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Analysis of enzymatic activity of the isolated microorganisms collected from local rice beer (Kiad)

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Abstract
The isolates were found to show variations in and exhibit the alcohol dehydrogenase enzyme activities which enable them to grow in the medium with high alcoholic content. Alcohol dehydrogenase (ADH) refers to a family of enzymes which catalyze the reversible oxidation of primary or secondary alcohols to aldehydes or ketones respectively. They display a wide variety of substrate specificities and play an important role in a broad range of physiological processes.

Keywords: Alcohol dehydrogenase, aldehydes, ketones

Introduction
Alcohol dehydrogenase (ADH) refers to a family of enzymes which catalyze the reversible oxidation of primary or secondary alcohols to aldehydes or ketones respectively. They display a wide variety of substrate specificities and play an important role in a broad range of physiological processes. In this study, the alcohol dehydrogenase enzyme production from the microorganisms isolated from Local Rice Beer (*Kiad*) was isolated and analyzed [1].

Materials and Methods
1. Assay of alcohol dehydrogenase
The reaction velocity is determined by the method of A. Vallee and Hoch (1955) in which the rate of absorbance at 340nm resulting from reduction of NAD^+ is measured. One unit reduces one micromole of NAD^+ per minute at 25°C under the specified conditions.

2. Isolation of the enzyme
The cultures in bulk were taken in a centrifuge tube for centrifugation. The supernatant was discarded from all the tubes. The pellet formed in all the tubes was then washed and centrifuged twice with autoclaved distilled water. Lyophilisation (Freeze-drying) of the pellet was then carried out in a Lyophiliser. The freeze-dried pellet was then stored at -20°C for future use. After isolation of the enzymes was done, the lyophilised enzymes were then dissolved at 1 mg/ml in 0.1 M phosphate buffer (pH 7.5). Immediately prior to use, all enzymes were diluted to a concentration of 0.25 units/ml in 0.1% albumin. The UV-Vis Spectrophotometer was then set to 340 nm and 25°C. 1.5 ml of 0.1 M Pyrophosphate buffer, 0.5 ml of 2.0 M Ethanol and 1.0 ml of 0.025 M NAD was pipetted into each cuvette and incubated in spectrophotometer for 3-4 minutes at 25°C to achieve temperature equilibrium and blank rate was established. At zero time, 0.1 ml of appropriately diluted enzyme was then added into the cuvette and the absorbance at 340 nm (A_{340}) was recorded for 3-4 minutes. Similarly for the rest diluted enzymes, same procedure as mentioned above was used and the absorbance at 340 nm (A_{340}) was taken for all [2].

3. Calculation of the enzyme
Calculation was done by adopting the method of Blandino *et al*. (1997).
(A.) $dA/dt\ (min^{-1}) = [Rate]_{experimental} - [Rate]_{control} = dA/dt$

(B.) $\text{Activity} = \dfrac{1000 \times TV \times D \times dA/dt}{\varepsilon \times V \times CF}$

Activity: Volumetric Activity (U/L).

TV: Total volume in cuvette (1000 µl).
D: Dilution of the cell extract= 8. (For example, if 50µl of cell extract were add to 950µl distilled water prior to using a volume of cell extract in the assay, then D=20).
V: Volume of cell extract used (50 µl).
ε: Molar extinction coefficient for NADH (6.22 L/mmol for a path length of 1.0 cm).
CF: Concentration Factor of cell extract= 5 (For example, if a 100 ml sample is concentrated to a 2 ml volume for the French Press, then CF=50).

(C.) Specific Activity = $\frac{\text{Activity}}{\text{Protein Concentration}}$

Activity: Volumetric Activity, as calculated in #2 above (IU/L)

Protein Concentration: Protein concentration, as calculated in total protein concentration (µg/ml) by Bradford method below.

Specific Activity: (IU/mg protein)

Similarly, estimation of protein by Bradford method was also performed.

4. Estimation of proteins by Bradford method
Bradford assay is a quantitative method for the estimation of proteins. Coomassive dye in Bradford's reagent reacts with protein sample and gives a blue color solution, where absorption shifts from 465 nm to 595 nm. OD can be measured at a maximum wavelength of 595 nm. The process includes the following steps which includes for protein determination, the enzymes were dissolved in 0.1 M phosphate buffer (pH 7.5). 11 test tubes were taken and different volumes of BSA (µl) from 50 µl to 500 µl were added in test tubes and distilled water (µl) was also added to make up the volume to 500µl. Bradford's reagent was then added in each test tubes. Eight more test tubes were taken and in each tube 100µl of protein sample, 400µl of distilled water and 2.5ml Bradford's reagent was added. Incubation for all the tubes was done at room temperature in dark for 10 minutes. OD was then taken at 595nm. A standard curve was then plotted with the standard protein concentrations on the X-axis and absorbance (OD) at 595nm on the Y-axis. The concentrations of the unknown samples were then calculated from the graph.

Results and discussion
1. Results for the alcohol dehydrogenase enzyme assay
Absorbance was taken in kinetic/time at 340nm. The total time taken for measurement of absorbance is 4 minutes (Table 1).

Table 1: Observation table for alcohol dehydrogenase enzyme assay.

Samples	Absorbance at 340 nm at different time intervals 'T'					
	T_0	T_1	T_2	T_3	T_4	Average absorbance (O.D.)
A	0.046	0.000	0.057	0.068	0.126	0.059
B	0.388	0.434	0.339	0.357	0.367	0.337
C	0.243	0.291	0.299	0.259	0.212	0.261
D	0.000	0.027	0.105	0.052	0.046	0.046
E	0.129	0.229	0.043	0.186	0.165	0.150
F	0.000	0.000	0.000	0.032	0.014	0.010

The calculated alcohol dehydrogenase activity and specific activity was shown in Table 2.

Table 2: Calculation table for alcohol dehydrogenase activity.

Samples	Average absorbance	Activity (IU/L)	Specific activity (IU/mg protein)
A	0.059	7588.424	151.768
B	0.337	4334.405	63.741
C	0.261	3356.913	167.846
D	0.046	591.640	7.215
E	0.150	1929.260	53.590
F	0.010	128.617	3.215

From the table above, it was found that the alcohol dehydrogenase activity is more in Sample B (*Escherichia coli*) and less in Sample F (*Pseudomonas cepacia*) whereas the specific activity of the enzyme isolated was found to be higher in Sample C (*Citrobacter species*) and the least in Sample F (*Pseudomonas cepacia*).

2. Estimation of proteins by Bradford method
The stock concentration i.e., Bovine Serum Albumin used was 100 µg/ml and the absorbance (OD) was taken at 595nm for both the known and unknown samples in protein estimation (Table 3 and Table 4).

Table 3: Observation table for estimation of proteins by Bradford method.

Test tubes	Vol of standard BSA (µl)	Vol of D/W (µl)	Conc. of BSA (µg/ml)	Vol. of Bradford's reagent (ml)		OD at 595 nm
Blank	0	500	0	2.5ml	Incubate at room temperature for 10 mins in dark	0.000
1	50	450	5			0.023
2	100	400	10			0.035
3	150	350	15			0.042
4	200	300	20			0.042
5	250	250	25			0.052
6	300	200	30			0.057
7	350	150	35			0.053
8	400	100	40			0.068
9	450	50	45			0.081
10	500	0	50			0.104

Table 4: Observation table for unknown samples of protein estimation.

Test tubes	Vol of Protein sample (µl)	Vol of D/W (µl)	Conc. of BSA (µg/ml)	Vol. of Bradford's reagent (ml)		OD at 595 nm
Sample A	100	400	?	2.5 ml	Incubate at room temperature in the dark for 10 minutes	0.052
Sample B	100	400	?			0.072
Sample C	100	400	?			0.021
Sample D	100	400	?			0.086
Sample E	100	400	?			0.038
Sample F	100	400	?			0.042

With respect to the results obtained above i.e., from Table 3 and Table 4, a standard curve (Figure 1) was plotted with standard protein concentration on the X-axis and absorbance (OD) at 595nm on the Y-axis.

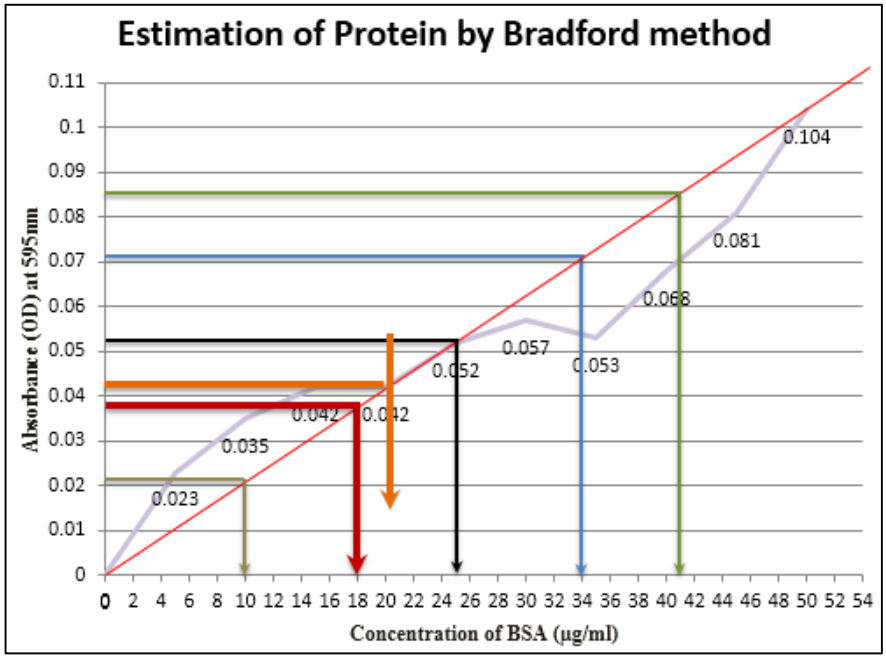

Fig 1: Graphical representation of protein estimation by Bradford method

Indicates unknown concentration of sample A.
Indicates unknown concentration of sample B.
Indicates unknown concentration of sample C.
Indicates unknown concentration of sample D
Indicates unknown concentration of sample E.
Indicates unknown concentration of sample F.

From the graph plotted above, the concentration (µg/ml) of the unknown samples used was found out to be as follows:

a) Sample A: 25×2= 50 µg/ml
b) Sample B: 34×2= 68 µg/ml
c) Sample C: 10×2= 20 µg/ml
d) Sample D: 41×2= 82 µg/ml
e) Sample E: 18×2= 36 µg/ml
f) Sample F: 20×2= 40 µg/ml

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