The Pharma Innovation Journal 2018; 7(6): 103-106

www.ThePharmaJournal.com

The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2018; 7(6): 103-106

www.thepharmajournal.com
Received: 24-04-2018
Accepted: 26-05-2018

Anushree Lokur
Ramnarain Ruia Autonomous College, Department of Microbiology, L. Nappo Road, Matunga, Dadar (East), Mumbai, Maharashtra, India

Correspondence
Anushree Lokur
Ramnarain Ruia Autonomous College, Department of Microbiology, L. Nappo Road, Matunga, Dadar (East), Mumbai, Maharashtra, India

β - Galactosidase assay on microfluidic paper-based analytical devices (µPADs)

Anushree Lokur

Abstract
Enzyme assays using Microfluidic Paper-Based Analytical Devices (µPADs) are effective yet economical alternatives to conventional tube based enzyme assays. The current work involves standardisation of β-Galactosidase (β-gal) assay on µPADs using Chlorophenol red-β-D-galactopyranoside (CPRG) as a substrate. The assay was standardised with respect to substrate concentration, time of incubation, working range of enzyme and the limit of detection on the device. Presence of β-gal producers (coliforms) in spiked food samples using this assay was demonstrated as a proof of concept.

Keywords: µPADs, enzyme assay, β – Galactosidase, CPRG, coliforms

1. Introduction
Devices made of patterned papers are called Microfluidic Paper-Based Analytical Devices (µPADs). These have gained popularity in the last decade as an attractive solution for low-cost analytical testing.
Paper is an ideal platform for enzymatic assays using chromogenic substrates as it provides the ideal background contrast for evaluating colour changes. As a substrate material, paper has many unique advantages over traditional device materials such as power-free fluid transport via capillary action, a high surface area which in turn helps to improve the detection limits for colorimetric methods and the ability to store reagents in an active form within the fibre network.
Different methods are available for the fabrication of µPADs. The µPADs used in this work have been fabricated using photolithography with indigenous materials.
The current work involves standardisation and proof of concept testing of β-Galactosidase (β-gal) assay on this device.
Chlorophenol red-β-D-galactopyranoside (CPRG) is a chromogenic substrate used for β-gal assay. CPRG is 10 times more sensitive than o-nitrophenyl-β-D-galactopyranoside (ONPG), which is the most commonly used substrate. (Eustice *et al*., 1991). CPRG is an analogue of galactose and thus β-gal can catalyse its hydrolysis. Samples are incubated with CPRG and β-gal converts the yellow-orange CPRG substrate into the red chromophore chlorophenol red, yielding a dark red solution (Sicard *et al*., 2014) [10]. There is a good contrast between positive and negative test (red and yellow), and easy for observation on paper. Hence, it is the substrate of choice for this work.
As a proof of concept, β-gal assay was used to demonstrate the presence of coliforms in spiked food samples. Coliform bacteria are commonly used indicators of sanitary quality of food and water. The enzymatic assays for detection of total coliforms are based on the hydrolysis of substrates by β-gal activity, an enzyme found in coliforms.

2. Materials and Methods
2.1 Fabrication of µPADs
µPADs were fabricated by FLASH method given by Martinez et.al using locally available SU8 equivalent. Whatman filter paper no. 3 with microtiter well type design was used for this purpose.

2.2 Substrate and enzyme preparation
Substrate used was Chlorophenol Red-β-D galactopyranoside (CPRG, Sigma Aldrich). A stock (11mgmL^{-1} i.e. 18.8M, 8X) of CPRG was prepared in phosphate buffer pH 7.5. Four

substrate concentrations were used for standardisation - 0.24mM (0.1X), 1.17 (0.5X), 2.35 (1X), 4.7 (2X). Enzyme used was pure β-Galactosidase (1500 UmL^{-1}, Sigma Aldrich). It was diluted to give the range of 0.01-10 UmL^{-1} with buffer pH 7.5.

2.3 Assay for determining working range of enzyme

A pilot assay for determining working range of enzyme was done. Three enzyme concentrations namely 0.1,1 & 10 UmL^{-1} were used for initial screening to check feasibility of this assay on paper. Since all enzyme concentrations gave visually detectable results, a pilot assay was done with enzyme concentrations in the range of 0.01-10 UmL^{-1}. This assay was done in triplicates with all four (0.1X, 0.5X, 1X, and 2X) substrate concentrations. Results were recorded after 1 h of incubation at 37 °C.

The range of concentrations that gave appropriate results was used for further assay with 8 replicates for determining optimum substrate concentration and time of incubation.

2.4 Assay for Determining Optimum Substrate Concentration and Time of Incubation

Enzyme concentrations in the range of 0.1-1 UmL^{-1} were used for this assay based on the results of pilot assay. Results were recorded after every 15 minutes up to 90 minutes of incubation at 37 °C. This assay was done in 8 replicates and the data was processed for statistical analysis. Graphs of Average invert grey intensity (AIGI) v/s Substrate Concentration at time intervals of 15, 30, 45 and 60 minutes were plotted to decide optimum substrate concentration and time of incubation for the assay.

2.5 Determination of Limit of Detection of the enzyme

The same set of experimental data for determination of optimum incubation time in the preceding section was used for the determination of LOD. Limit of detection was defined as the lowest concentration of enzyme giving test results that are significantly different from control.

2.6 Demonstration of coliforms from spiked food samples using β-Galactosidase assay

Food samples were prepared and spiked as per the protocol mentioned in the Appendix.

3. Results and Discussion

3.1 Pilot assay for determining working range of enzyme on µPADs

Average invert grey intensity readings for enzyme concentrations in the range of

0.01- 0.1 were not consistent as the reddish colour seen for positive reaction was barely visible. Therefore this range was not considered for further experimentation. Enzyme concentration range 0.1 - 1.0 UmL^{-1} gave good gradation and consistent results especially with 1X substrate concentration. Hence, this range was used for the assay.

3.2 Assay for Determining Optimum Substrate Concentration & Time of Incubation

Box plots for all the readings confirmed that there were hardly any statistical outliers. There is no abnormal value in the data.

Substrate concentration 0.1X did not show much colour even after 60 minutes of incubation. Statistical analysis of the data shows that none of the readings obtained for 0.1X substrate concentration are significant according to *t* -Test except for highest enzyme concentration

1UmL^{-1} at 15 minutes. Hence substrate concentration 0.1 X cannot be used.

For substrate concentration 0.5 X, all enzyme concentrations show positive result visually and readings are significant for all concentrations of enzyme at 15, 45 and 60 minutes, except for 30 minutes. At 30 minutes, the values are significant beyond 0.4UmL^{-1}. The graph of average invert grey intensity against enzyme concentration (UmL^{-1}) shows linearity at all time intervals and the R^2 values for 45 and 60 minutes are both above 0.9, but the colour of the positive result at 0.5X concentration is light, contrast between positive and negative is not much. Therefore, this substrate concentration is also not considered as qualitative examination is based on visual detection.

For Substrate concentrations1X and 2X, all enzyme concentrations show significant values for all time intervals. Graph of mean invert grey intensity against concentration of enzyme show Michaelis -Menten type of kinetics. With substrate concentration 1X, for all the time intervals tested, maximum invert grey intensity is at enzyme concentration 0.6 UmL^{-1} while for 2 X it is at 0.2UmL^{-1}, beyond which it is more or less constant (Table 1, 2). Hence substrate concentration to be used will depend upon the concentration of enzyme to be tested. A combination of time and substrate concentration should to be taken into account before finalising the optimum conditions.

It was decided to use Substrate concentration of 1X (2.35 mM) for further work as the graph shows good linearity over a wider range of enzyme concentrations and R^2 value of 0.77 which is acceptable.

Even though substrate concentrations 1X and 2X gave clearly visible results within 15 minutes, optimum incubation time can be 60 minutes as the colour obtained at 60 minutes is dark and hence gives higher values of mean invert grey intensity. Limit of detection remains the same even after continued incubation. The sets were incubated up to 90 minutes and readings were taken at 75 and 90 minutes. It was observed that the readings did not increase beyond 60 minutes of incubation, on the contrary they decreased. Therefore, these readings were not considered for statistical analysis. This could be due to the fact that all proteins suffer denaturation, and hence there is loss of catalytic activity, with time.

Hence Optimum Substrate concentration for pure β-Galactosidase on µPADs is 1X and Optimum Time of Incubation is 60 minutes.

3.3 Limit of Detection of the enzyme

The lowest enzyme concentration that can be detected visually with this method is 0.03 UmL^{-1}.

3.4 Demonstration of coliforms from spiked food samples using β-Galactosidase assay

All spiked samples except coriander showed positive results. Coliform count of spiked coriander sample was 3.0×10^3 $CFUmL^{-1}$. The amount of enzyme produced by this cell number may be below the detectable limit of this device i.e. 0.03 UmL^{-1}. Samples with low coliform counts may be enriched for a longer duration to increase the number of cells and therefore the amount of enzyme produced, to a detectable level. The coliform counts of all other spiked samples were above 10^7 $CFUmL^{-1}$. Spinach and coriander samples showed green colour on the spot when loaded but it did not interfere with the observation of the results. The red colour of positive test was predominant as the incubation progressed (Figure 1).

Table 1: Results and statistical analysis of assay for determining optimum substrate concentration and time of incubation for µPADs (Substrate concentration 1X and time of incubation 15 and 30 minutes)

Enzyme Concentration (UmL^{-1})	AIGI (n = 8)		AIGI (T – C)		S.D.		p-value	
	Time (minutes)		Time (minutes)		Time (minutes)		Time (minutes)	
	15	30	15	30	15	30	15	30
Control	76.13	83.94			2.58	3.44	-	-
0.1	80.32	87.88	4.19	3.94	2.51	3.55	0.005	0.0410
0.2	85.46	93.88	9.33	9.94	2.85	3.80	8.120 e-06	8.319 e-05
0.3	90.44*	99.99	14.31	16.05	4.29	5.86	1.015 e-07	3.015 e-05
0.4	90.74	99.92	14.61	15.98	2.20	3.89	1.015 e-08	5.598 e-07
0.5	93.36	102.22	17.23	18.28	3.78	4.72	1.401 e-07	8.096 e-07
0.6	96.40	105.65	20.27	21.71	7.25	8.70	4.523 e-05	9.727 e-05
0.7	93.06	94.04	16.93	10.1	4.27	4.07	7.851 e-07	0.0001
0.8	94.84*	95.57	18.71	11.63	2.65	3.21	9.483 e-10	6.494 e-06
0.9	97.36	96.03*	21.23	12.09	4.74	5.09	2.976 e-09	1.74 e-05
1	100.88	97.24	24.75	13.3	5.53	6.00	4.862 e-07	0.0002
*Outlier								

Table 2: Results and statistical analysis of assay for determining optimum substrate concentration and time of incubation for µPADs (Substrate concentration 1X and time of incubation 45 and 60 minutes)

Enzyme Concentration (UmL^{-1})	AIGI (n = 8)		AIGI (T – C)		S.D.		p-value	
	Time (minutes)		Time (minutes)		Time (minutes)		Time (minutes)	
	45	60	45	60	45	60	45	60
Control	79.79	81.05			3.40	0.85	-	-
0.1	83.52	87.88	3.73	6.83	3.43	3.55	0.0462	0.0008
0.2	88.67	93.88	8.88	12.83	4.26	3.80	0.0004	1.849 e-05
0.3	94.10	99.99	14.31	18.94	5.59	5.86	5.525 e-05	3.191 e-05
0.4	93.94	99.92	14.15	18.87	4.13	3.89	3.703 e-06	1.338 e-06
0.5	96.40	102.22	16.61	21.17	5.66	4.72	1.567 e-05	2.881 e-06
0.6	98.57	105.65	18.78	24.6	8.10	8.70	0.0001	8.54e-05
0.7	92.78	104.39	12.99	23.34	3.62	6.64	3.556 e-06	1.889 e-05
0.8	93.28	105.06	13.49	24.01	3.11	6.49	9.584 e-07	1.33 e-05
0.9	94.44*	104.96	14.65	23.91	4.64	7.52	1.749 e-06	3.833 e-05
1	95.32	106.33	15.53	25.28	6.40	8.74	9.339 e-05	7.369 e-05
*Outlier								

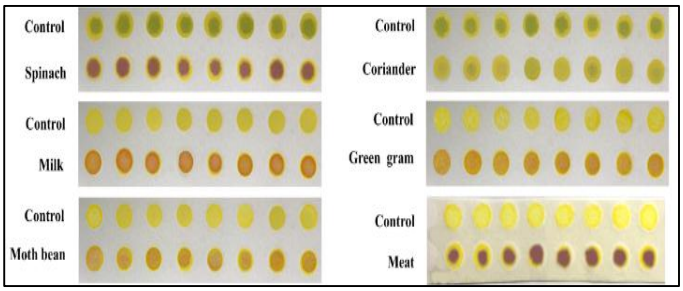

Fig 1: Demonstration of coliforms from spiked food samples

Conclusion

β-gal assay on µPADs is a good alternative to conventional tube assays. The amount of reagents required is in micro liters, it does not need any sophisticated instruments, glassware or trained personnel for the test. Qualitative test is based on visual colour detection, while quantitative analysis requires just a smartphone to capture and analyze the image. The paper can be safely disposed of by incineration.

Therefore, µPADs are an effective tool for enzyme assays. Commercial enzyme producing industries can use these µPADs at the site of fermentation plant for quick, continuous monitoring of enzyme production. They can also be used for the detection of microorganisms from samples like soil, food and water. These devices can be used for the detection and assay of any enzyme with chromogenic substrate.

Appendix

Protocol for Assay on µPADs

	Substrate (µL)	Enzyme (µL)	Diluent (µL)
Test	5	5	-
Control	5	-	5

The papers were incubated at 37 °C for the predetermined time in a moist chamber. A photograph was taken with a smart phone or the papers were scanned. The invert intensity was measured using ImageJ™ software (Miura, 2013) [7]. Readings for control were subtracted from readings of the test.

Statistical Analysis

The results were statistically analysed using Stata/IC 14.1. A box plot of average invert grey intensity for each β – Galactosidase concentration was drawn. This was done to identify outliers and to compare distribution of the data. Outliers were removed based on the readings of the box plot.

A *t*-test was conducted to test whether the invert grey intensity for a particular concentration of β - Galactosidase is significantly different from that of the control. If the value for a particular reading was not significantly different at 5% level of significance from the control mean, it was excluded from the analysis.

Spiked Food Sample Preparation

10 g of chopped solid food samples such as vegetable, meat or food sample was weighed and added to a flask containing 40 ml Ringer's Solution (Vegetable samples were taken along with the leaves and 2 inch stalks). This was then mixed in a blender for 2 minutes. Remaining 50 ml of Ringer's solution was added. This food sample was used for further enrichment or direct use. For liquid food samples, 10^{-1} dilution in Ringer's Solution was used. 1mL of positive control culture (*E. coli* ATCC25922, known β-gal producer) suspension of variable cell numbers was added to 10 mL of such food preparation. Viable count was performed on Mac Conkey's agar for each sample. The samples used in the current work were – Spinach, Coriander, Milk, Green Gram, Moth Bean and Meat.

Procedure for enrichment and preparation of Cell Lysate for β Gal Assay

Spiked samples were inoculated at 10% inoculum level in sterile Lactose broth tubes (5 mL) for enzyme induction. It was incubated at 37 °C for 1.5 h. The culture broth was centrifuged; the pellet was washed with sterile saline and lysed using 200 µL of buffer and 8 µL of Toluene (W. Jackson, & De Moss, 1965) [12]. The lysate thus obtained was used for the assay on µPADs.

Acknowledgment

The author thanks Ramnarain Ruia Autonomous College for their support and for the facilities provided.

References

1. Eaton A, Clesceri L, Greenberg A. Standard methods for the examination of water and wastewater, 19th edition. Washington: APHA American Public Health Association, 1995.
2. Fiksdal L, Tryland I. Application of rapid enzyme assay techniques for monitoring of microbial water quality. Current Opinion in Biotechnology, 2008; 19(3):289-294. http://dx.doi.org/10.1016/j.copbio.2008.03.004
3. http://dx.doi.org/10.1111/j.14321033.1971.tb01402.x
4. Husain Q. β - Galactosidases and their potential applications: a review. Critical Reviews in Biotechnology. 2010; 30(1):41-62. http://dx.doi.org/10.3109/07388550903330497
5. Manafi M, Kneifel W, Bascomb S. Fluorogenic and Chromogenic Substrates used in Bacterial Diagnostics. Microbiological Reviews. 1991; 55(3):335-348.
6. Martinez A, Phillips S, Wiley B, Gupta M, Whitesides G. FLASH: A rapid method for prototyping paper-based microfluidic devices. Lab on a Chip. 2008; 8(12):2146. http://dx.doi.org/10.1039/b811135a
7. Miura K. Basics of Image Processing and Analysis, 2013.
8. Nikaeen, Pejhan A, Jalali M. Rapid Monitoring Of Indicator Coliforms in Drinking Water by an Enzymatic Assay. Iran. J. Environ. Health. Sci. 2009; 6(1):7-10.
9. Rompré A, Servais P, Baudart J, de-Roubin M, Laurent P. Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. Journal of Microbiological Methods. 2002; 49(1):31-54. http://dx.doi.org/10.1016/s0167-7012(01)00351-7
10. Sicard C, Shek N, White D, Bowers R, Brown R, Brennan J. A rapid and sensitive fluorimetric β - Galactosidase assay for coliform detection using chlorophenol red-β-d-Galactopyranoside. Analytical and Bioanalytical Chemistry. 2014; 406(22):5395-5403. http://dx.doi.org/10.1007/s00216-014-7935-0
11. Tenu J, Viratelle O, Garnier J, Yon J. pH Dependence of the Activity of beta-Galactosidase from Escherichia coli. European Journal of Biochemistry. 1971; 20(3):363-370.
12. Jackson WR, De Moss J. Effects of Toluene on Escherichia coli. Journal of Bacteriology. 1965; 90(5):1420-1425