# STUDY ON IMMOBILIZATION OF ENZYME ALPHA-AMYLASE IN TRADITIONAL ALCOHOL WINE FERMENTATION

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## Abstract

Enzyme immobilization offers an innovative approach for reuse, preservation, and optimization of production efficiency and costs in the food and biofuel industries. In this study, amylase enzymes immobilized in Ca-alginate membranes were utilized in the fermentation of traditional sticky rice wine. The morphology and activity of immobilized amylase beads were maintained effectively at a 2% concentration of both carrier material and enzyme solution. After seven days of fermentation, fermentation efficiency reached an ethanol concentration of 55% v/v. The activity of immobilized amylase retained 60% of its activity after four consecutive fermentation cycles. These results suggest that immobilized amylase beads have promising applications in sticky rice wine production, replacing free amylase, which is difficult to recover and reuse.

**Keywords:** α-amylase, enzyme immobilized, ethanol fermentation, Na-alginate

## 1. Introduction

Amylase, a glycoside hydrolase enzyme, plays a crucial role in the initial stages of the fermentation process (Sundaram et al., 2014). In this stages, carbohydrates from the substrate (vegetable starch) are converted into smaller sugar molecules such as glucose and maltose, which serve as a carbon nutrient source for microorganisms (Souza, 2010). In traditional alcohol fermentation, this enzyme can be supplied indirectly by fungal species present in the environment or by using commercial sources of amylase (Souza, 2010; Mobini-Dehkordi et al., 2012). However, the enzyme's activity is susceptible to changes due to environmental conditions, including preservation factors (e.g., pH, temperature), as well as storage and fermentation environments (Bayramoğlu et al., 2004; Eed, 2012; Homaei et al., 2013). Furthermore, the issue of enzyme reuse needs to be evaluated for optimal production cost purposes, as the nature of the enzyme allows it to reactivate after catalyzing a reaction. Free enzymes are often dissolved in the fermentation environment and are challenging to recover and reuse, whereas immobilized enzymes can be easily reused and offer a more viable solution (Homaei et al., 2013; Sheldon et al., 2021). Various immobilization methods have demonstrated effective applications for living cells and proteins such as enzymes (Kim et al., 2014; Sheldon et al., 2021). Among these, biopolymers are considered a good material for enzyme encapsulation due to their

safety, efficiency, ease of use, and low cost (Homaei et al., 2013). This study will evaluate industrial amylase enzymes for efficiency in wine fermentation when immobilized in Na-alginate polymer membranes.

## 2. Materials and methods

### *2.1 Materials*

The study utilized α-amylase enzyme (from ICFOOD Vietnam Company) and Na-alginate as the carrier material.

### *2.2 Methods*

#### *Enzyme immobilized in Ca-alginate*

The amylase enzyme was immobilized in Alginate using the method described by Kumar et al. (2006), with minor modifications. Na-alginate solutions were prepared with concentrations of 1.5%, 2.0%, and 2.5% (w/v), and amylase solutions of 1%, 1.5%, 2%, and 2.5% (w/v) in 0.01M acetate buffer. The Na-Alginate solutions were thoroughly mixed with the amylase solutions at 4:1 (v/v). Then, the Alginate-amylase mixture was dropped into a 0.2M $CaCl_2$ solution to form immobilized enzyme beads. The immobilized enzyme beads were immersed in a 0.2M $CaCl_2$ solution for 30 minutes. After 30 minutes, the immobilized enzyme beads were filtered through filter paper, washed twice with distilled water, and stored in capped glass vials immersed in deionized water at 0-4°C.

#### *Method for determining enzyme activity*

The activity of α-amylase enzyme was measured using the spectrophotometric method with DNS (Dinitro salicylic acid) reagent (Miller et al., 1959) and the maltose standard curve $y = 5x + 0.137$ ($R2 = 0.993$) at a wavelength of 540nm. The enzyme activity in the experimental samples was determined according to the following procedure: 0.5ml of 1% starch solution (supplemented with 10% acetate buffer) and 0.5g immobilized enzyme beads were mixed in a test tube, followed by incubation at 40°C for 15 minutes. Then, 1ml of DNS was added, and the mixture was incubated at 100°C for 5 minutes and cooled to room temperature. Subsequently, 8ml of distilled water was added, and the absorbance spectrum was measured at a wavelength of 540nm. The control sample was conducted similarly to the test sample, only replacing the immobilized enzyme beads with sterile water.

#### *Methods of hydrolysis, fermentation, and determination of reducing sugar concentration*

The sticky rice was hydrolyzed using the boiling method. A mixture of 100g of sticky rice and 20g of immobilized enzyme was placed into a 250ml glass flask, followed by the addition of 400ml of distilled water. The reaction mixture was incubated at 40°C for 30 minutes. After hydrolysis, the solution was filtered through filter paper, and the reduced sugar content was determined using the DNS method. For the control sample, 4ml of a 2% amylase enzyme solution was used instead of the immobilized enzyme beads.

The amount of reducing sugar was calculated using the formula: $X = a.n.V$, where:

X: The amount of sugar in the solution to be determined (g)
a: the amount of reducing sugar in the sample (g)
n: dilution factor of the solution
V: volume of the measured solution (ml)

Fermentation experiments were performed in 500ml conical flasks containing 100g of hydrolyzed sticky rice, 20g of immobilized enzyme beads, and 1g of yeast. The pH was adjusted to 4.5 using citric acid, and fermentation was carried out at 28°C to 30°C for seven days. The fermented solution was distilled to recover and evaluate the quality of sticky rice wine. The control sample followed the same procedure, with 4ml of a 2% amylase enzyme solution (diluted in acetate buffer) replacing the immobilized enzyme beads.

***Statistical analysis method***

The experimental data were statistically analyzed using one-way ANOVA with the Tukey test in GraphPad Prism version 9.00 software.

## 3. Results and discussion

### *3.1 Effect of enzyme concentration and carrier concentration on immobilized enzyme activity*

The enzyme concentration is a critical parameter in the polymer membrane method immobilization. The results of post-immobilization activity demonstrate significant differences among the various tested enzyme concentrations. Specifically, immobilized enzymes at a concentration of 2% exhibit the highest activity, reaching 346.6 IU, while immobilized beads at enzyme concentrations of 1%, 1.5%, and 2.5% demonstrate activities of 140.8 IU, 225.8 IU, and 168.4 IU, respectively. At 2% enzyme concentration, the enzyme activity is 2.46 times higher than 1% and 1.53 times higher than 1.5%, with statistical significance at $p < 0.001$ (*Figure 1*). This substantial discrepancy underscores the impact of enzyme concentration in immobilization, where 2% of enzymes exhibit effective beads without excessive reduction compared to free enzymes.

Similarly, immobilized enzyme beads at a 2% carrier material concentration demonstrate sustained enzyme activity. At this concentration, the activity of immobilized beads reaches 439.1 IU, which is 1.14 and 1.24 times higher than at other concentrations ($p < 0.001$) (*Figure 1*).

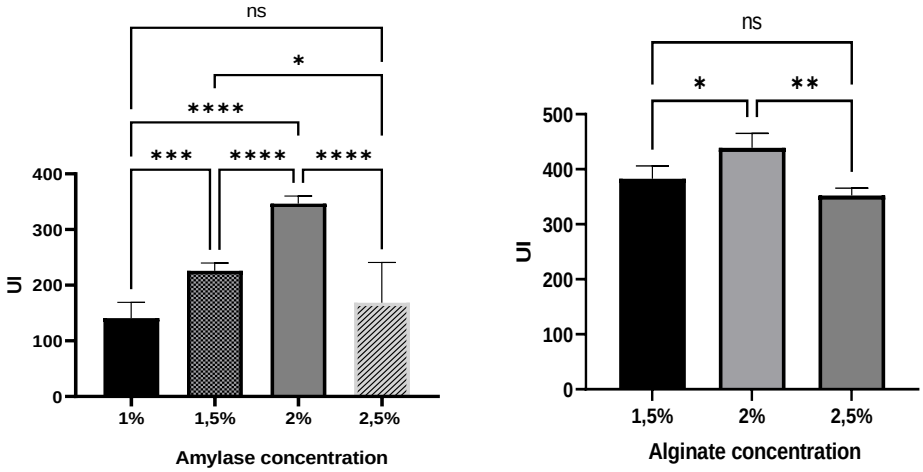


***Figure 1***. The effect of carrier and enzyme concentration on immobilized enzyme activity ($p<0.001$).

These results align with other studies, highlighting that the two most critical factors in enzyme immobilization are enzyme concentration and carrier material concentration,

both of which significantly influence the efficiency of the immobilization process and the activity of immobilized amylase beads (Talekar et al., 2012). In this study, the optimal concentrations for enzyme and carrier were determined to be 2%. Lower concentrations of the enzyme and carrier lead to decreased enzyme activity. This reduction in activity is attributed to the instability of the membrane structure and the large pore sizes present in the immobilized granules, which result in losses during the immobilization and storage processes (Zaborsky, 1973). Conversely, excessively high concentrations of enzymes and carriers lead to decreased activity as the dense membrane structure impairs substrate access to the active site (Talekar et al., 2012).

***Effect of immobilized enzyme on rice starch hydrolysis efficiency***

The immobilized amylase beads hydrolyzed starch to produce an average of 15.1mg/ml of maltose, while the free amylase solution hydrolyzed to yield 15.07mg/ml of maltose (*Figure 2*). The hydrolysis process lasted for 30 minutes at 40°C. The maximum maltose yield reached 15.14mg/ml in hydrolysis reactions involving immobilized enzyme beads and free enzymes. These results indicate that immobilized enzymes at 2% carrier and enzyme concentration perform comparably to free enzymes regarding starch hydrolysis efficiency. Immobilized enzymes have been shown to maintain higher activity and stability under conditions that typically reduce enzyme function, such as variations in temperature and pH (Pandya et al., 2005; Karam et al., 2017). Therefore, the immobilized amylase beads retain activity equivalent to free enzymes and present a viable, cost-effective alternative for reducing production costs.

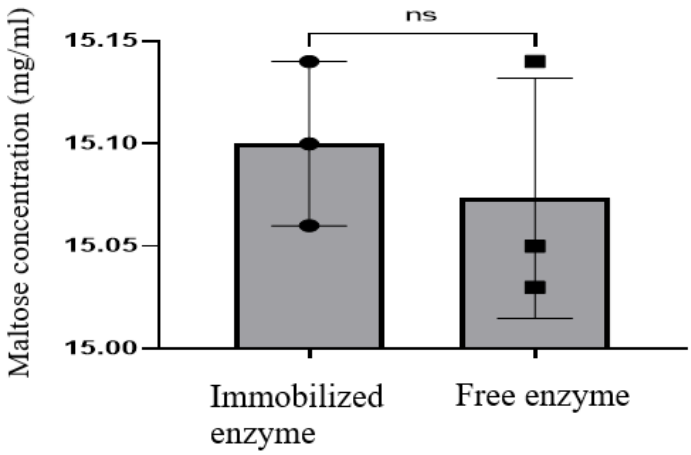


***Figure 2***. The concentration of reducing sugars produced after the hydrolysis of glutinous rice starch (ns: not significant).

***Stability of immobilized enzyme during hydrolysis***

The hydrolysis process was repeated multiple times using immobilized amylase beads. Initially, the maximum yield reached 15.1mg/ml in the first hydrolysis, followed by a gradual decrease to 12.04mg/ml, 10.77mg/ml, and 8.92mg/ml in subsequent hydrolysis rounds (*Figure 3*). The activity of the immobilized amylase beads consistently declined by 10-20% with each successive hydrolysis cycle. The lowest maltose yield was observed in the second hydrolysis, with a reduction of up to 21% compared to the first hydrolysis.

The stability of the immobilized amylase beads in the sticky rice starch hydrolysis reaction across multiple cycles was moderate. This decrease in activity can be attributed

to factors such as the granules' pore size, the polymer network's structure encapsulating the enzyme molecules, and external conditions like pH and temperature (Ertan et al., 2007). During the recovery of enzyme beads, some enzymes dissolved in the hydrolysis solution, primarily those molecules bound only to the outer membrane of the beads. The study results demonstrated that over 60% of the activity of the immobilized amylase beads was maintained after four fermentation cycles.

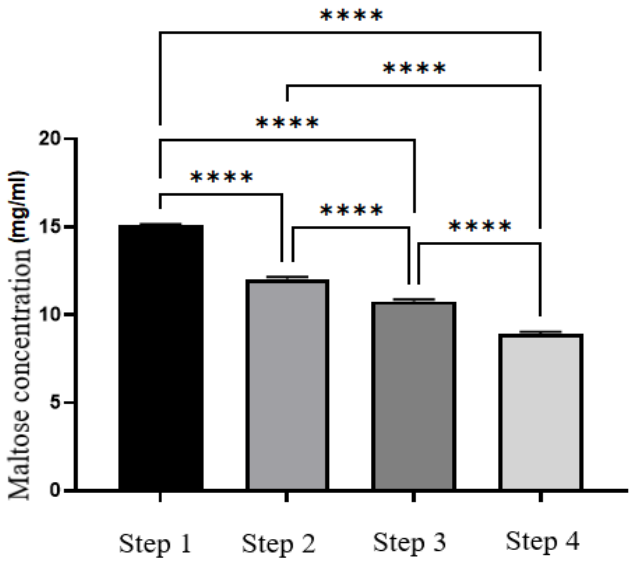


***Figure 3***. Stability of immobilized enzyme beads in rice starch hydrolysis ($p < 0.001$).

***Application of immobilized enzyme bead in sticky rice wine fermentation***

Starch hydrolysis containing reducing sugars was supplemented with yeast to initiate glutinous rice alcohol fermentation. The fermentation process exhibited similar outcomes to alcohol fermentation using yeast supplemented with immobilized and free amylase. The fermented glutinous rice alcohol reached a concentration of 55% v/v, with a delicate aroma, no sediment, and no off-flavors (*Figure 4*). These findings suggest that immobilized amylase beads perform efficiently, providing adequate sugar levels for yeast fermentation into ethanol, comparable to the effectiveness of free amylase enzymes.

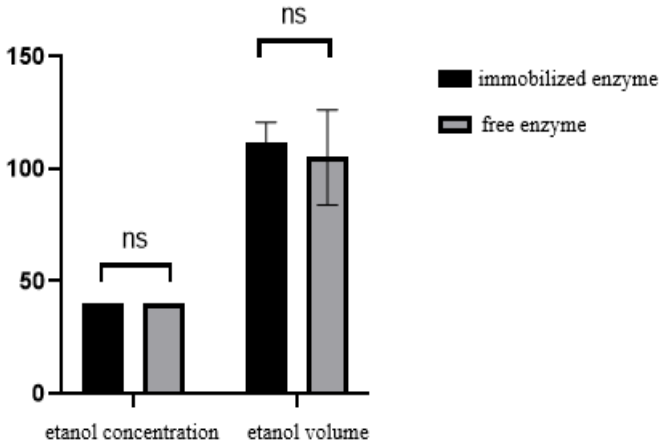


***Figure 4***. Results of immobilized enzyme application in sticky rice wine fermentation.

## 4. Conclusion

The study determined that an enzyme and Alginate carrier concentration of 2% in the immobilized form is sufficient for traditional sticky rice alcohol fermentation. The immobilized enzyme beads exhibit mechanical stability and maintain consistent fermentation activity across four consecutive fermentation cycles, comparable to free enzymes. Immobilizing enzymes in alcohol fermentation presents a promising solution for optimizing the efficiency and utilization of fermentation resources.

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