Vol-3, Issue-2 PP. 101-106 ISSN: 2394-5788

GLOBAL JOURNAL OF ADVANCED RESEARCH
(Scholarly Peer Review Publishing System)

BIOREMEDIATION OF DISPOSED X-RAY FILM FOR ENZYMES PRODUCTION

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ABSTRACT

Annually, the production of X-ray films sheets can utilize up to 1000 tons of total silver chemically produced worldwide and being wasted when these films are used. To avoid waste, the biological methods are used to study the effect of disposed X-ray on production of two different types of enzyme by using microbial power. Firstly, production of CMCase enzyme by using disposed X-ray as a carbon source. Secondly, production of nitrate reductase enzyme responsible to catalyse the reduction of silver nitrate inside X-ray film. Both bring the disposed X-ray as a substrate. Different bacterial isolates were used for production of both enzymes and being optimized by using several parameters. The protein and enzyme assay were analysed using optical density measurement. CMCase production found to be optimal in 140 rpm incubator with lactose as carbon source by 1.365±0.026 (U/ml), malt extract as nitrogen source by 0.485±0.028 (U/ml), pH 9.0 by 0.129±0.007 (U/ml), 1.5 g substrate concentration by 0.217±0.007 (U/ml), 2 ml inoculum size by 0.143±0.029 (U/ml), and thiamine for vitamin by 0.208±0.041 (U/ml). While nitrate reductase production, the most potent isolates achieved optimum condition in static incubation condition by 1.182±0.000µg/ml, 0.5g substrate concentration by 1.500±0.020µg/ml with 15 days incubation periods in darkness, NaNO3 as nitrogen source by 166.773±0.054µg/ml and pH 3.0 by 0.773±0.001µg/ml. This study proved that the power of most potent isolates successfully used the disposed X-ray as substrate to produce valuable by-products using green technology to reduce environmental pollution.

Keywords

Bioremediation; Disposed x-ray film; CMCase; Nitrate reductase.

1. INTRODUCTION

Nowadays, the increasing number of industrialization sector has caused a lot of pollution around the world. That occurs due to lack of awareness about the harmful effects especially to aquatic environment. When the water pollution occurs because of chemical, physical, radioactive or pathogenic substances, the large scales illness and deaths will occur. Common examples of chemical water pollutants are mercury, certain nitrogen compound, chlorinated organic molecules and various acids [1]. To remove and neutralized pollutants from contamination, the bioremediation should be done. These mean that the natural treatment that uses organisms to breakdown hazardous substances into less or non-toxic substances. In this study, the bioremediation of disposed x-ray films were used for enzymes production. Firstly, production of CMCase (EC 3.2.1.4) by using disposed x-ray as a carbon source. Cellulose which act as a carbon source plays an important role as a substrate that enzyme can process to produce reducing sugar. Cellulose will be degraded by microorganisms that was isolated from waste. The importance of this research is to decrease hazardous waste and produce valuable

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material. The x-ray film that was used as substrate is not a common waste. It is a waste that may cause potential pollutants if it not properly handled.

The x-ray film may come from dental offices, photographic processors and metal plating industries. Cellulose that was known to be part of the x-ray film is being wasted and disposed. Then, the unknown microorganisms were isolated from various type of source to help in biodegradation process. Microbial cellulases are mostly available and convenience as microorganisms can simply grow on easily obtained media such as food industries and agriculture [2].

Secondly, production of nitrate reductase enzyme responsible to catalyse the reduction of silver nitrate inside x-ray film. As we knows, the disposed x-ray films consumes about 1000 tons of total silver which chemically produced worldwide annually and being wasted when these films were used.

The production of nitrate reductase (NR; EC 1.6.6.1-3) can catalyzes NAD(P)H reduction of nitrate to nitrite [3]. By using the biological methods instead of physical and chemical, the production of this enzyme consume lower cost and less time consuming. The unknown isolate were used in this research to study about their ability to produce nitrate reductase enzyme in the optimum condition by using disposed x-ray films as substrate.

Silver wastes may cause potential pollutants if it not properly handled. In this research, the hazardous waste which is x-ray films (sheets) were used as substrate because it consume about 1000 tons of total silver chemically produced worldwide annually. This silver is being wasted when these films are used and disposed. Then, the unknown microorganisms were isolated from various type of source to precede this research.

2. MATERIALS AND METHODS

1.1 Isolation of Bacterial Isolates

The bacteria labelled GL7 was isolated from water (Gambang Lake, Kuantan), CL4C and CL8A from spoiled food (rotten chicken liver) and PS1 from soil (Panching soil, Kuantan) were grown on nutrient agar under aerobic condition at 37 °C for 24 h. A single colony was isolated from a dozen of Petri-dishes exposed. Then, it was subcultured in nutrient broth for inoculation.

1.2 Pre-treatment of X-ray film sheets

The substrate (x-ray film) containing target materials which are AgNO3 and cellulosic material was cut into small pieces (0.3 x 0.3 cm) and 4 N NaOH was added until it covered the substrate surface and mixed well using the orbital shaker for overnight at 150 rpm at room temperature. It was then washed several times using tap water to remove the colour of substrate and the pH was adjusted to pH 7.0. After that, the mixture was left to dry in the oven overnight.

1.3 Inoculum preparation

Nutrient broth was prepared prior to incubation of microbial isolates. The nutrient broth was measured with 50 ml measuring cylinder and poured into 100ml conical flask before autoclaved for 20 min at 121 °C. Using aseptic technique, microbial isolates were inoculated using inoculation loop from slant agar to the nutrient broth. Finally, the microbial isolate was incubated at 37 °C 72 h in shaker incubator with 140 rpm.

1.4 Preparation of modified minimal media (MM9)

Four stock solutions need to be prepared, which are solution A, B, C and D. Stock solution A consists of 25.6 g/l of Na2HPO4.7(H2O) (Merck), 6 g/l of KH2PO4 (Merck), and 1 g/l of NaCl (Merck). All chemicals that have been weighed was put off in 500 ml distilled water, mixed well and top up until 1 L. Then, the 500 ml of solution A stock was transferred into another 1 L beaker and added with 2 ml of solution B (1.0 M MgSO4 solution), 0.1 ml of solution C (1.0 M CaCl2 solution) and 20 ml solution D (20% D-Glucose solution) as carbon source. Top up the stock solution with distilled water until reached 1 L amount.

1.5 Synthesis of silver nanoparticles

Fifty ml of complete modified production media (MM9) was added into 100 ml conical flasks. After that, all the parameters controlling on nitrate reductase enzyme productivity were prepared for each isolate.

1.6 Substrate concentration (pre-treated x-ray films)

Fifty ml of complete MM9 were prepared and for each flasks and different substrate concentration were prepared which were 0.2 g, 0.5 g, 0.8 g, 1.0 g and 1.5 g for each isolates.

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1.7 Nitrogen source

Different sodium nitrate (NaNO3) concentrations were studied which were 0 g, 0.01g, 0.02 g, 0.03 g, 0.04 g and 0.05 g. Each concentration was added into all flasks for each isolates that contain 50ml of MM9.

1.8 pH values

For pH values, adjust the MM9 to pH 3, pH 4, pH 5, pH 6, pH 7, pH 8 and pH 9 for each isolates.

1.9 Carbon sources

Seven types of carbon sources namely starch, lactose, sodium acetate, maltose, yeast extract, sucrose and CMC. Around 20% 1 ml carbon source was added to each separated flasks containing 50 ml M9 media and 1.0 g substrate.

1.10 Inoculum sizes

To check for optimum volume of isolates, 0 ml, 2 ml, 5 ml, 7 ml and 10 ml inoculum was added to 5 different flasks containing 50 ml M9 media and 1.0 g substrate.

1.11 Vitamins

Around 100 ppm vitamins namely pyridoxine, thiamine and β-carotene was weighed and added to 3 different flasks containing 50 ml M9 media and 1.0 g substrate.

Then, it was autoclaved at 121 °C for 15 min. After that, remove 5 ml of mixture in both flask and replaced with 5 ml of bacterial inoculum respectively. The controlled mixture was also prepared. Finally, all the flasks were incubated at 37 °C for fifteen days in darkness and static condition. Next, the mixture was centrifuged for 5 min at 5000 rpm and at 4 °C to separate the microbial cell with the supernatant.

1.12 Protein assay

The supernatant was undergo protein assay analyses by UV-vis spectrophotometer at 700 nm was used according to Lowry's method [4].

1.13 Nitrate reductase assay

The enzyme activity was measured using [5] method by putting the substrate for the enzyme (nitrate) and then the amount of nitrite after 60 minutes was measured. The net increase in nitrite at 60 min is the amount of nitrate reductase activity. The reagents used as assay medium were 30 mM KNO3 and 5 % propanol in 0.1 M phosphate buffer pH 7.5. Then, for nitrite assay reagents, sulfanilamide solution: 1 % (w/v) in 25 % (v/v) HCl and N-(1-napthy) ethelenediamine dihydrochloride solution (NEED): 0.02 % (w/v) in distilled water was prepared.

Six tubes each containing 10 ml of the each unknown culture supernatant, taken from the 15 days reaction in darkness were prepared. Tubes 'a', 'b' and 'c' (triplicates) were place in a boiling water bath sample to kill the enzyme as soon as 10 ml assay medium was added (i.e. at time-zero). All the tubes considered as control. After 5 min boiled, the tubes were kept at room temperature until the nitrite was measured. Next, 10 ml of assay medium were added to tubes 'c, d, e' (triplicates) and incubate in the darkness for 60 min before placing the tubes in the boiling water bath for 5 min. After the samples cooled, 5 ml of sulfanilamide solution and 5 ml of NEED solution was added and quickly mixed. After 20 min the absorbance was measured at 540 nm using UV-VIS spectrophotometer. The amount of nitrite found in tubes 'a','b' and 'c' were subtracted from that in tubes 'c, d', and 'e' to calculate the amount produced by enzyme activity during 60 min [6] . Nitrite standard curve was prepared by placing known amounts of nitrite (0 to 200 µg/ml NO2) followed by 5 ml of sulphanilamide and 5 ml of NEED solution. After mixed and incubated for 20 min at room temperature, the absorbance was measured at 540 nm. The unknown concentration of nitrite produced was calculated based on the standard curve produced.

1.14 CMCase assay

Dinitrosalicylic colorimetric (DNS) method is a method used to detect the presence of free carbonyl group (C=O) or reducing sugars [7]. Using this method, the equivalent enzyme used in the reaction can also be detected. Dinitrosalicylic Acid Reagent Solution 1 % was prepared by mixing 10 g or of dinitrosalicylic acid, 2 g phenol, 0.5 g sodium sulfite and 10 g sodium hydroxide in 1 L of distilled water. Potassium sodium tartrate solution, 40 % was prepared by dissolving 40 g of potassium sodium tartrate (Rochelle salt) in 100 ml distilled water. A procedure for 0.1 M phosphate citrate buffer pH 6.6 was carried out by mixing 36.4 ml 0.2 M Na2HPO4 and 13.6 ml of 0.1 M citrate in a 100 ml beaker. This buffer was labelled and stored at room temperature before further use.

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One ml of cell-free filtrate was incubated with 1 ml of 2 % (w/v) CMC in 0.1 M phosphate citrate buffer (pH 6.6) at 40 °C for 2 hours. For the blank solution, distilled water is used instead of cell-free filtrate. The resulted reducing sugars were determined using DNS method. About 1.5 ml of DNS reagent was added to 1.5 ml sample in a capped tube to avoid the liquid from evaporating. The mixture was heated at 90 °C for 5-15 min to develop the red-brown color. One ml of 40 % potassium sodium- tartrate solution was added to stabilise the color. After cooling to room temperature, the absorbance was measured using spectrophotometer at 575 nm and recorded in a table. Three replicates of each sample were prepared to give more accurate results.

3. RESULTS AND DISCUSSION

Data recorded in Table 1 showed the parameters that have highest CMCase activity values for CL8A and PS1 isolates. For PS1, 0.251 ± 0.018 U/ml was recorded for starch which separated it from other carbon sources lactose, sodium acetate, maltose, yeast extract, sucrose and CMC. For CL8A, lactose with 1.365 ± 0.026 U/ml was found to significantly induce CMCase activity than the rest of carbon sources. All of the tested sources were prepared fresh to maintain their effectiveness of degrading simple and complex cellulose into reducing sugar.

Among all other nitrogen sources, only malt extract expressed a high value to enzyme production for both PS1 0.137 ± 0.011 U/ml and CL8A 0.485 ± 0.028 U/ml. Yeast extract, beef extract and malt extract were identified as organic nitrogen sources while ammonium sulphate, sodium nitrate, potassium nitrate and ammonium chloride were inorganic nitrogen sources. The result was in accordance to [8] that organic nitrogen source has greater possibility to produce more cellulose.

Effect of different pH on CMCase activity was also carried out which from 3 to 9 and pH 8 and pH 9 were recorded as the highest for PS1 0.125 ± 0.006 U/ml and CL8A 0.129 ± 0.007 U/ml respectively. An analysis with Avicelase also confirmed that pH range from 4 to 9 contribute to enzyme stability [9].

X-ray film has been used as substrate which act as feeder to the microbe in the media. However, to react effectively, pretreatment has to take place before hydrolysing the cellulose. According to [10] breaking down crystalline structure is the role of the pretreatment. However, different isolates need different amount of substrate to actively contribute to CMCase production. Seven different amount of substrate were weighed, 0.2 g, 0.5 g, 1.0 g, 1.5 g, 2.0 g, 2.5 g and 3.0 g. PS1 exhibited 0.181 ± 0.008 U/ml with 2.5 g while CL8A exhibit 0.2173 ± 0.007 U/ml with only 1.5 g.

Total of 5 different inoculum sizes have been tested which were 0 ml, 2 ml, 5 ml, 7 ml and 10 ml. Two ml of inoculum was recorded as the highest for both PS1 and CL8A by 0.231 ± 0.007 U/ml and 0.143 ± 0.029 U/ml respectively. The volume of microorganisms is not correspond to enzyme productivity as only small volume of inoculum needed for the enzyme to have higher yield.

Finally, 3 different types of vitamins were used to boost enzyme activity namely, pyridoxine, thiamine and β-carotene. Among them, thiamine had a good reaction and showed higher result for both PS1 and CL8A with 0.145 ± 0.014 U/ml and 0.208 ± 0.004 U/ml respectively.

Table 1. Optimum parameters for PS1 and CL8A isolates

No.	Parameters	Bacterial isolates	
		PS1	CL8A
1	Carbon sources	Starch	Lactose
2	Nitrogen source	Malt extract	Malt extract
3	pH value	8	9
4	Substrate concentration (g)	2.5	1.5
5	Inoculum sizes (ml)	2	2
6	Vitamin	Thiamine	Thiamine
Enzyme activity U/ml		0.934 ± 0.020	4.559 ± 0.018
Protein content mg/ml		0.895 ± 0.016	0.841 ± 0.010

According to Table 2, for production of nitrate reductase enzyme for unknown isolate labelled CL4C, the result revealed that in static incubation reach the optimum condition with the concentration 0.773 µg/ml. The amount of net increases in all samples was calculated by subtraction of amount of sample concentration and their control produced during 60 min [6]. For substrate concentration and incubation periods, CL4C sample optimised at 0.5 g substrate and 15 days incubation periods with the protein content 0.765 ± 0.010

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mg/ml. For pH values, CL4C sample reach the optimum at pH 3 with the protein content 1.047 ± 0.004 mg/ml. This revealed that this unknown isolate might be acidophilic or "acid loving" bacterium because it experience optimal growth at low pH. Even they can live in very low pH environments; their internal pH is closer to neutral values [11]. For nitrogen source, the concentration of 0.03 g sodium nitrate ($NaNO_3$) give the optimum concentration produced in the sample compared to other concentration with the protein content of 0.922 ± 0.010 mg/ml. The nitrogen source is requiring in bacterial growth as they used to form amino acids, DNA and RNA. The nitrate is a salt that can dissociate to give NO_3^- to the bacterial [12].

Table 2. Optimum parameters for production of nitrate reductase (NR) enzyme for isolate labelled CL4

Parameter	Optimal Parameters for NR Enzyme concentration (µg/ml) for CL4C		
	Control	Sample	Net increases
Incubation condition	3.273 ± 0.000	4.045 ± 0.001	0.773 ± 0.001
Substrate Concentration	4.273 ± 0.015	5.773 ± 0.035	1.5 ± 0.02
Incubation period			
pH values	2.273 ± 0.000	3.045 ± 0.001	0.773 ± 0.001
Nitrogen Source	3.909 ± 0.001	170.682 ± 0.055	166.773 ± 0.054

Table 3 revealed the result for optimum parameters of unknown isolate labelled GL7. Same as CL4C sample, the sample reach the optimum incubation condition also in static. But for substrate concentration, 0.8 g substrates reach the optimum with the 15 days incubation periods. The protein content is 0.998 ± 0.021 mg/ml. Then, for pH values, the sample produced high concentration of enzyme at pH 8 with the protein content 0.990 ± 0.008 mg/ml. This unknown isolate can be considered as neutrophils. Lastly, for nitrogen source, GL7 sample also achieved optimum condition at 0.03 g $NaNO_3$ which was same with CL4C and the protein content was 1.082 ± 0.058 mg/ml.

Table 3. Optimum parameters for production of nitrate reductase (NR) enzyme for isolate labelled GL7

Parameter	Optimal Parameters for NR Enzyme concentration (µg/ml) for GL7		
	Control	Sample	Net increases
Incubation condition	3.227 ± 0.004	4.409 ± 0.004	1.182 ± 0.000
Substrate Concentration	3.909 ± 0.004	4.727 ± 0.007	0.818 ± 0.003
Incubation period			
pH values	3.045 ± 0.000	3.545 ± 0.008	0.500 ± 0.008
Nitrogen Source	3.636 ± 0.002	153.500 ± 0.052	149.864 ± 0.050

4. CONCLUSION

Additional work on purification of enzyme can determine whether a large scale production can be done in the future. Different cellulosic material derived from different sector other than agriculture and clinical can be explored to get higher yield of CMCase. This studied proved that, the bioremediation of disposed x-ray films are able to produce various enzymes by using microbial power. The several unknown bacterial isolated were successfully produced CMCase and nitrate reductase enzymes after the parameters were being optimized by using disposed x-ray films as their carbon source.

5. ACKNOWLEDGMENTS

A grateful acknowledgement is given to Faculty of Industrial Sciences and Technology, University Malaysia Pahang (UMP) for the financial support (RDU 130611).

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