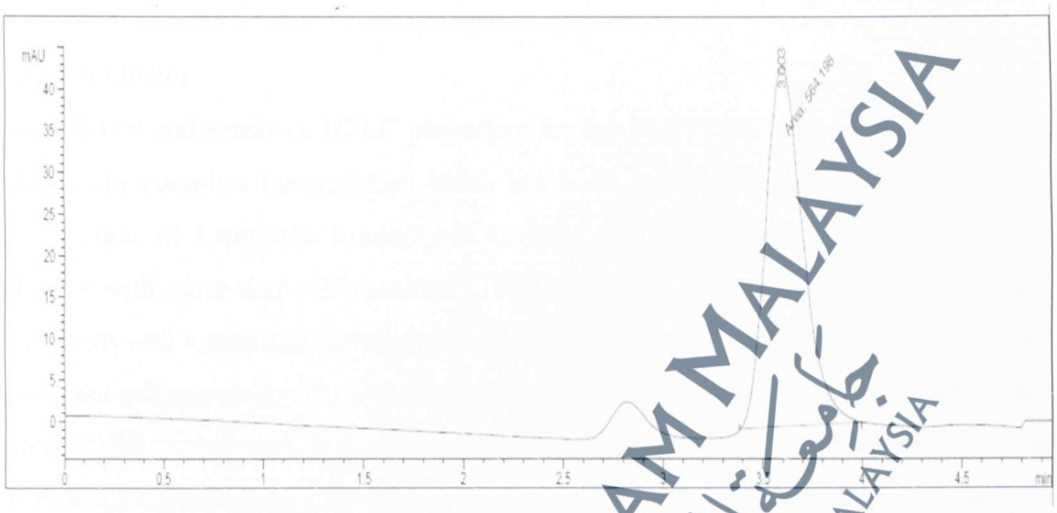
Table 8: Recoveries of glucose (means ± SD, n=5).

Glucose concentration (g/L)	Average	Recovery (%)	RSD (%)
0.4	0.38 ± 0.05	94.31	12.20
5.0	4.80 ± 0.04	96.02	0.91
8.0	7.82 ± 0.14	97.78	1.81
10.0	10.08 ± 0.27	100.79	2.72
20.0	20.17 ± 0.63	100.87	3.10
30.0	29.51± 0.57	98.36	1.94
40.0	39.08 ± 0.79	97.69	2.02
60.0	57.55 ± 4.53	95.92	7.87
80.0	78.22 ± 1.81	97.77	2.32

The recovery measures the closeness between the theoretically added amount and the experimental value. It was performed by spiking the glucose solution with a known concentration. The recovery of the sample at different concentrations was above 94% with RSD values below 8% except for the glucose concentration at 0.4 g/L and 60 g/L respectively, as shown in Table 8. The results indicated that glucose can be fully recovered using the column proposed in this study.

4.3.6. Chromatography of Glucose in Culture Broth of *B. subtilis* ATCC 21332

Figure 8: HPLC Chromatogram for glucose in complex fermentation broth at 32 h



The proposed method was also tested with glucose used for fermentation process from culture broth contain of Cooper's media which glucose as the main carbon source. The media proposed supplies the microorganisms with all the nutrients the microorganisms need to grow and produce the various fermentation products (Cooper *et al.,* 1981). From numerous information scripture in literature, microorganism may produce range of compounds such as raw substrates (Chen *et al.,* 2008b), fermentation products (Isa *et al.,.,* 2007), microorganisms and its derivative components (Pursell *et al.,* 2004), chemical additives added to the fermentor (Cooper *et al.,* 1981; Isa *et al.,* 2007) and gases such as oxygen and other metabolic gases. The complex nature of culture broth (Oka *et al.,* 1993) makes identification and quantification of glucose difficult to achieve. However by using this method, it shows good resolution for the chromatogram obtain as shown in Figure 8. It has overall similarity to the glucose chromatogram shown in Figure 6 in terms of the elution time and separation of glucose peak respectively, with a total elution time of approximately five minutes. Determination of the glucose concentration in the culture broth of *B. subtilis* ATCC 21332 was conducted by summing the area under glucose peaks in the chromatrogram (Figure 6) and then calculated using the equation of $y = 45.363x$, obtained from

Figure 7. The glucose concentration in the culture broth of *B. subtilis* ATCC 21332 at 32 h as shown in Figure 8 was 14.94 ± 0.58 g/l, as determined by performing the injection in triplicate.

4.4. Conclusion

An efficient and sensitive HPLC procedure for qualitative and quantitative analysis of glucose in complex fermentation broth has been successfully developed with total elution time of 5 minutes. Linearity of analysis was achieved up to at least 80 g/L of glucose with more than 95% recovery. The capability of detecting glucose with high sensitivity and without prederivatization is a major advantage of this method proposed using the column over other detection methods besides no modification for HPLC that using VWD as detector. Broth and culture solutions are simply centrifuged or filtered to remove particulates, diluted, and then injected to the system. The rapid analysis where take 5 minutes for each analysis renders this technique ideal for fermentation broth optimization during process development and assessment of the fermentation condition for fermentation process. The high sensitivity of this method permits lower detection limits and less sample waste. Therefore, this method also exhibited the potential to monitor metabolically related substrates and products in cell cultures, which could be useful for optimizing product yields and kinetic study of fermentation process.