Talanta 121 (2014) 210–214

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Rapid monitoring of glycerol in fermentation growth media: Facilitating crude glycerol bioprocess development

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ARTICLE INFO

Article history:
Received 16 July 2013
Received in revised form
5 December 2013
Accepted 11 December 2013
Available online 9 January 2014

Keywords:
Monitoring
Fermentation
Glycerol
HPLC
TLC
Bioprocess

ABSTRACT

Recently, the need for crude glycerol valorisation from the biodiesel industry has generated many studies for practical and economic applications. Amongst them, fermentations based on glycerol media for the production of high value metabolites are prominent applications. This has generated a need to develop analytical techniques which allow fast and simple glycerol monitoring during fermentation. The methodology should be fast and inexpensive to be adopted in research, as well as in industrial applications. In this study three different methods were analysed and compared: two common methodologies based on liquid chromatography and enzymatic kits, and the new method based on a DotBlot assay coupled with image analysis. The new methodology is faster and cheaper than the other conventional methods, with comparable performance. Good linearity, precision and accuracy were achieved in the lower range (10 or 15 g/L to depletion), the most common range of glycerol concentrations to monitor fermentations in terms of growth kinetics.

1. Introduction

During the last decade the biodiesel industry had an exponential growth in terms of production. As a result, large amounts of the main byproduct, crude glycerol, were generated. Crude glycerol has caught the attention of several researchers as a potential feedstock to generate value-added products. Obviously, there is a clear need for valorisation from environmental and economic perspectives. Applications include catalytic conversions to obtain monomers and polymer precursors [1–3], glycerol carbonate [4,5], energy [6–8], methane production [9], animal feed [10–12], and other miscellaneous applications. Additionally, the potential usage of crude glycerol as carbon source in fermentation processes has gained notorious attention. A large number of different bioprocesses are being tested and developed in order to produce value-added products [3,13–15]. Bioproducts include again monomers and polymer precursors [16–20], polyhydroxyalkanoates [21–23], pigments [24,25], organic acids [26–28]; and fatty acids as AA, EPA and DHA [29,30].

All the efforts in bioprocess development based on crude glycerol require analytical methods to quantify glycerol. Cell growth kinetics are substrate-dependent, so the calculation of growth related parameters essential to the fermentation design require carbon source quantification. Nowadays, many labs and companies determine glycerol concentrations using techniques based on separation (liquid chromatography), enzymatic techniques and potentiometric methods. When developing fermentations, the carbon source monitoring requires reliable, inexpensive and particularly fast methodologies to quantify the residual glycerol. A large number of samples is typically generated, and rapid monitoring is crucial to the decision-making process during batch, fed-batch or continuous fermentation.

High performance liquid chromatography (HPLC) is widely used to analyse glycerol content in fermentation media. HPLC methods offer high accuracy. Enzymatic based kits are also popular in glycerol based fermentations [29–32]. Enzymatic kits might offer less accuracy compared to HPLC, and post a direct cost per sample analysed. Both methods can reach high accuracy but they are time consuming with a response delay often critical for fermentation monitoring close to real time. Current HPLC methodologies spent around 30 min per sample considering sample preparation injection and peak analysis. Depending on the column, the time can be reduced from 30 down to 15 min per sample. Enzymatic kits are faster than HPLC methods, but the cost per each assay is relatively high. Therefore it discourages exhaustive monitoring of the fermentation, due to the amount of samples. Potentiometric methodologies are simple and cheaper than HPLC and enzymatic kits. However, this methodology needs larger volumes of samples and could not be suitable for monitoring different fermentations the at same time. The nature of the samples to analyse can be crucial in the methodology selection. Medium composition (e.g. high salinity and organic compounds

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0039-9140/$ - see front matter © 2014 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.12.022

interfering with the methods) or metabolites generated may introduce errors or invalidate the methodology. For the specific case of enzymatic kits, the interference of the metals present in the medium in some of the enzymatic reactions could also mislead the determination. Potentiometric methods could not be applicable in presence of organic compounds (e.g. coming from Yeast extract or metabolites produced) containing more than two hydroxyl groups on adjacent carbon atoms [33].

The current work presents a method developed to monitor either pure or crude glycerol residual concentration from fermentation samples. The method relies on a DotBlot assay (DB) coupled with image processing algorithms. A method presenting several analogies but using Thin Layer Chromatography (TLC) was reported to quantify residual glycerol in biodiesel samples [34]. Here, the simple DB developed assay has specific staining adapted and validated for bioprocess development in aqueous medium with high salinity. Subsequent image processing and analysis has the advantage of rapid output with enough accuracy for bioprocess monitoring with a short response time.

The new method was proofed during crude glycerol fermentations, comparing the three methods (HPLC, enzymatic kit and DB with the image processing method). Such benchmarking test was established in the hardest conditions (i.e. marine microorganism fermentation) since salinity of the medium may interfere in the quantification.

2. Materials and methods

2.1. Analytical techniques

2.1.1. HPLC analysis

For crude glycerol determination using HPLC, a calibration curve was prepared by a series of dilutions in the range of 0–10 g/L. Dilutions were prepared with artificial seawater media instead of water to reproduce the conditions of fermentation, mimicking salinities found in the ocean where the microorganism naturally grows. In this case, 18 g/l of sodium chloride and other salts in minor amounts were used. Citric acid (Sigma-Aldrich, Spain) was added as internal standard.

An HPLC 1100 series from Agilent Technologies® equipped with a Transgenomic™ ICSep COREGEL 87H3 column and a refraction index detector (RID), was used for glycerol analysis. A column temperature of 80 °C was used. The mobile phase was 0.04 N sulphuric acid in MilliQ water at 0.4 ml/min. The concentration of sulphuric acid was higher than the usual conditions to avoid column damage from the cationic load of seawater. The injection volume was 10 μl of sample. Chromatograms were analysed with ChemStation Software from Agilent Technologies®.

2.1.2. Analysis by enzymatic kit

An enzymatic glycerol determination kit was used, K-GCROL kit (Megazyme International). The kit uses tablets containing nicotinamide–adenine dinucleotide (NADH), adenosine-5′-triphosphate (ATP) and phosphoenolpyruvate. Glycerol is phosphorylated by ATP to L-glycerol-3-phosphate in the reaction catalysed by glycerokinase. The adenosine-5′-diphosphate (ADP) formed in the reaction is reconverted by phosphoenolpyruvate (PEP) with the aid of pyruvate kinase into ATP with the formation of pyruvate. In the presence of L-lactate dehydrogenase, pyruvate is reduced to L-lactate by oxidising NADH which is stoichiometric with the content of glycerol and can be measured by the decrease in absorbance at 340 nm.

2.1.3. Analysis by DotBlot assay

Plates of silica gel 60 F_{254} (Merck, Germany) with 10 cm of height and variable length were used. Two microliters of supernatant samples were spotted. Spots must be separated by at least 7 mm, since concentrated samples suffer a considerable diffusion on DB. After spotting, the samples must be immediately dried by heated air. To visualise the spots, a solution of potassium permanganate (Sigma Aldrich, Spain) 37 mM, potassium carbonate 0.29 M (Panreac, Spain) and 0.05% NaOH (Panreac, Spain) in MilliQ water was added. Then, spots were scanned with HP ScanerJet 5100 (resolution of 1200 ppi). The scanned DotBlot layer was processed and the resulting image was analysed using Matlab® (Mathworks Inc., MA).

2.1.4. Growth media

Glycerol based growth medium used marine artificial water with yeast extract 0.1%, tryptone 0.1% and streptomycin sulphate 25 μg/mL [35]. Pharma-grade glycerol, also referred as pure glycerol for molecular biology was purchased from Fisher Scientific Spain. In crude glycerol samples, raw glycerol used was gently provided by Transportes Ceferino Martínez S.A. It is obtained as the residual fraction from transesterification of used cooking oil (UCO) to biodiesel through an alkali catalysed process, with a capacity around 5000 ton/year. The crude glycerol generated has a dark brown colour, having a glycerol content of ca. 80%.

2.1.5. Microorganism and fermentation

Aurantiochytrium limacinum (ATCC MYA-1381) culture was used in this study. *A. limacinum* cultures were grown in a bench scale batch 2.5 L bioreactor, Minifors, Infors (Switzerland), using 1.5 L of artificial seawater. Crude glycerol was added as carbon source at a concentration of 10 g/L. Spent fermentation media samples of 10 mL were taken, starting 16 h after inoculation.

2.1.6. Sample preparation

For residual glycerol determination, fermentation samples were centrifuged at 4000 rpm (Sigma 3-16K, Germany), during 5 min at 5 °C. The supernatant was collected for direct analysis or stored at −80ºC to avoid possible zoospore proliferation, until the sample was analysed.

2.1.7. Comparison of methods during the fermentation

A fermentation of *A. limacinum* was monitored in order to evaluate DotBlot capabilities to measure crude glycerol compared to the other methods. Immediately after pumping the samples from the reactor, they were kept in a cold room (ca. 5 °C). All the samples were analysed at the same time. Three replicas were analysed for each method.

3. Results and discussion

To our best knowledge, a general method to analyse residual free glycerol in harsh conditions (artificial seawater and usage of byproducts) has not been reported. In the present work a DotBlot methodology that enables glycerol detection and quantification is described and compared to conventional HPLC and enzymatic methods. Results from sample and reagents preparation, as well as sample processing were evaluated in terms of linearity, precision and accuracy. Initially, artificial marine medium (AMM) with known crude glycerol concentrations was tested to validate the methods. Then, the three methods were simultaneously used to monitor residual crude glycerol concentration from a culture using AMM in a labscale bioreactor.

3.1. Glycerol determination by HPLC

A typical chromatogram analysing a standard of known concentration of crude glycerol in AMM media (without cells), has a peak at a retention time of 11.2 min attributed to salts and other

compounds of the artificial seawater medium. Then, a peak appearing at 20.8 min retention time corresponds to glycerol (see Supplementary material S1). The glycerol peak is separated with near-baseline resolution, without any interference from other components of the medium. Therefore, this is a specific and selective method for crude glycerol. Then, in order to evaluate specificity and selectivity samples were compared in terms of the recovery % (Table 1). No significant interference was detected. Thus, the method could be considered as specific and selective for glycerol regardless the source and medium composition.

To evaluate the linearity, accuracy and repeatability of the entire sample processing and HPLC-RID analysis, a calibration curve of eight different glycerol concentrations (triplicates) was performed. AMM with crude glycerol samples were quantified and found linear ($R^2 > 0.999$). Table 1 summarizes the results of plot Response units (RIU) vs. glycerol concentration (g/L). The slope ± standard error was 171,259 ± 2150 and intercept ± standard error was 54,940 ± 12,059. The method is linear in the range of 0.68–12.5 g/L (plot included in Supplementary materials S2). In order to evaluate accuracy, the % recovery was calculated. Such % recovery corresponds to the % difference between the value the method determines and the known concentration (weighted) of the standard used. % Recovery was between 1.2% and 11.6%. Repeatability was examined by measuring the variation between 6 replicas at each concentration (Table 2). Repeatability ranged between 0.4% and 2.3% (CV) at low and high glycerol concentrations respectively. The detection limit was found to be 0.2 g/L and the quantification limit was 0.68 g/L of crude glycerol. Consequently, this method can detect and quantify minimal glycerol concentrations from a culture and detect the complete depletion of the carbon source.

HPLC is an excellent technique in terms of precision and accuracy. However, it requires a considerable time to analyse each sample. The HPLC-RID methodology described in the present work, typically takes 30 min per sample. When monitoring cultures that might last several days, many samples should be collected and analysed, delaying the outcome when fast decision-making is required. The chromatographic time can be reduced down to 15 min using a different column. The HPLC-RID method had a suitable performance when analysing high salinity samples. Additionally, crude glycerol contaminants did not interfere with the analysis.

3.2. Glycerol determination by enzymatic kit

Many labs nowadays prefer the choice of a glycerol detection kit based on enzymatic reactions. These kits offer faster quantifications than those based on the HPLC methods. In this work, K-GCROL (Megazyme International) kit was used. In about 20 min the residual glycerol concentration of a fermentation sample could be determined. In order to compare the results obtained with HPLC determinations, the enzymatic method was validated in the same manner. Results from sample and reagents preparation, as well as sample processing were evaluated in terms of linearity, precision and accuracy. Analogous samples compared to the HPLC method were analysed and the results obtained are summarised in the Table 3.

Specificity and selectivity were evaluated by comparing the results of the same concentration of crude and pure glycerol diluted (Dilution factor 1:100) in Milli-Q water. A good agreement was observed amongst the results (Table 1), which showed good recovery values. The method was found to be linear in the range of 0.01–0.1 g/L of crude glycerol ($R^2 > 0.998$). Therefore fermentation samples have to be conveniently diluted, to fall into this range. The slope ± standard error was 0.0101 ± 0.0003 and intercept ± standard error was −0.0003 ± 0.0018 Absorbance units (AU) vs. glycerol concentration (g/L) [plot included in Supplementary materials S2]. The enzymatic kit is specific for glycerol, but it was not conceived to test high salinity media samples. However, the dilution required to fall in the analysis range allows acceptable conditions to host the enzymatic process and did not interfere with the enzymatic reactions. Accuracy and repeatability were also evaluated. % Recovery was always ≤4.58. The maximum variability was found to be 0.71%. This corresponds to a variation of 0.008 g/L for the most diluted samples. The detection limit was 0.008 g/L, and the limit of quantification was set to 0.1 g/L. Enzymatic kit could be considered an accurate and precise method under the conditions assayed.

While offering similar performance, enzymatic kits are faster than HPLC methodologies. Nevertheless, considerable manual sample processing is required dissuading the usage of kits when large number of samples have to be analysed. Kits are a good option for punctual glycerol determination, but offer drawbacks for bioprocess development and fermentation monitoring. The cost of enzymatic kits for glycerol determination might represent

Table 1
Specificity and selectivity of three methods (HPLC-RID, enzymatic kit, DotBlot assay) for glycerol determination, comparing recovery values (%) with different conditions.

	Recovery (%)	SD	CV (%)
HPLC-RID			
Commercial glycerol + H_2O	99.8	0.6	0.6
Crude glycerol + H_2O	97.6	1.1	1.1
Commercial glycerol + Medium	98.3	0.9	0.9
Crude glycerol + Medium	96.9	1.5	1.5
Enzymatic kit			
Commercial glycerol + H_2O	100.1	0.4	0.4
Crude glycerol + H_2O	99.8	0.5	0.5
Commercial glycerol + Medium	99.2	0.4	0.4
Crude glycerol + Medium	99.7	0.6	0.6
DotBlot assay			
Commercial glycerol + H_2O	98.7	0.9	0.9
Crude glycerol + H_2O	97.8	1.2	1.2
Commercial glycerol + Medium	97.5	1.4	1.4
Crude glycerol + Medium	97.1	1.1	1.1

Table 2
Precision and accuracy for glycerol quantification by HPLC-RID.

Glycerol (g/L)	Mean response	SD	CV (%)	Concentration found (g/L)	Recovery (%)
0.5	1.57E+05	2.15E+03	1.4	0.56	88.40
1.0	2.30E+05	5.32E+03	2.3	0.99	98.73
2.5	4.76E+05	2.12E+03	0.4	2.43	97.17
5	8.93E+05	6.36E+03	0.7	4.88	97.64
7.5	1.33E+06	7.07E+03	0.5	7.42	98.88
10	1.79E+06	7.07E+03	0.4	10.14	98.64
		Mean (%)	0.9	**Mean (%)**	96.57

Table 3
Precision and accuracy for glycerol quantification using a commercial Kit.

Crude glycerol (g/L)	Mean Response	SD	CV (%)	Concentration found g/L	Recovery (%)
1	0.01	1.41E−03	0.71	0.98	98.46
2.5	0.03	7.07E−04	0.37	2.53	98.89
5	0.05	1.77E−03	0.41	5.10	98.01
7.5	0.07	7.07E−04	0.45	7.16	94.42
10	0.10	7.07E−04	0.51	10.13	98.73
		Mean (%)	0.5	**Mean (%)**	97.90

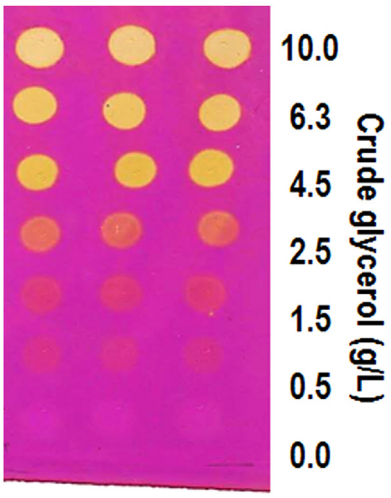

Fig. 1. DotBlot assay with three replicas of glycerol samples in culture medium (in rows).

an extra drawback. The cost could be considerable, especially for those studies focused on media, strains screening, or bioprocess development, where extensive sampling and analysis.

3.3. DotBlot assay validation

In the present work, a silica gel thin layer was used as a support to perform a DotBlot assay. DotBlot was the fundamental technique used to monitor glycerol consumption. In a DotBlot, the elution was eliminated due to the absence of unsaturated compounds or alcohols secreted by the microorganisms used. To visualise the spots a permanganate solution was chosen, oxidising glycerol and forming yellow spots (Fig. 1). The intensity and size of the spot was found to be proportional to glycerol concentration. In order to accelerate the reaction the spots were heated to 100 °C.

Once the DotBlot was stained, it was scanned and the resulting image analysed using Matlab®. Image $f(x,y)$ analysis was divided in three differentiated stages: normalisation, segmentation and calculation. Normalisation prepares the image for the segmentation process over the spots and guarantees repeatability to the method. The normalisation results are strongly dependent on the quality of the staining process. Therefore, it is important to use the same permanganate solution in every batch. For this reason the same permanganate solution was used, preserved using potassium carbonate to ensure freshness until the end of the culture. Then, segmentation was applied. Segmentation is typically needed to apply the suitable Matlab® function to calculate the area and intensity of each spot. The segmentation process differentiates spots and background of the image $f(x,y)$ based in a global threshold (T value).

$$f'(x,y) = \begin{Bmatrix} 1 & if\ f(x,y) \geq T \\ 0 & if\ f(x,y) < T \end{Bmatrix}$$

Finally, the calculation step determines the area and intensity values of each labelled spot. The method needs to be experimentally calibrated and validated using known concentrations of glycerol.

The validation was performed with crude glycerol. Results are detailed in Table 4. As seen in Fig. 1 the first row belongs to samples of 10 g/L glycerol, generating the brightest spots. Next rows of spots correspond to glycerol decreasing concentrations. The last row of spots corresponds to samples of artificial seawater where glycerol was already depleted (no colour change). Consequently, the methodology could be considered as specific for

Table 4
Precision and accuracy for glycerol quantification using Dot Blot assay.

Crude Glycerol g/L	Mean Response	SD	CV (%)	Concentration found g/L	Recovery (%)
1	3.8	0.08	2.11	0.77	76.8
2.5	4.6	0.29	6.30	2.63	94.6
5	5.7	0.11	1.93	5.20	96.0
7.5	6.7	0.12	1.79	7.53	99.6
10	7.7	0.40	5.19	9.87	98.7
		Mean (%)	3.5	**Mean (%)**	93.14

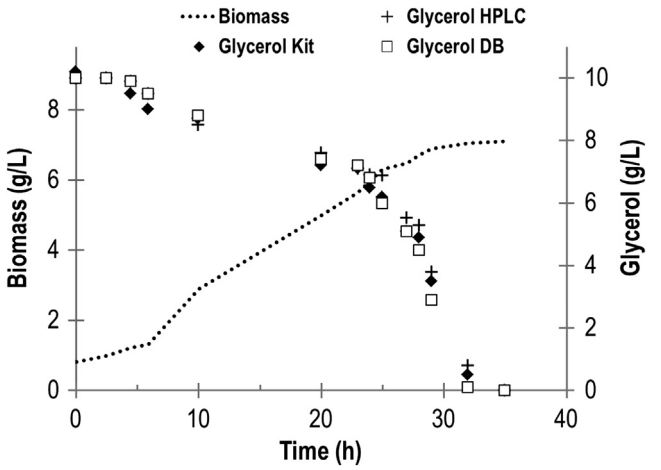

Fig. 2. Crude glycerol fermentation monitoring comparing the three methods (Biomass (g dry weight/L), residual crude glycerol (g/L) determined by each method).

glycerol, without interference due to other components of the growth media. Furthermore, the specificity was evaluated comparing samples using pure glycerol and crude glycerol diluted in water or medium. The results are summarized in Table 1. Recovery values were stable over all the samples, confirming specificity and selectivity of the methodology.

DotBlot assay was found to be linear from 1.5 g/L to 10 g/L ($R^2 > 0.998$). The slope ± standard error was 0.428 ± 0.012 and intercept ± standard error was 3.471 ± 0.075 in response arbitrary units vs. glycerol concentration (g/L). Table 4 summarizes the results evaluating repeatability and accuracy between replicas and mean % recovery is calculated. The maximum variability among samples was below 7% (e.g. variation for the most diluted samples would be equal to 0.141 g/L). % Recovery was ≤5.3% for samples above 2.5 g/L glycerol. The limit of detection was 0.5 g/L and the quantification limit was 1.5 g/L of glycerol. Below the threshold of 1.5 g/L the method became less accurate in terms of quantification, but still good enough for glycerol monitoring in fermentation.

3.4. Method comparison using fermentation broth

In order to evaluate if DotBlot capabilities are the same than HPLC and kit methodologies, *A. limacinum* crude glycerol consumption was monitored during the fermentation. Several samples were taken at different times during the cultivation. Samples were analysed independently using each methodology. All samples were processed at the same time to reduce variability.

As can be seen in Fig. 2, all methods determined similar residual glycerol concentrations. The DotBlot assay gave slightly lower values towards the end of the culture, compared to HPLC-RID and kit. The total time needed to analyse all the samples by HPLC-RID was 6 hs. Approximately, 3 h were needed using enzymatic kits. With the DotBlot method just 15 minutes were needed. Therefore, the three

methods offer similar results, but Dotblot allows faster determinations, reducing sample manipulation, to those researcher or bioprocess engineers in charge of fermentations.

4. Conclusions

HPLC and enzymatic kits are common and suitable methods to analyse glycerol in fermentation media. HPLC-RID methodology was accurate and precise but the slower, besides requiring an initial investment in equipment. Kits are faster and presented the lowest LD and LQ of the three methods compared, besides adding just a direct cost per sample analysed. The DotBlot with the image analysis method developed and validated was proved to be a suitable method for a fast monitoring of crude glycerol during fermentations. The DotBlot assay was clearly the fastest method to quantify glycerol in fermentation samples. Despite it was not as precise and accurate as HPLC or enzymatic kits, allowed a satisfactory monitoring of glycerol concentration during the fermentation. To the best of our knowledge, such methodology was not applied before fermentation monitoring. DotBlot was the cheapest and fastest method while ensuring proper precision and accuracy. It enables the quantification of glycerol concentration in about a minute. Additionally, a high amount of replicas can be processed in the same assay. The method can be applied for other types of microbial cultures using glycerol as carbon source. The DotBlot assay, besides simplifying monitoring and operational growth related parameters determination, enables high-throughput screening of microorganisms, due to the low volume needed for this assay (just 2 μL per replica). In screening as well as process development, a relative large number of cultures take places simultaneously. The DotBlot assay allows simultaneous quantification of glycerol from the different cultures.

Acknowledgements

The chromatographic unit at Institut Químic de Sarrià (IQS) is acknowledged for the support provided. SA acknowledges INTERQUIM S.A. for the doctoral scholarship provided.

Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.12.022.

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