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Production and Kinetics of Isoamyl Acetate from Acetic Anhydride using Candida Antarctica Lipase B in a Solvent-Free System

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Isoamyl acetate is the most essential esters in food industry. Studies on isoamyl acetate production were expanded since 1976 through having 1174 patents that contains isoamyl acetate on that year. Most researches have used hexane or heptanes as solvent which are not preferable due to their toxic properties. Therefore, the risk and unfavorable conditions were removed by introducing solvent-free system. In this study, isoamyl acetate was produced using *Candida Antarctica* Lipase-B (CALB) by esterification reaction between acetic anhydride and isoamyl alcohol in a solvent-free system. The parameters studied were acid-alcohol molar ratio (0.1 – 2), reaction time (2-6 h), temperature (30-50 °C) and mass of enzyme (4-12 wt%). The isoamyl acetate production was maximum (41.6-fold) at acid to alcohol molar ratio of 2, mass of enzyme of 5.78 wt%, at time of 3 h and 44 °C reaction temperature. An enzymatic kinetic model based on modified Michaelis-Menten equation was developed and fitted to the non-linear regression to the experimental data. The model developed was in agreementwith the experimental data with R^2 value equals to 0.999.

1. Introduction

Esters are one of the most important classes of organic compounds in industry. It can be extracted from natural sources (Hari Krishna et al., 2001) orsynthesized by reaction between alcohols and carboxylic acids (esterification),and interexchange of acyl groups(Llauro and Michel, 2006).Different physical properties of acid and alcohol will result in different usage of esters. For instance, esterification products of long-chain acids (12-20 carbon atoms) with long-chain alcohols are used as lubricant and plasticizer for high-precision machinery. Esters resulting from the reaction of long-chain acids with short-chain alcohols (3-8 carbon atoms) are used as additives in food, detergent, cosmetic and pharmaceutical product. While the products of short-chain (2-8 carbon atoms) acids and alcohols are important in flavor and fragrance (Zaidi et al., 1995). Among all of the most desired esters by industry, isoamyl acetate hasthe commercial importance, its strong banana flavor properties making annual demand of 74 tonnes in USA alone (Welsh et al., 1989) and the demand is getting high over the years.
Traditionally, isoamyl acetate was produced by extraction of banana flavor from natural sources(Prapulla et al., 1992). It often has difficulties with short of supply. Then researchers found an alternative way by using fermentation method, but the practice is either too scarce or expensive for commercial exploitation (Hari Krishna et al., 2001). Because of constrains faced by industry, researchers taking the alternative routes of isoamyl acetate production vianatural esterification processes. The routes are also facing difficulties such as longer time required to produce ester but with low yield. Then chemical synthesize by using strong acid and alcohol were introduced. However, those synthesized by chemical methods use polluting liquid acids as catalysts, require post treatment and also costly for industrial application (Hanen Ghamgui et al., 2006). Recently, a new method of esters production by the application of lipase has been found and it gave more economic benefits compared to other previous methods due to its mild operation conditions, degree of purity of the products and their acceptability in the food industry (Rocha et al., 1999).

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Many works were performed by commercial lipase such as *Candida Antarctica(Cvjetko et al., 2012), Rhizomucormeihei(Güvenç et al., 2002), Aspergillus*sp. *Rhizopusarrhizus(Marlot et al., 1985), MucorMeihei(Hari Krishna et al., 2000)*and *Thermomyceslanuginosus(Fernandez-Lafuente, 2010)* in organic solvents.Despite the best yields in organic solvent, the toxicity of the solvent remains a problem for many applications. In addition, some organic solvents are too expensive to allow profitable commercial scale-up (Yahya et al., 1998). Hence a solvent- free system isintroduced in this study, giving advantage by the absence of solvent removal at the downstream processing; fewer components would be present in the reaction mixture at the end of reaction. Solvent-free system would reduce the production cost and minimizing the environmental impact cause by the solvent toxicity.

Several mechanisms have been proposed for lipase-catalyzed reaction (Brockman, 1984). The majority of these mechanisms were developed for the case of soluble lipases acting on insoluble substrates and for single substrates reaction. The simplest kinetic model applied to describe lipase catalyzed reaction is based on Michaelis-Menten mechanism(Richardson and D.B, 1985). The kinetic equation can be represent as equation (1)

$$r = \frac{r_{max}[S]}{KM+[S]} \quad (1)$$

Where $r_{max}$is the rate observed when the lipase is saturated with substrates, *KM* is the Michaelis-Menten constant.

However, this equation only fit for single substrates reaction. Hence new kinetic equation will be carry out by using modified Michaelis-Menten equation.

2. Material and methods

2.1 Equipment

The experiments were carried out in a 100 ml stopped rubber shake flask, which was incubated in Incu-Shaker Mini (Benchmark Scientific, New Jersey) at 150 rpm. Temperature was set between 30 °C to 50 °C. Working pressure was at ambient pressure condition.

2.2 Chemicals

Isoamyl alcohol was supplied by Merck Co., while acetic anhydride (reagent grade, ≥98 %) was supplied by Sigma Aldrich. All substrates were used without any pre-treatment. Immobilized enzyme from *Candida Antarctica*, Novozyme 435 (specific activity ≥ 10,000 *U/g*, recombinant, expressed in *Aspergillus Niger)* supplied by Sigma Aldrich.

2.3 Esterification process

The reaction were carried out without any organic solvent in 100 ml stopped rubber shake flask with working volume of 15 ml. Acetic anhydride and isoamyl alcohol was added into the flask until 0.1 alcohol to acid ratio. Then 4 wt% of immobilized enzyme from Candida Antarctica was inserted into the mixture at 30 °C. The reaction mixture was then incubated in an Incu-shaker mini (Benchmark, New Jersey) at 150 rpm for 360 min. Samples were withdrawn periodically and analyzed using gas chromatograph until 6 h of reaction time. Experimental procedures were repeated for 8 % and 12 % weight of enzyme, at 40 °C and 50 °C temperature and acid over alcohol mole ratio of 1 and 2.

2.4 Analysis

Aliquots of the reaction mixture were withdrawn at different times. Samples were analyzed by gas chromatograph (7820A) supplied by Agilent Technologies, USA, equipped with a hydrogen flame ionization detector and a SGE BP21 (FFAP) column (60 m x 0.32 mm x 0.25 μm). Helium was used as carrier gas at flow rate of 5 mL/min. After injection of samples, the temperature of oven was kept at 100 °C and linearly increased to 140 °C. The rate of temperature increase was set at 70 °C/min, and was kept at 140 °C for the remaining time of analysis. Injector and detector temperatures were set at 200 °C and 250 °C, respectively. Quantification of data was done by calibration with standards samples. The retention times ofpeaks were as follows: isoamyl acetate, 2.26 min; isoamyl alcohol, 2.38 min; acetic anhydride, 2.48 min; and acetic acid, 3.21 min.

3. Results and Discussion

3.1 Effect of enzyme amount

The effect of enzyme concentration was studied at 4-12 %(w/w) for 0.1 acid-alcohol molar ratio at 30°C. The isoamyl acetate concentration against reaction time depicted in Figure 1 for two different amount of enzyme. The initial reaction rate increased with increasing amount of enzyme from 4 % to 12 %. Although

the equilibrium was reached using 12 % of enzyme at 1.5 h of reaction time, longer reaction time (3.3 h) required for reaction using 4 % of enzyme. This result is in accordance with the behaviour of enzyme which is reacting as a catalyst to lower the activation energy hence accelerates the chemical reaction. This behaviour has also been reported earlier(Romero et al., 2005). The maximum ester concentration reached was 1.09 mol/L at 1.6 h.

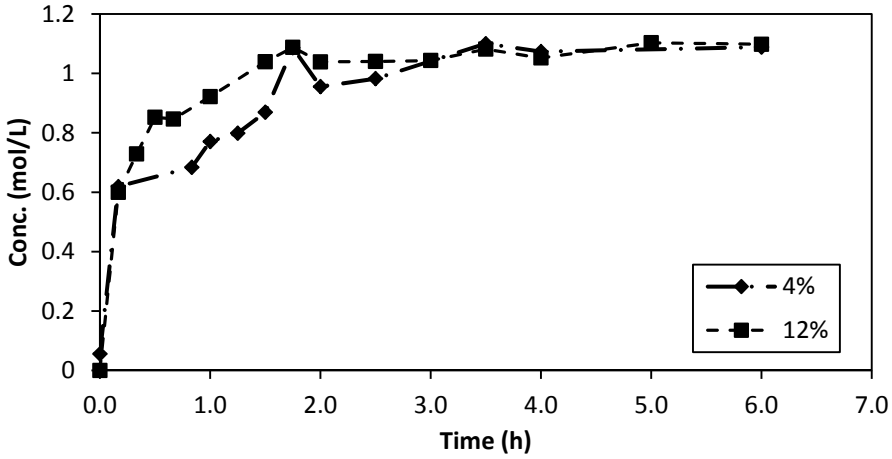

Figure 1: Effect of enzyme concentration on the ester production

3.2 Effect of temperature

Effect of temperature in the ester yield may affect the reaction efficiency. A temperature rise could have a positive effect on the kinetic constant as defined in transition state theory (Romero et al., 2005). On the other hand, high temperature reaction condition may disturb the enzyme tertiary structure, hence losing its catalytic activity. Figure 2 shows the result for Isoamyl acetate production which was carried out for an enzyme concentration of 8 %, acid-alcohol molar ratio of 1, over the temperature range between 30-50 °C. The initial reaction rate increased with increasing temperature from 30 to 50 °C. After 0.3 h of reaction time, production rate of ester for higher temperature, which are 40 and 50 °C, were decreased compared to reaction at 30 °C. Ester concentration after 5 h was 3.3 mol/L for 30 °C reaction temperature, whereas at temperature 40 and 50 °C, the ester concentration only reaches 2.90 mol/L. This shows that a temperature rise would have a positive effect on the kinetic thus give higher initial production of ester. Conversely, longer reaction time at higher temperatures may slightly disrupt the enzyme tertiary structure and losing its catalytic activity. Although thermal inactivation of the enzymes was not observed as the curve showed an increasing trend with increasing time, CALB still give low ester production compared to lower temperature reaction condition. At the given reaction conditions, the esterification reaction reached equilibrium after 5 h of reaction time.

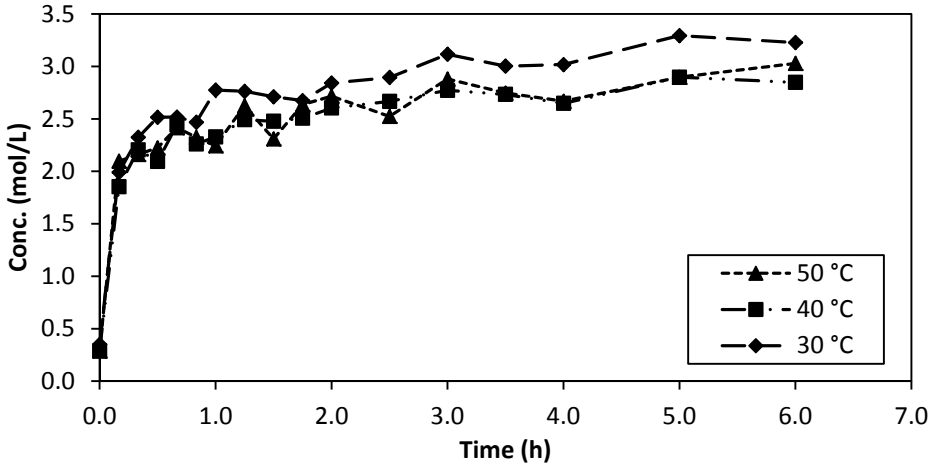

Figure 2: Effect of temperature on the ester production

3.3 Effect of acid and alcohol ratio

The effect of acid-alcohol molar ratio was studied by fixing enzyme concentration at 8 % and reaction temperature at 40 °C. Acid-alcohol molar ratios were varied at low alcohol concentration, equimolar, and excess in alcohol, which the ratios were 0.1, 1 and 2 respectively. The result for this experiment condition is shown in Figure 3. A maximum yield of isoamyl acetate (3.12 mol/L at 5 h) was achieved when alcohol in excess over the acid ratio. The plot also shows that excess in alcohol led to higher isoamyl acetate production yields due to the availability of excess nucleophile in the reaction.

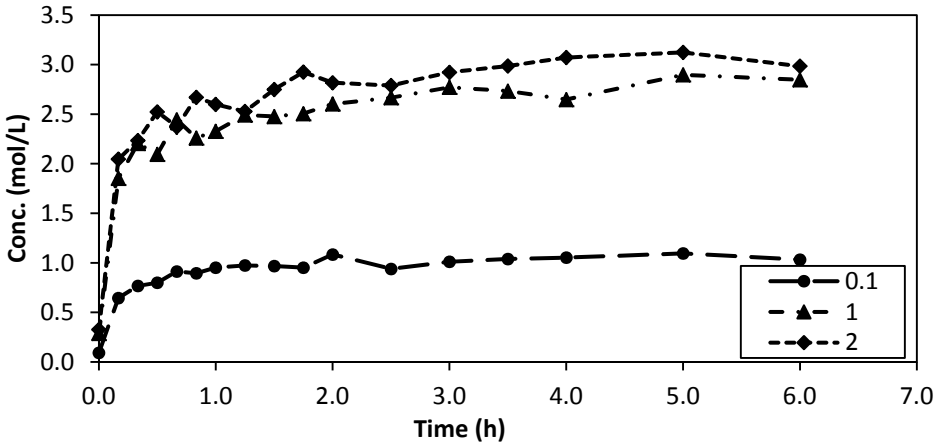

Figure 3: Effect of acid-alcohol ratio on the ester production

3.4 Kinetic synthesis

Generally, isoamyl acetate enzymatic esterification follows the general Michaelis-Menten equation as in equation (1). However, the above equation is only valid for reaction with a single substrate and a single product, Cleland (1963) has introduced the modified Michaelis-Menten equation for two substrates as given below;

$$r = \frac{r_{max}[A][B]}{KM_B[A]+KM_A[B]+[A][B]} \tag{2}$$

where*r* is the reaction rates of the process, $r_{max}$is the maximum initial reaction rates, [A] and [B] indicates the concentration of acetic acid and isoamyl alcohol respectively, and KM_A and KM_B are the kinetic constant for the process.

Initial reaction rates were determined from initial acetic anhydride and isoamyl alcohol concentration which was calculated using equation (3).

$$r = \frac{[A]_i+[B]_i}{t-t_0} \tag{3}$$

where $[A]_i$ and $[B]_i$ are the initial concentration of acetic anhydride and isoamyl alcohol respectively, and $t - t_0$ is time taken for the reaction to take place.

Table1: initial concentration of acid and alcohol

Initial acid, $[A]_0$(mol/L)	initial alcohol, $[B]_0$ mol/L)	reaction rate,r(mol/L.h)
0.85	8.46	3.103
4.90	4.90	3.267
6.71	3.37	3.360

In order to determine the other constant, equation (2) above need to be rearrange as;

$$\frac{1}{r} = \frac{KM_B[A]+KM_A[B]+[A][B]}{r_{max}[A][B]} \tag{4}$$

Rearrange equation (4) to $y = mx + c$ form,

$$\frac{1}{r} = \frac{KM_B[A]+KM_A[B]}{r_{max}[A][B]} + \frac{1}{r_{max}}$$

$$\frac{1}{r} = \frac{KM_B[A]+KM_A[B]}{r_{max}[B]}\left(\frac{1}{[A]}\right) + \frac{1}{r_{max}} \tag{5}$$

In order to obtain kinetic constants, r_{max} need to be found by substituting$\frac{1}{[A]} = 0$ in equation (6), which results in equation (6);

$$\frac{1}{r_{max}} = 5.39842 - 0.22299(T) - 0.20920(t) + 2.606x10^{-3}(T^2) + 0.071422(t^2) + 7.258x10^{-4}(T\,t) \quad (6)$$

Since there is two constant in the modified Michaelis-Menten equation, the constant value ($KM_A$and KM_B), were found by substituting equation (6) into equation (5) and it was solved using nonlinear equation solver in POLYMATH with initial value of both constant is equals to one.

Table 2: Kinetic constant

Variable	Initial guess	Value	95% confidence
KM_B	1	$1.29x10^{-08}$	$5.25x10^{-08}$
KM_A	1	$-1.02x10^{10}$	$4.18x10^{-11}$

From POLYMATH, the final result for KM_A and KM_Bwerefound to be equal to $-1.022x10^{-10}mol$ and $1.288x10^{-8}mol$ respectively as shown in Table 2. The fitting of the model was excellent with $R^2$and adjusted R^2 equals to 0.999. This shows that the theoretical model and the actual value are in a very good agreement. The plot of experimental value and calculated value of reaction rate were shown in Figure 4.

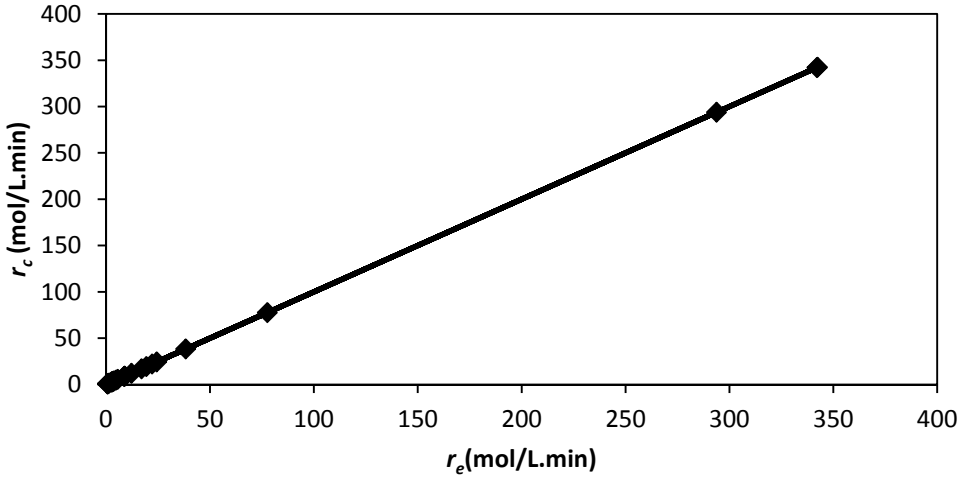

Figure 4: Plot of calculated and experimental value of reaction rate(r_e is experimental reaction rate and r_c is calculated reaction rate)

4. Conclusion

The production of isoamyl acetate, a banana flavour ester which widely used in food industry, was carried out in a solvent-free system. A commercial immobilized lipase CALB was used as biocatalyst. The tested parameters were reaction temperature (30-50 °C), acid-alcohol molar ratio (0.1-2) and mass of enzyme (4-12 %). An isoamyl acetate concentration was maximum (41.6-fold) at acid alcohol molar ratio of 2, mass of enzyme of 5.78 wt%, at time of 3 h and 44 °C reaction temperature. An enzymatic kinetic model based on modified Michaelis-Menten equation was developed and fitted to the non-linear regression to the experimental data. The model developed was matched with the experimental with R^2 value equals to 0.999.

Acknowledgement

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