OPTIMIZATION OF ENZYMATIC SYNTHESIS OF ETHYL VALERATE IN STIRRED TANK REACTOR

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UNIVERSITI SAINS ISLAM MALAYSIA
Nilai

March 2015
I hereby declare that the work and report in this thesis entitled "Optimization of Enzymatic Synthesis of Ethyl Valerate in Stirred Tank Reactor" is my own research except for quotations and summaries which have been duly acknowledged. I allow this work to be reviewed by other students and anyone else for study, teach and research purpose. I also agree to allow Universiti Sains Islam Malaysia to make copies of this work for the same purposes.

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APPROVAL

This thesis, entitled “Optimization of Enzymatic Synthesis of Ethyl Valerate in Stirred Tank Reactor” submitted to the Faculty of Science and Technology, USIM and was accepted as part of fulfilment of the requirement for the degree of Master of Science with Honours.

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ABSTRAK

KAJIAN MENGOPTIMUMKAN PENGHASILAN ETIL VALERATE MENGGUNAKAN KAEDAHL SINTESIS ENZIM DALAM TANGKI BERPUISING REAKTOR


Sintesis peringkat awal etil valerate dijalankan dalam tindakbalas skala kecil dengan jumlah isipadu 3,5 mL menggunakan botol kecil bertutup skru. Pengoptimuman kajian tindakbalas menggunakan kaedah konvensional iaitu memvariasikan satu parameter pada satu masa telah dijalankan. Hasil peratus pertukaran yang tinggi >70% telah dicapai pada optimum masa tindakbalas 45 minit, suhu tindakbalas 37°C dan jumlah enzim 10% v/v pada kelajuan putaran tetap 150 rpm.

Sintesis tindakbalas kemudian diteruskan dengan pengoptimuman oleh RSM berdasarkan 5 peringkat, tiga-pembolehubah rekaan pusat komposit berputar (CCRD) untuk menilai kesan interaktif bagi parameter-parameter yang penting di dalam pemprosesan pada skala yang lebih besar iaitu masa tindakbalas (20-60 min), suhu tindakbalas (40-60°C) dan amaun enzim (5-15% v/v). Secara amnya, dengan peningkatan secara serentak bagi amaun enzim, kelajuan putaran dan suhu tindakbalas akan meningkatkan hasil. Skala tindakbalas telah ditingkatkan kepada 40x dengan jumlah isipadu pada 340 mL. Peratusan penukaran yang tinggi iaitu 69.41% diperolehi di bawah keadaan optimum, di mana ia telah dibandingkan dengan baik dengan nilai anggaran maksimum iaitu 70.88%.

Kajian kinetik terhadap tindakbalas juga telah dijalankan dimana kadar tindakbalas pada kepekatian substrat berbeza telah dikaji. Hasil keputusan menunjukkan tindakbalas mengikut mekanisma Ping-pong Bi-bi; tindakbalas mematuhi kinetik Michaelis-menten untuk substrat yang dilihat daripada hubungan antara kadar tindakbalas permulaan dan kepekatan alkohol.

Bagi menilai reaktor sebagai alat mencampur, kesan oleh empat jenis pengaduk telah digunakan untuk mengkaji prestasi reaktor. Jenis pengaduk itu ialah Rushton turbine, anchor, propeller dan hydrofoil. Keputusan menunjukkan tidak banyak perbezaan antara pengaduk tersebut, antara 83% hingga 89% peratus penukaran etil valerate. Akhir sekali, spektroskopi inframerah (FTIR) digunakan untuk mengenalpasti kehadiran etil valerate selepas proses esterifikasi. Puncak dilihat sekitar 1200 cm⁻¹, menunjukkan C-O kumpulan ester. Manakala lajur pancaran sekitar 1680 cm⁻¹ merujuk kepada C=O kumpulan ester dan sekitar 3000 cm⁻¹ menunjukkan C-H alipatik.
Enzymatic synthesis of ethyl valerate, a flavour ester was successfully carried out by lipase-catalyzed esterification reaction between valeric acid and ethanol. In this study, commercial immobilized lipase from Candida antarctica (Novozym 435) was used as biocatalyst. The study was divided into four parts which are the optimization of reaction synthesis at small scales using conventional method, optimization using Response Surface Methodology (RSM) in Stirred Tank Reactor (STR), kinetic study and reactor study. Finally was verification of the product of the reaction.

Preliminary synthesis of ethyl valerate was carried out in a small scale reaction with a total volume of 3.5 mL using screw-capped vials. Optimization reaction study via conventional method of varying one parameter at-a-time approach was carried out. A high percentage conversion yield of >70% was achieved at optimum reaction time of 45 min, reaction temperature of 37°C and amount of enzyme of 10% w/w at fixed agitation speed of 150 rpm.

The reaction synthesis was further optimised by Response surface method (RSM) based on five-level, three-variable central composite rotatable design (CCRD) to evaluate the interactive effects of important parameters in larger scale processing which are reaction time (20-60 min), reaction temperature (40-60°C) and amount of enzyme (5-15% w/w). Generally, simultaneously increasing amount of enzyme, reaction temperature and amount of enzyme would improve the yields. The reaction was scaled-up to 40x with a total volume of 340 mL. A high percentage conversion of 69.41% was achieved under the optimum condition, which compared well with the maximum predicted value of 70.88%.

Kinetic study of the reaction was also carried out, whereby the rates of reactions at different concentrations of substrate were studied. Result shown that the reactions follows Ping-pong Bi-bi mechanism which means the reaction obeyed Michaelis-Menten kinetics for single substrate as observed from the relationship between initial rate and varying alcohol concentrations.

In order to evaluate reactor as a mixing device, the effect of four types of impeller mixing were used to study the reactor performance. The impellers are concave Rushton turbine, anchor, propeller and hydrofoil. The result shows there are no significant different of the conversion yield of ethyl valerate. Finally, FTIR was used to verify the presence of ethyl valerate after esterification reaction. The peaks observed around 1200 cm⁻¹ corresponding to C-O stretch of the ester group. While wavelength around 1680 cm⁻¹ corresponding to the C=O stretch of the ester group and around 3000 cm⁻¹ corresponding to aliphatic C-H stretch.
المحفوظ

تحسين من توليف الأنزمية من الألف كالبارات في مفاعل حزام أنثر

هذا التصنيف الأزمي من إثيل كالبارات، أجري استراح الكمية بنجاح على طريق تفاعل الأزمية ليباك المحفزة بين حمض الفالكيرك والإيثانول. في هذه الدراسة، تم استخدام بحث التجربة المقابل من المليمات من القطرة النباتية الجنوبية (Novozenyme 4350) بوصفها حائز بيولوجي. وقد تم تقسيم الدراسة إلى أربعة أجزاء ما هي الاستفادة المنطقية في الظليل من التوليف على مستويات صغيرة باستخدام الطريقة التلقائية الأتمية باستخدام الاستجابة المنطقية في قاعداً، دراسات الحركية والدراسة المفاعل، وأخيراً، تحليل وتصريف السطحية (STR) في مفاعلين آثار حزام (RSM) من منتج

ون Disability

وقد أجريت التوليف الأولي للإثيل كالبارات في ريد فعل على نطاق صغير مع إجمالى حجم 3.5 مل باستخدام قاعدة السحاب ونوج. وقد أجريت دراسة رد الفعل الأمثل عبر الطريقة التشريحيّة متفقة مع معايير واحدة على نهج واحد في مهلة. وقد حقق الأعلى العائد تحويل نسبة المنوية إلى 70٪ في وقت رد الفعل الأمثل من 45 دقيقة، ودرجات الحرارة رد فعل من 37 ⁰C وكمية الإنزيم 10٪ لث في سرعة الانفعالات التالية من 150 دورة في دقيقة.

كان رد فعل التوليف منصة عمل الأمثل طريقة سطح الاستجابة المركزية للتدوير تصميم مركب (CCRD). لقد قيم المتغيرات التفاعلية من المعلمات الحامة في مساحة نطاق أوعص ما

هي فترة رد الفعل (0-60 دقيقة) درجة الحرارة (لرد 40-40) °C. وكمية من إنزيم (5-15٪) لث. وتم عموماً في وقت واحد زيادة كمية من درجة الحرارة إنزيم التفاعل وكمية من إنزيم من شأنها أن يحسن الغلالة. ثم

تحجري رده الفعل يصل إلى 0X40 مع إجمالى حجم 340 مل. الوصول إلى نسبة منوية عالية من تحويل 69.41٪ تحت أفضل الظروف، أي قابلية المافاقة مع توقع أقصى قيمة من 70.88٪.

حمت معدلات الانتظار بعض التراكبات المختلفة مع إنزيمات للدروس في وقت لاحق في الدراسة الحركية للفعال بها Michaelis-Menten worksheets. وربما النتيجة أن ردد الفعل يلي بيغ بونغ الديدة ثنائيي بي المعنى، فإن رد الفعل يباع حركية أركنز واحداً كما لوحظ من العلاقة بين معدل الأولي وتركيزات التحول متفاوتة.

الفعال أداء تستعمل في دراسة المكرر خلط الترجمة أربعة أنواع من الاحتفاظ، وتأكيد كجهاز مفاعل من أجل تحكم 85.08٪ المروحة بعثة يعنى، رمسية 85.82٪. يعنى التوربينات راشوت مقررة نتيجة تظهر أن 84.22٪ عملية بعد إثيل أو الحاضر كالبارات مركب لتوصيف FTIR وأخيراً، تم استخدام يعنى الحوامة 52.12٪ حول الترجمة المصطلبات بينما استمر للقريب ستراع المتفاعلة تأتي 12000 cm⁻¹ 1/0 ± 0.02 سترادم القياسية لـ 16800 cm⁻¹ 3 وحول أستر للقريب ستراع CH دهني المواقي 1/0 ± 0.02
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**Publication**

Green Synthesis of Butyl Acetate, A Pineapple Flavour via Lipase-Catalyzed Reaction

S. Mat Radzi, W. A. F. Mustafa, S. S. Oloman, H. M. Noor

**Abstract**

Butyl acetate, a pineapple flavor, has been applied widely in food, beverage, cosmetic and pharmaceutical industries. In this study, Butyl acetate was successfully synthesized via green synthesis of enzymatic reaction route. Commercial immobilized lipase from Rhizomucor miehei (Lipozyme RMIM) was used as biocatalyst in the esterification reaction between acetic acid and butanol. Various reaction parameters such as reaction time, temperature and amount of enzyme were chosen to optimize the reaction synthesis in solvent-free system. The optimum conditions to produce butyl acetate were at reaction time 8 h, 35 °C and amount of enzyme 15% w/w of total substrate. And the yield obtained was 88%.

**Keywords**

Butyl acetate, immobilized enzyme, esterification, flavor ester, green synthesis.

1 Introduction

Green chemistry is the design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances. In the last decade, green chemistry has been recognized as a scientifically based new approach for environmental protection. From feedstock to solvents to synthesis and processing, green chemistry actively seeks ways to produce materials in a way that is more benign to human health and the environment. [1] Biocatalysts are one of the fundamental pillars and satisfies the green chemistry concept. Moreover biocatalysts are generally more efficient than chemical catalysts. For example, biocatalysts suffer from instability and substrate product inhibition. In addition, the process is also environmentally friendly compared to chemical production method.

Nowadays, there is an increasing demand for natural flavors. The worldwide market for natural ‘green notes’ is estimated to be 3-10 metric tons per year [2]. These flavors are used in foods, cosmetics and pharmaceutical industries. Traditionally, the flavors have been isolated from natural sources or produced by chemical synthesis. However, several problems were encountered for natural extract out of natural flavors due to the high production cost and limited supply to support worldwide demand. While flavor produced by chemical synthesis caused the bad side effect due to the use of hazardous chemicals. Moreover, other problems unaccounted are the separation process, high toxicity and instability. [3]

Flavor esters are short chain ester derived from a reaction between short chain acid and alcohol. The process involved is an esterification reaction whereby alcohol reacted with carboxylic acid and with the elimination of water. Ethyl, isobutyl, amyl and isoamyl acetates were frequently used as components in flavoring, and some of them are also in the natural flavors and perfumes. Flavor esters were generally produced by free and immobilized lipases from various sources in organic solvents. [4] Until now, there are several researches have been carried out in synthesizing flavor esters such as ethyl valerate, butyl acetate [2], isoamyl acetate [3] and benzyl acetate [6].

Biocatalysis appears to be attractive in the use of various ester preparations under milder conditions and may be given the natural label of the product. This includes the three principal techniques of biosynthesis that can be distinguished in the following ways: use of enzymes, use of microorganisms, plant cells and culture of tissues. Among these, application of enzymes is the most frequently used technique of biosynthesis. Flavor esters have been generally produced by lipases from various sources in organic solvents. However, solvent toxicity and high production costs are the major problems in most reactions [4]. To facilitate these, solvent free system offers more advantages because the absence of solvents facilitates downstream processing, thus offering significant cost saving and minimizing environmental impact.

There are a number of publications regarding the enzymatic synthesis of flavor esters in nonconventional media, particularly in the presence of solvents [2, 6-7, 8]. However, the number of articles that discovered the enzymatic synthesis of solvent free systems is considerably low due to the low production yield. Therefore, a study has to be embarked in order to determine the main effect governing enzymatic synthesis of flavor ester. The present paper focuses on the synthesis of butyl acetate, a pineapple flavor using immobilized lipase Rhizomucor miehei (Lipozyme RMIM) as biocatalyst using solvent free system. This is a preliminary study of synthesizing flavor esters before scaling up the reaction using an appropriate reactor system. The main objectives are to synthesize a flavor ester and to better understand the relationships between reaction variables (reaction time, temperature and amount of enzyme) and the yield of flavor (butyl acetate).

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II MATERIALS AND METHODS

A. Materials

Lipasezyme RMIM from *Rhizomucor miehei* immobilized onto Sepharose™ 4B, activated by sodium+ ion exchange gels) manufactured by Novozymes A/S (Denmark) was used as received without pretreatment. Substrate as acetic acid was obtained from Merck KgA (Germany) and butanol from Fisher Scientific Ltd. (UK). All other reagents used were of analytical grade and used as received.

B. Analysis of Butyl Acetate

The reaction system consisted of 30 mmol of acetic acid and 50 mmol of butanol and 5.4% (w/w) of total substances of Lipasezyme RMIM. All substrates were placed in screw-capped vials and the reaction assayed in triplicates. The reaction mixtures were maintained at 37°C in a horizontal water bath shaker with shaking speed of 150 rpm and continuously reacted for 24 hours. The product of butyl acetate was determined by mean method with 0.1 M of NaOH using modern automatic titrator (Metrohm, Switzerland).

C. Identifying reaction product

Preliminary detection of butyl acetate was tested using Thin layer chromatography (TLC). (Merck type DC-plastic plate, Kieselgel 60 F254). Hexane and dichloromethane were used as developing solvent system in ratio of 9:1 (v/v). Then the synthesized butyl acetate was analyzed using spectrophotometry methods of Fourier transform infrared-spectroscopy (FTIR) to confirm the product obtained.

D. Analysis of reaction product

The yield of butyl acetate produced was expressed as a percent (%w) of converted acetic acid and compared to the total fatty acid in the reaction mixture using the following equation:

\[
\text{Conversion(\%w) = } \frac{\text{Vol}_{\text{acetic acid in enyme}} - \text{Vol}_{\text{acetic acid in enzyme}}}{\text{Vol}_{\text{acetic acid in reaction mix}}} \times 100\%
\]

In this study, the butyl acetate produced was determined in terms of relative percentage conversion (%w).

E. Effect of reaction time

The reaction mixture was maintained at 37°C continuously using a horizontal water bath shaker with continuous shaking speed of 150 rpm. The reaction was assayed at various reaction times (2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, and 24 hours). The percentage conversion was determined as described earlier.

F. Effect of temperature

The reaction mixture was maintained at various temperatures (30, 37, 45, 50, 55, 60, 70, and 80°C) using a horizontal water bath shaker with continuous shaking speed of 150 rpm. The percentage conversion was determined as described earlier.

G. Effect of amount of enzymes

The reaction mixture was catalyzed by varying amount of enzymes (5%, 10%, 15%, 20%, 25%, and 30% w/w) of Lipasezyme RM at optimum temperature and reaction time. The percentage conversion was determined as described earlier.

III RESULTS AND DISCUSSIONS

A. Identification of reaction products

Preliminary detection of butyl acetate was carried out using thin layer chromatography (TLC) analysis. Hexane and dichloromethane were used as mobile phases in the analysis with a ratio of 9:1 (v/v). The pure synthesized butyl acetate, unreacted alcohol and acid were detected as brown spots on the silica gel plate when visualized by an iodine reagent [9]. Rf values for each spot were calculated by comparing the distance of solute and the distance of mobile phase. The particular distance for each substance spot was measured from the starting point of the substance spot. This analysis showed that the Rf values for butyl acetate, acetic acid and butanol were 0.62, 0.57 and 0.44, respectively. The synthesized butyl acetate was analyzed using Fourier transform infrared-spectroscopy. According to Fig 1, a strong peak was observed at 1742 cm⁻¹ (C=O) which indicated the presence of ester-carbon group (C=O). Similar finding was reported by Mat Razak, et al. (2003) [9] in the synthesis of oleyl oleate, a wax ester whereby the ester bond was detected at wavenumber at 1742 cm⁻¹.

B. Effect of time on the esterification reaction

Tuning enzyme activity is enhanced with the performance of an enzyme as the reaction progress. Such progress curves help determine the shortest time necessary for obtaining good yields and so enhanced cost-effectiveness of the process. This is a very important factor especially in large scale processing to obtain high performance of production yield. The profile of production of flavor ester at various time intervals is presented in Fig. 2. Generally, the relative percentage conversion of butyl acetate was increased with increasing reaction time. Temperature of the reaction mixture was maintained at 37°C and, thus, it was increased gradually from 3 hours to 18 hours. The initial reaction acceleration was due to the production of water needed for active conformation during reaction which increases lipase activity [10]. After 18 hours of reaction time, the relative percentage of ester started to decrease. This may be due to the large amount of water produced during the reaction occurs. Similar findings was reported by Mat Hadzi, et al. (2001) [11] in the synthesis of oleyl oleate by enzymatic alcoholysis reaction between triolein and oleyl alcohol.

C. Effect of temperature on the esterification reaction

The effects of temperature can be ascertained its effect on substrate solubility as well as its direct influences on the reaction [12]. Effect of varying reaction temperatures on enzymatic synthesis of butyl acetate is shown in Fig. 3. Initially, the relative percentage conversion of ester was increased with increasing temperature from 30-40°C. However, at higher temperatures, the relative percentage of yield was gradually decreased from 40-80°C. This is probably due to the reaction being of a critical temperature, where the temperature may have been deactivated [13]. As shown in the Fig. 3, the highest relative percentage of ester was obtained at 37°C. The yield started to
Fig. 1 Determination of MIC value by microdilution assay using MTT as an indicator. Treatment of (a) C. Harbort essential oil and (b) C. Stemoides on B. subtilis ATCC 21332. The blue color showed bacterial growth due to the blue formazan formed, while the pale yellow indicated no bacterial growth.

Fig. 2 Effect of reaction time on the esterification reaction of acetic acid and butanol by Lipoyxime RMIM. Reaction condition: temperature 57°C, amount of enzyme 3% (w/w) and agitation speed of 150 rpm.

Fig. 3 Effect of temperature on the esterification reaction of acetic acid and butanol by Lipoyxime RMIM. Reaction condition: reaction time 18 hours, amount of enzyme 5% (w/w) and agitation speed of 150 rpm.

D. Effect of amount of enzyme on the esterification reaction

From an applied point of view, the substrate concentration should be as high as possible to obtain a higher degree of esterification. Simultaneously, from an economic point of view, the amount of enzyme used should be as low as necessary to obtain the desired product. The influence of varying amount of enzyme on the esterification reaction of acetic acid and butanol is shown in Fig. 4. The result shows a slight increase in the relative percentage conversion of esters when 10% (w/w) to 25% (w/w) of enzyme was used and drastically decreased at 30% (w/w) of enzyme was used. Torres and Orero (2001) [18] had also indicated that the use of large amounts of enzyme could significantly increase the fraction of acyl donor molecules to form acyl-enzyme complexes. Moreover, these active sites would not be exposed to the substrate and remain inside the bulk of enzyme particles without contributing significantly to the reaction. According to Karra-Chabouni et al. (2006) [2], in the presence of high amount of lipase, not all active sites are exposed to the substrates and resulted molecules of the enzyme tend to aggregate together. However, too small amounts of enzyme may have been insufficient for complete substrates conversion within the specified reaction period [19]. They also suggested that another factor also contribute to the lost of enzyme activity such as pH, reaction-medium and temperature. Foresti and Ferreira (2005) [20] also reported that agglomeration using immobilized lipases in a solvent-free system leads to aggregate formation and inhomogeneous enzyme distribution.
CONCLUSION

Three parameters were chosen to optimize the synthesis of
lauryl palmitate, namely reaction time (R), reaction
temperature (T) and amount of mixture (E). The optimum
yield of lauryl acetate (68 %) can be obtained under optimal
conditions at 15 hours of reaction time, temperature of 137°C
and amount of mixture of 24 % (w/w of total substrate). This
work suggests that lauryl palmitate as a wax ester can be
produced at a very high yield via enzymatic reaction route.

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REFERENCES

role of catalysis in the design, development and implementation of green

Gargouri Y., "Production of fatty esters by immobilized Staphylococcus
acclamatus lipase in a solvent-free system", J. Process Biochem., vol. 41,
pp. 1682-1686, 2006

"Production of fatty acids by lipase from novel of Rhizopus oryzae", J.

soybean oil using unmodified lipase in a solvent-free system", Process

synthesis of soybean oil using immobilized lipase from Rhizopus

"Lipase catalyzed synthesis of benzy acetate in solvent-free medium
4041-4044, 2006

[7] R. Sharma, Y. Chau and U.C. Banerjee, "Production, purification,
characterization, and application of lipases", J. Biotechnol. Adv., vol. 19,
pp. 617-652, 2001


[9] S. Mut Rada, B. Maharan, B. S. Abu Bakar, A. Anakgo, M.
Radiainz, A.B. Moid Basyari and A.B. Raja Noor Zulphi, "High
performance enzymatic synthesis of dodecyl oleate using immobilized
252-258, 2005

the lipase catalyzed synthesis of benzyl butyrate transesterification",

Abdul Rahman and A.B. Salah, "Enzymatic hydrolysis of triolein to
2001

esterification of soybean oil deodorizer distillate", J. Sci. Food Agric.,
vol. 81, pp. 1193-1199, 2001

Kumaradavan, "Response surface methodology study on lipase-
39, pp. 1511-1518, 2003

Mysdok, "Biochemical and molecular characterization of lipase
produced by Rhizopus oryzae, a comparative study between s-lipase and
solvent-free system", J. Enzyme Microbiol. Technol., vol. 39, pp. 166-
172, 2006

Enzymatic synthesis of soybean oil with immobilized Candida
antarctica lipase in supercritical carbon dioxide", Supercritical Fluids,
vol. 33, pp. 73-84, 2005

Rahman, "Palm-Based Fatty Alcohols from Specialty Entre Alternative
Universiti Putra Malaysia Press, Serdang, pp. 23-39, 2005

fatty and dicarboxylates", J. Appl. Food. Chem., vol. 49, pp. 6786-
6794, 2001

[18] C. Terzer and C. Ottor, "Fast III. Direct enzymatic esterification of
milk fat mixtures by lipase in organic solvent", Enzyme, Microbiol.

Rahman, "Palm Based Fatty Esters from specialty Entre Alternative

mediated by lipase from Candida antarctica B adsorbed on polystyrene