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Estimation of Kinetic Parameters during Enzymatic Hydrolysis in Immobilized Mode

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Abstract : Whey is the major pollutant of the dairy industry which creates significant disposal problem because of its high BOD value. One option is to use whey permeate as an inexpensive feedstock for ethanol production. But as *Saccharomyces cerevisiae*, the most popular microorganism used in fermentation cannot directly convert lactose to ethanol and with the helpother yeast strains it is not economically feasible. Enzymatic hydrolysis of lactose is a good solution because the hydrolyzed products can be consumed by lactose maldigesters. The enzyme β-galactosidase hydrolyzes lactose into glucose and galactose. Despite the high cost of enzyme attachment, immobilized β-galactosidase systems remain more economically feasible than free enzyme systems, as these processes may be performed continuously and offer the possibility of reutilizing the enzyme. In this context, the present work has been undertaken with an objective to suitably hydrolyze whey lactose with the help of enzyme entrapped calcium alginate beads. The lactose concentration was estimated using DNSA (Dinitro salicylic acid) method. The concentration of the glucose was measured using GOD-POD test. On the basis of the data analyzed, Hanes-Woolf plot was used to determine the kinetic parameters. The maximum rate of hydrolysis was found to be 0.556 mM/lit.min.
Keywords : Lactose, whey, enzymatic hydrolysis, Immobilized mode, kinetic study, Hanes-Woolf plot.

Introduction

Whey represents about 85–95% of the milk volume and retains 55% of milk nutrients. Among the most abundant of these nutrients are lactose (4–5% w/v), soluble proteins (0.6–0.8% w/v), lipids (0.4–0.5%w/v) and mineral salts (8–10% of dried extract). Whey also contains appreciable quantities of other components, such as lactic (0.05% w/v) and citric acids, non-protein nitrogen compounds (urea and uric acid) and B group vitamins[1].

The disposal of whey remains a significant problem for the dairy industry. As whey contains 5 to 6% dissolved solids including 3 to 5% lactose, the biological oxygen demand (BOD:38,000 to 40,000 ppm) is high. Generally, whey must be treated prior to discharge into the environment[2]. Large volumes are produced—in Canada, 3,000,000 tonne of lactoserum are produced annually[3].

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The disaccharide lactose can be recovered from whey permeate, but world market demand is exceeded by lactose availability. One option that is carried out industrially by several cheese producers is to use whey permeate as an inexpensive feedstock for ethanol production[2]. With the appropriate yeast strains (e.g., *Kluyveromyces marxianus*) lactose may be fermented directly into ethanol. Compared with the production of ethanol from glucose by traditional *Saccharomyces cerevisiae* strains, organisms fermenting lactose exhibit inferior production rates and yields[4]. Production of ethanol from lactose would be a more attractive process if the 90 to 93% product yields associated with *Saccharomyces cerevisiae* could be realized; however, *S. cerevisiae* is unable to ferment lactose.

Hence it is required to hydrolyze lactose into its 2 monomeric sugars, glucose and galactose, which are readily and efficiently fermented. There are 2 means by which this can be accomplished, acid-catalyzed hydrolysis and enzymatic hydrolysis. Acid hydrolysis at high temperatures generates compounds that are inhibitory to yeasts[5].

Enzymatic hydrolysis of lactose is an important biotechnological process because the hydrolyzed products can be consumed by lactose maldigesters[5]. The enzyme β- galactosidase hydrolyzes lactose into glucose and galactose[6]. The soluble enzyme is normally used for batch processes while the immobilized form lends itself to continuous operation[7]. Despite the high cost of enzyme attachment, immobilized enzyme technology is beneficial due to the ability to confine an enzyme in a well-defined, predetermined space that provides opportunities for applications unique to immobilized enzymes. The benefits are as follows[9]:

1. Retaining inside a reactor to avoid the loss and consequent replacement as they are costly and expensive.
2. In retaining the activity for longer periods of time rather than the enzymes in solution
3. Increase catalytic efficiency for the multi-step conversion
4. To get the large throughput of the substrate.

Basically there are two methods used for the immobilization of the enzymes:Chemical methods and Physical methods. This project deals with the physical method of immobilization only.The present work has been undertaken with an objective to suitably hydrolyze whey lactose with the help of immobilized enzyme technology where the enzyme binds with substrate via physical methods of immobilization. The reaction will be modeled using Hanes-Woolf equation and model parameters will be determined and reported.

Methodology

Estimation of Sugar by Dinitrosalicylic Acid (DNSA) Method

In the laboratory, casein whey was prepared following iso-electric precipitation of casein protein of milk. The casein whey thus obtained has a pH of 5.78 and it was straw colored(Fig.1).The lactose content of the whey thus formed was measured using DNSA (Dinitro salicylic acid) method[10]by measuring the absorbance of the solution at 540nm with the help of the calibration curve. Since lactose is a reducing sugar, it responds to the test wherein 3,5-dinitrosalicylic acid (DNS) is reduced to 3-amino,5-nitrosalicylic acid under alkaline conditions and colour of the solution changes from yellow to orange-red (Fig.2,3). The lactose standard curve (Fig.6) was used to determine the concentration of lactose in whey.

Figure1: Whey (pH 5.78)

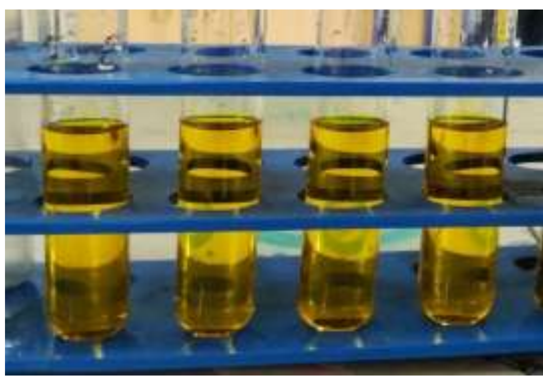


Fig.2: Initial colour of the solutions for DNSA test

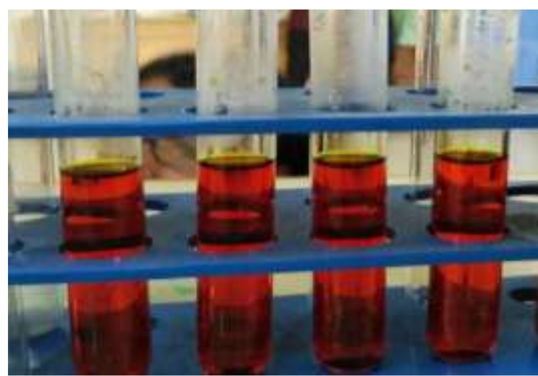


Figure3: Final colour of the solutions for DNSA test

Substrate Preparation

Lactose was used as substrate for hydrolysis study. For preparation of 0.1 M Phosphate buffer, chemicals used were K_2HPO_4 (obtained from Central Drug House, New Delhi) and K_2HPO_4 (obtained from SRL, Mumbai) and this buffer was used for preparation of lactose and enzyme solutions; Sodium hydroxide (NaOH) and Phosphoric acid (H_3PO_4) used for adjustment of pH were obtained from MERCK, Mumbai; β-galactosidase enzyme (Biolacta FN5, EC 3.2.1.23) isolated from *aspergillus oryzae* was obtained from Sigma-Aldrich. GOD-PERID kit (Span Diagnostics Ltd., Surat, India) is a reagent set used for determination of glucose, based on enzymatic method using Glucose oxidase and Peroxidase enzymes. Sodium alginate, obtained from SRL, Mumbai and calcium chloride dehydrate, obtained from MERCK, Mumbai were used for preparation of alginate beads.

Preparation of enzyme entrapped calcium alginate beads

Lactose hydrolysis was performed in a batch mode with 5 sample lactose solution of concentration 5,10,15,20&25 g/l respectively .ß-galactosidase enzyme in immobilized form with concentration (1mg/mL) was used as the catalyst for the hydrolysis reaction. The enzyme was mixed with aqueous solution of sodium alginate (2% w/v). The enzyme/alginate mixture was drawn into a plastic syringe and then added drop wise into 100ml aqueous solution of calcium chloride. The concentration of $CaCl_2$ in the solution was 3% w/v. The enzyme entrapped beads were left in the calcium chloride solution to harden for about 30 minutes. The alginate will be ionically cross-linked by the calcium ions. The calcium alginate beads (Figure 4) were separated from the solution using a strainer and washed thoroughly with distilled water three times to remove excess $CaCl_2$. The beads were dried using tissue paper and then exposed to open air for about 1 hour for drying before use.

Fig.4Enzyme entrapped calcium alginate beads

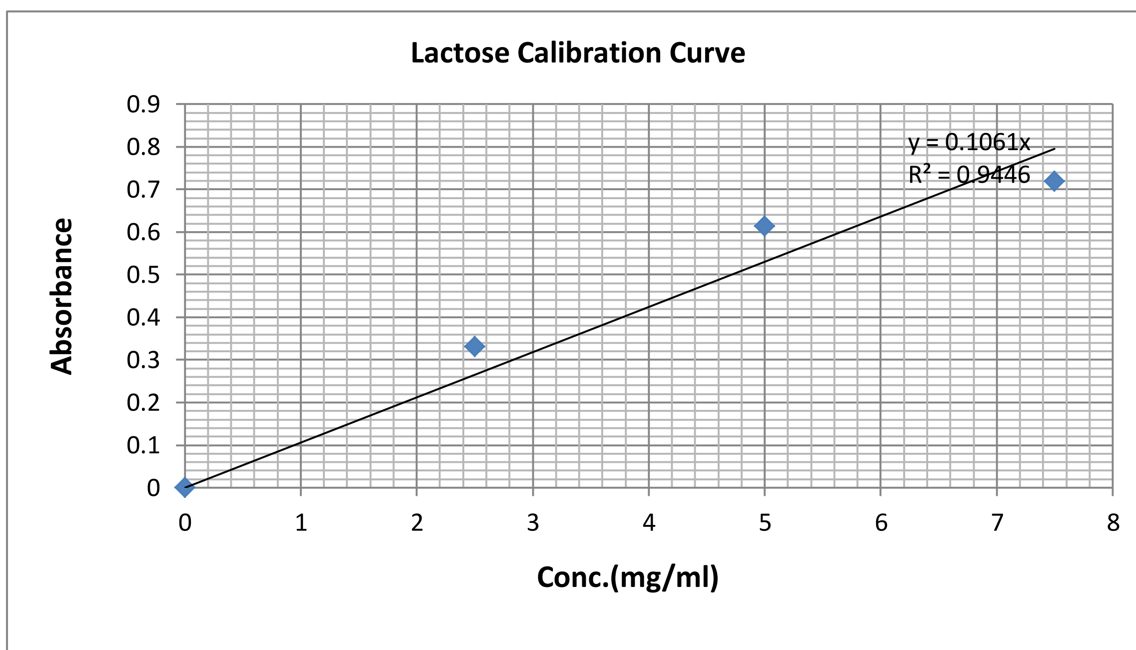


Fig.5: Colour of the solutions for GOD-POD Test

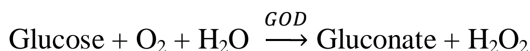
The Enzymatic Hydrolysis Of Lactose in Immobilized Mode

The calcium alginate beads with the entrapped enzyme were then placed in the 5 lactose solutions taken in 5 separate beakers and then incubated at 30ºC and pH6.75. Samples were collected after incubation period of 15 minutes in sample vials and then introduced into the boiling water for 5 minutes to stop the hydrolysis reaction by deactivating the catalyst for the reaction. The samples collected contained the hydrolyzed products: glucose and galactose as well as unconverted lactose. The amount of glucose formed was measured with the help of GOD-POD method[11] and the UV-Vis spectrophotometer.

Glucose Estimation By GOD-POD method

The Glucose (GOD-POD) kit is based on Trinder's method. Glucose is oxidized by Glucose oxidase and produces Gluconate and hydrogen peroxide. The hydrogen peroxide is oxidatively coupled with

4aminoantipyrine and phenol. The intensity of the coloured complex (quinoneimine) is proportional to the glucose concentration in the sample and can be measured photometrically at 540nm.



$$\text{Glucose} + O_2 + H_2O \xrightarrow{GOD} \text{Gluconate} + H_2O_2$$

$$2H_2O_2 + \text{Phenol} + \text{4- Aminoantipyrine} \xrightarrow{POD} \text{Red Quinonimine} + 4H_2O$$

Results and Discussion

Using the calibration curve (Fig.6), the lactose content of raw whey was measured .The lactose content in whey was found to be 2.6 % (2.6 gms per 100 ml). Then enzymatic hydrolysis was led to occur with the enzyme entrapped beads. Hanes-Woolf method[12] was used to determine the kinetic parameter of the hydrolysis reaction. In biochemistry, a Hanes–Woolf plot is a graphical representation of enzyme kinetics in which the ratio of the initial substrate concentration [S] to the reaction velocity v is plotted against [S]. It is based on the rearrangement of the Michaelis–Menten equation. The equation for Hanes-woolf plot is shown below:

$$\frac{[s]}{R} = \frac{Km}{Vmax} + \frac{[S]}{Vmax}$$

where K_m is the Michaelis–Menten constant, $V_{\max}$ is the maximum reaction velocity, [s] is substrate concentration and R is reaction velocity.As is clear from the equation, perfect data will yield a straight line of slope $1/V_{\max}$, a y-intercept of $K_m/V_{\max}$ and an x-intercept of $-K_m$. From Fig.7the values of V maxand $K_m$have been estimated and those are found to be 0.556mM/l.min and 52.91 mM/lit respectively.

Fig.6: Lactose Calibration Curve

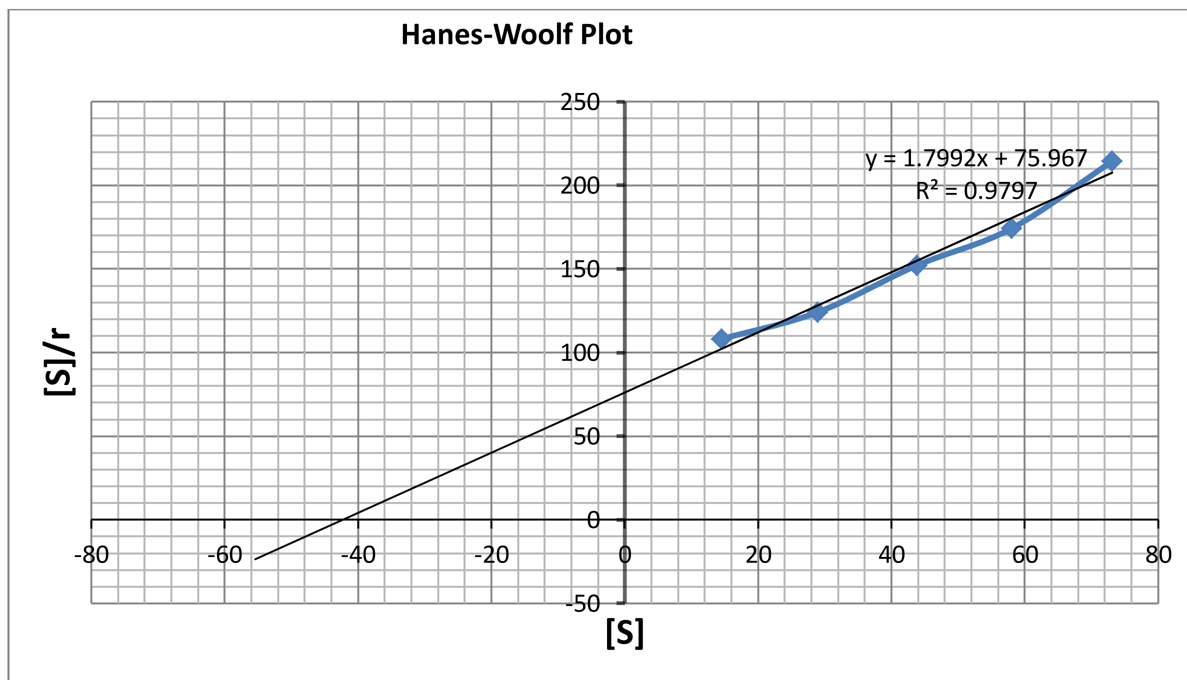


Fig.7:Hanes-Woolf plot

The catalytic efficiency of the reaction was calculated by the following Equation: $k_{cat} = \frac{V_{\max}}{[E_0]}$

Where $V_{\max}$ is maximum reaction velocity, $[E_0]$ is total enzyme concentration and k_{cat} is catalytic efficiency which is found to be 62.75 min^{-1}.

The same beads were used for three times for enzymatic hydrolysis with lactose concentration 10 & 20 g/l and ultimately the rate of reaction became 0.0616 and 0.0655 mM/L.min respectively.

Conclusion

In this study, the lactose content of casein whey, a dairy effluent has been estimated to be 2.6%. The kinetic study of enzymatic hydrolysis reaction using Hanes-Woolf method reveals that the parameters $V_{max}$and K_m have been found out to be 0.556mM/lit.min and 52.91 mM/lit respectively.$K_m$is a reflection of the affinity of enzyme for its substrate and is characteristic for aparticular enzyme-substrate system. The smaller the value of K_m, the more stronglythe enzyme binds the substrate. Though the conversion was less than the free mode[13] but immobilized technology is advantageous because it can be reused again, reduces wastage of enzymes as they are costly and expensive and also retain the activity for longer periods of time than the enzymes in solution.

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