# PRODUCTION OF ISOAMYL ACETATE WITH CANDIDA ANTARCTICA LIPASE B (CALB) AS ENZYME IN A SOLVENT-FREE SYSTEM USING MINIATURISED INTENSIFIED REACTOR

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**by**

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*Sathiswaran Sangaran*
*June 2018*

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| CALB | Candida antarctica Lipase B |
| RSM | Response Surface Methodology |
| OFAT | One-Factor-At-one-Time |
| GC-FID | Gas Chromatography- Flame Ionized Detector |
| CCD | Central Composite Design |
| DoE | Design of Experiment |
| Re | Reynolds Number |

**PENGHASILAN ISOAMIL ACETAT MELALUI ESTERIFIKASI BERMANGKIN DALAM SYSTEM TANPA PELARUT DENGAN MENGGUNAKAN REACTOR MINIATUR**

## ABSTRAK

Isoamil acetat adalah salah satu ester yang mempunyai kewangian sama dengan buah pisang. Isoamil acetat selalunya digunakan sebagai perasa tambahan khasnya dalam industry pemakanan. Pada zaman dahulu, kajian tentang isoamil acetat telah dilakukan secara tradisional, iaitu menggunakan kaedah pengestrakan ester diikuti dengan penghasilan isoamil acetat dengan proses tindak balas kimia. . Kini, proses esterifikasi telah berkembang maju kearah penghasilan isoamyl acetat dengan menggunakan pemangkin. Hal ini kerana, pelanggan lebih suka akan isoamyl acetat yang dihasilkan dengan proses semula jadi berbanding dengan proses tindak balas kimia. Selain itu, process intensifikasi juga sedang menjadi popular dalam kalangan penyelidik. Hal ini kerana proses intensifikasi boleh dilakukan dalm reactor miniatur yang mempunyai kelebihan untuk menghasilkan isoamil acetat yang berkualiti dalam masa yang singkat berbanding reactor kontemporari. Oleh itu, eksperimen ini telah dikhususkan untuk megkaji penghasilan isoamyl acetat dengan menggunakan pemangkin *Candida antarctica* Lipase B (CALB) dalam reactor miniatur. Faktor factor yang memberi impak kepada penghasilan isoamil acetat telah dikaji seperti suhu tindak balas, nisbah asid-alkohol dan penggunaan pemangkin dalam proses esterifikasi. Faktor-faktor tersebut dikaji menggunakan kaedah penkajian satu–faktor-pada-suatu-masa (OFAT). Tambahan pula, process pengoptimuman pun telah dilakukan menggunakan Kaedah Sambutan Permukaan (KSP). Keputusan menunjukkan bahawa Kaedah OFAT lagi tepat daripada kaedah KSP.

**PRODUCTION OF ISOAMYL ACETATE WITH CANDIDA ANTARCTICA LIPASE B (CALB) AS ENZYME IN A SOLVENT-FREE SYSTEM USING MINIATURISED INTENSIFIED REACTOR**

## ABSTRACT

Isoamyl acetate is a type of ester that has similar fragrance to banana. It is widely being used as banana flavouring in food industries. Initially, isoamyl acetate was extracted using tradition method from plants and fruits, and then followed by chemical synthesis. Over the time, the esterification process is conducted using enzyme because consumers highly prefer naturally synthesized ester compared to chemically synthesized ester. On the other hand, lately process intensification is gaining momentum among the researchers. This is because the maximum conversion and yield can be achieved in shorter time in miniaturised intensified reactors compared to the large conventional reactors. Thus this study is conducted to investigate the performance of isoamyl acetate production using enzyme *Candida antarctica* Lipase B (CALB) in miniaturised intensified reactors. The effect of operating parameters such as reaction temperatures, acid-alcohol ratio and presence of enzyme on the final concentration and conversion of isoamyl acetate produced was studied using One-Factor-At-A-Time (OFAT) method and also Response Surface Methodology (RSM) to optimise the results. The results of OFAT show that it is more reliable than RSM method because the regression coefficient value is very closer to unity which is more than 0.91. The optimum operating condition obtained through OFAT is 50°C for temperature and 0.6 for acid-alcohol ratio. The optimum temperature with enzyme is 40°C.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Introduction of isoamyl acetate process

Esters are naturally occurring organic compound which contains –COOR as functional group. Esters are commonly prepared by the reaction of carboxylic acid and alcohol with elimination of water. However, esters are can be produced by replacing carboxylic acid with acid anhydrides, acid chlorides, amides, nitriles, unsaturated hydrocarbons, ethers, aldehydes, ketones, alcohols, and esters (via ester interchange). Esters are widely used for various purposes in chemical industry. For example, Butyl and hexyl acetates are excellent solvents for polyurethane coating systems. Ethyl, isobutyl, amyl, and isoamyl acetates are often used as components in flavouring, and isopropyl, benzyl and methyl acetates are main additives in perfumes (Aslam et al., 1993) . Most of the esters have a very pleasant smell which is similar to food and flavours. Due to this property of this ester, it is commonly used for flavouring purposes in food industry to enhance the taste of the food (Romero et al., 2005a). There are many types of esters that are being used for flavouring and flavouring purposes.

Table 1.1 Esters that gives fruity fragrance

| **Esters** | **Flavours or Fragrances** | **References** |
|---|---|---|
| Butyl butyrate | Pineapple | (Varma and Madras, 2008) |
| Ethyl cinnamate` | Cinnamon | (Sharma et al., 2011) |
| Isoamyl acetate | Banana | (Romero et al., 2005b) |
| Butyl acetate | Apple | (Young et al., 1996) |

The demand for the natural products in food industry have paved path to the increasing demand of ester to enhance the flavour of the food produced. Besides that it also gives a very pleasant smell. Thus, production of esters was widely explored for the past few decades to meet the world demand (Longo and Sanromán, 2006).

Ester is abundant in flowers and plants. Years ago, ester was obtained by extraction from its natural resources. However, this traditional method had several limitations. It was very expensive to obtain esters through natural extraction and only small amount was able to extract. This method was not able to satisfy the global demand as well as too expensive for commercial exploitation (Krishna et al., 2001).

Due to the limitation of traditional method, synthetic flavours were developed by researchers through chemical synthesis (Yang et al., 2010). This chemical synthesis was done through Fischer esterification mechanism. Fischer esterification is an equilibrium reaction which uses sulphuric acid as catalyst whereas other esterification routes do not involve equilibrium. One of the reactants is used in excess depends on the cost and also availability to favour the production of esters. The mechanism starts with protonation of carboxyl group, attack by the nucleophilic hydroxyl, a proton transfer and loss of water followed by loss of catalysing proton to give ester. The finding through this research was chemically synthesized esters gives higher yield compared to naturally extracted product. However, the main disadvantage was consumers prefer foods that are labelled natural (Schrader et al., 2004). Moreover, food that uses chemically synthesized flavouring was disallowed to label as natural. A separation unit was needed to separate undesired product which is environmentally unfriendly and it was money consuming. Catalyst used in this method which is sulphuric acid is

considered very harmful because it can cause severe burns when it comes in contact with skin and cause corrosion to the mechanical parts of the reactor (Joseph et al., 2005).

Synthesis of esters in organic solvents was also widely investigated by researchers. They found that this method gives highest yield compared to other method (Cvjetko et al., 2012). The common organic solvent used was n-hexane to produce ester isoamyl acetate. However, this method also had some disadvantages such as toxicity of organic solvent to humans, need of downstream process to separate ester from organic solvent, and high cost of organic solvent was not suitable for large scale industry (Azudin et al., 2013a). Various problems encountered with the methods mentioned above, such as polluting liquid acid, harmful chemical esters, uneconomical methods and so on. As a solution to all these problems, a biotechnological route in solvent free system was proposed. The advantage of the process was, it increases the yield of ester. This is because biocatalyst provides high enantioselectivity to obtain pure compounds, stable in thermal condition, stable in organic solvent and no need cofactors (Pohar et al., 2009). Esterification is usually performed with immobilized enzyme reactors. This criterion increases the stability and enables to reuse the enzyme.

Currently, miniaturised intensified reactors are gaining wide recognition for its advantageous performances such as high surface area to volume ratio and also effective heat and mass transfer. This causes researchers to extend their study by producing ester in miniaturised intensified reactor with immobilized enzyme in solvent free system. This method gives higher yield of ester compared to conventional batch scale method. This is because this reactor gives better selectivity, improves yields over shorter period

of time, improves process control, more safe to use, gives flexible production (Mills et al., 2007).

## 1.2 Problem statement

Esters are chemical compound which has fruity and fragrance smell. It is widely used in food industry for flavouring purposes to enhance the taste of the food. Initially, esters are extracted traditionally from natural resources. However, shortage of supply was a main problem because the amount can be extracted traditionally is very small. Moreover, fruits like banana need to attain maturation before extraction can be done. Moreover, traditional extraction is expensive. Commercial exploitation could not be done because the higher price and limited yield. On the other hand, demand for esters keeps increasing though the supply is tight. This pushes the researchers to work on more alternative processes which are suitable for commercial exploitation. The research mainly focuses on producing large amount of ester in shorter time (Ghamgui et al., 2006).

Over the time, the researchers developed synthesis of ester chemically through Fischer esterification mechanism. In this mechanism, one of the reactant is used in excess depends on the cost and also availability. Acid catalyst is used to increase the rate of reaction.

The advantage of this method is that it offers higher yield compared to traditional extraction. However, there are several drawbacks in this method. Firstly, consumers preferred food labelled natural and ester produced through this method is not allowed to be labelled as natural (Schrader et al., 2004). Acid used as catalyst is very

harmful to human and it may corrode the mechanical parts of the plant (Joseph et al., 2005). The separation of desired product from unreacted reactant and acid was money consuming. Hence, researchers opt for other method which promises higher product yield with high economic benefit and a more pure product.

Ester was produced through microbial fermentation. Fermentation process is conducted by preparing a culture medium and grows the fungus in order to prepare inoculum. Wild type microorganisms were able to produce ester with higher concentration. However, the problem was it takes a longer time for the fungus to grow and can only extract ester after almost 7 days.

As an alternative route, synthesis of ester using enzyme was introduced. Isoamyl acetate was synthesized from isoamyl alcohol and acetic anhydride using Candida Antarctica Lipase B as enzyme to increase the rate of reaction. The yield obtained through this method was higher. However, the problem was the reaction cannot be conducted at higher temperature because denaturation will occur. Moreover, enzymes are expensive. Due to the limitations to conduct the reaction at low temperature, more enzymes were needed to increase the reaction. Eventually, it increases the cost of the whole process. Over the time, researchers found a way to increase the rate of reaction. It was proven that conducting the whole process in organic solvent was able to increase the rate of reaction. The most common organic solvent used is n-hexane (Romero et al., 2005a) and n-heptane (Pohar et al., 2009). The advantage is it gives phenomenally higher yield compared to natural and chemical synthesis of ester. However, it also had some disadvantages. For instance, toxicity of organic solvent, need of downstream process to separate pure product from solvent and organic solvent is too expensive to be used in large scale industry (Ghamgui et al., 2006).

Due to the organic solvent's nature as mentioned above, solvent free system is introduced. Though the yield is not up to the yield obtained using organic solvent, there are several advantages of using solvent free system. For instance, solvent free system is cost saving due to elimination of solvent, process becomes simpler due to elimination of downstream process to separate pure product from solvent, safer method because there is no toxicity due to organic solvent (Azudin et al., 2013a)

However, it takes longer time to synthesis ester in solvent free system and the yield obtained was relatively lower. Thus, researchers started to study about using miniaturised intensified reactor (MIR) to synthesis ester. The use of miniaturised intensified reactor is to carry out process intensification. Conventional method takes longer time to complete the reaction. In miniaturised intensified reactor, the reaction time is lower and it enables us to obtain pure product in shorter time. This is because miniaturised intensified reactor offers better mixing, higher heat and mass transfer, improves process control, safer to use because it involves small amount of chemicals only and scaling up can be done by increasing the number of miniaturised intensified reactors (Mills et al., 2007). The yield for ester in miniaturised intensified reactor is also found to be higher.

In conclusion, to overcome the problem mentioned above and also to find a better solution in ester synthesis, enzymatic synthesis of ester in solvent free system using miniaturised intensified reactor is studied in this experiment.

Table 1.2 Advantages and disadvantages of isoamyl acetate synthesis method

| Synthesis method | Advantage | Disadvantage |
|---|---|---|
| Tradition extraction from natural sources | • Natural product | • low product yield<br>• Cannot commercialise this method due to very small of production<br>• Too expensive<br>• Need large amount of raw material<br>• Need to wait to extract until the fruit reached maturation |
| Chemical synthesis | • Higher yield compared to traditional method | • Labelled as not natural<br>• Need downstream process<br>• Use highly corrosive acid |
| Fermentation process | • Wild type microorganisms give higher yield of ester. | • It takes longer time to produce ester. |
| Enzymatic synthesis in organic solvent | • Final product is labelled as natural<br>• Higher yield is observed | • Toxicity of solvent is still unsolved problem because it is harmful for humans<br>• Organic solvent is too expensive to be used in large scale industry<br>• Need downstream process to separate product from organic solvent |
| Solvent free esterification | • Comparably higher yield<br>• Final product is considered natural<br>• Cheaper production cost because organic solvent is not used<br>• No need downstream process because no solvent is used<br>• Environmental friendly | • Longer time needed to produce desired product |

Continued.

| | |
|---|---|
| Esterification in miniaturised intensified reactor | • Effective mixing<br>• Effective heat transfer and mass transfer<br>• Higher yield<br>• Shorter reaction time<br>• Improve process control<br>• Safer to use |

### 1.3 Research objective

Objective of the research are:

i) To study the mixing behaviour of fluids in miniaturised intensified reactor

ii) To identify the effect of manipulated operating conditions such as flow rate, temperature, acid-alcohol ratio and presence of enzyme in the production of isoamyl acetate in solvent free system using miniaturised intensified reactor.

iii) To compare the production of isoamyl acetate in solvent free system using miniaturised intensified reactor with enzyme and without enzyme.

iv) To find optimum operating condition for the synthesis of isoamyl acetate in solvent free system using miniaturised intensified reactor by conducting optimization using Response Surface Method.

### 1.4 Scope of study

In this study, enzymatic synthesis of isoamyl acetate in a solvent free system using miniaturised intensified reactor with the presence of *Candida Antartica Lipase B* (CALB) as enzyme is studied. Isoamyl alcohol and acetic anhydride is used as reactants to produce isoamyl acetate. The experiments were conducted in miniaturised intensified reactor under various experimental conditions. The parameters investigated were

reaction temperature with a range from 30-50°C, acid-alcohol ratio with a range of 0.6-1.0 and presence of enzyme. The reaction time was kept constant for all set of experiment. Optimization was done using Response Surface Methodology (RSM) to find the optimum operating condition for production of isoamyl acetate with highest yield.

As mentioned earlier, this experiment was conducted in miniaturised intensified reactor. Water bath is used to maintain the reaction temperature and syringe pump is used to maintain the acid-alcohol ratio which is inferred by the flow rate values. Samples were drawn after the reaction time is completed and was analysed using Flame Ionization Detector Gas Chromatography (GC-FID).

Optimization process was done using Design of Experiment (DoE) software. The method used is Response Surface Methodology and the design used is a central composite design (CCD) was used to do optimization. This is done to find the optimum operating condition which gives highest yield for the production of isoamyl acetate.

## 1.5 Organization of thesis

This thesis is divided into five chapters as follows:

**Chapter 1:** This chapter includes the introductory part of this thesis. It gives a brief introduction about production of isoamyl acetate in solvent free system by CALB using miniaturised intensified reactor. This chapter covers problem statement, scope of study and objectives of this study.

**Chapter 2:** This chapter describes the previous works and literature reviews on synthesis of isoamyl acetate. This explains the various methods conducted before applying solvent free system as well as using enzyme as biocatalyst to increase the production. Moreover, this chapter also covers the theories and researches behind the application of miniaturised intensified reactor to produce isoamyl acetate.

**Chapter 3:** This chapter covers the methodology used to conduct this study. It also describes the materials, chemicals, equipment, experiments and analysis required to carry out enzymatic synthesis of isoamyl acetate in solvent free system using miniaturised intensified reactor.

**Chapter 4:** This chapter covers the results and discussion part. The experimental results are analysed and discussed. The optimization process also is discussed in this chapter. Optimum operating condition which gives highest yield of isoamyl acetate was studied.

**Chapter 5:** This chapter covers conclusion and also recommendations for improvement purposes in the next study.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Flavouring Industry

Flavours has been used since many years ago mainly in food industries to enhance the taste of food. Over the years, flavouring industry has undergone tremendous changes in order to produce flavours efficiently. In those days, flavours compound ranging from single to complex substances are obtained by natural resources especially plant sources. However, due to elucidation of structure, low concentration of desired compound makes the extraction complicated and expensive (Schrader et al., 2004). Furthermore, since the flavours is extracted from the plants, factors such as weather condition and plant diseases causes difficulties in extracting the flavours from plants. To overcome this problem, flavours were synthesised chemically. Though chemically synthesised flavours seem to give more products in shorter time, it had several disadvantages. Firstly, consumers prefers foods that is labelled natural (Schrader et al., 2004) and food that uses chemically synthesized flavouring were not allowed to label as natural. Next, chemical reaction can produce undesired product which is environmentally unfriendly (Longo and Sanromán, 2006). Separation of desired product from undesired product and unreacted reactant is money consuming. The disadvantages of both method paved path to production of flavours based on microbial biosynthesis. This method uses enzyme and the enzyme is obtained from microbial reaction. This enzyme acts as biocatalyst which provides and alternative route which requires lower activation energy to produce flavours from reactants. The advantages of this method are it has higher ecological acceptability and economically beneficial (Krishna et al., 2000, Romero et al., 2005a, Güvenç et al., 2002)..

## 2.2 Esterification process

Esterification is process of producing esters. Esterification process occurs when a carboxylic acid reacts with alcohol. Most of the esterification process needs catalyst to produce esters. Catalyst is used to increase the rate of reaction of esterification process. The reaction occurs in a way that –OH from carboxylic acid and +H from alcohol is removed to form water which is a by-product of this process .

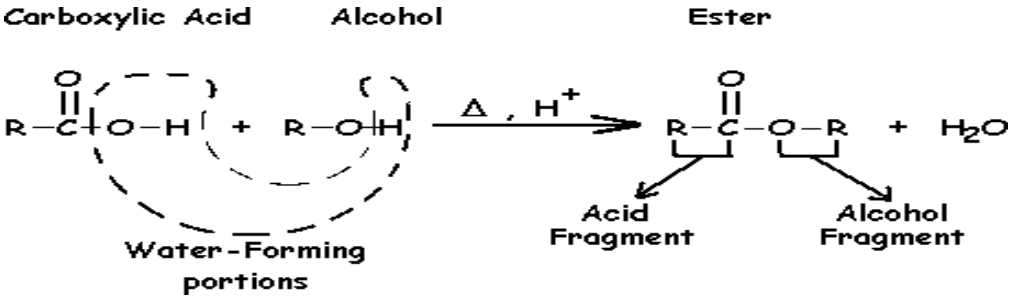



Figure 2.1 Molecular equation of isoamyl acetate esterification

The most common method to produce esters is to heat the reactants and remove the by-product which is water. Esters can also formed through other reactions such as using acid anhydrides, acid chlorides, amides, nitriles, unsaturated hydrocarbon, ethers, aldehydes, ketones, alcohols and also esters (through ester interchange). According to the studies, esterification reaction will not be a complete reaction because it involves a reversible equilibrium. To achieve 100% conversion, the desired product (ester) and undesired product (water) need to undergo separation. Esterification can be carried out through both batch and continuous process.

## 2.3 Isoamyl Acetate

Food is a basic need for human. It gives us important nutrition that is required by the body. Food is also important to maintain a good health. However, when a society is been given with more than adequate food supply by the manufacturers of food, other

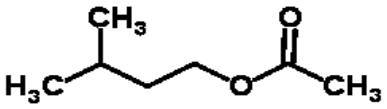
issues relating to the consumers' food choice have grown in importance. One of the issue the taste of the food. People always like tasty food. Thus, manufacturers look for ways to enhance the taste of the food they manufacture. One of the ways to enhance the taste of the food was using food flavouring. There are several importances in using flavouring to the food. Firstly, flavouring started to give different sensory experience. People started to consume more when a food tastes good. Next, some food which has high nutritional value does not have a good taste. Thus, food flavours were added to that food to make people consume more healthy food. Besides that, flavour of food also becomes a factor in consumer's food choice. Thus, common flavours which are popular and also taste good were added to the food to attract consumers to consume the food (Clark, 1998).

The global consumption of flavours is increasing year after years. According to a research, it has been found that 27,000 tonnes of flavours was used in 2014. Flavouring is used widely in dairy products and also beverages. One of the flavours that is widely used in food industry is isoamyl acetate.

Isoamyl acetate is a type of isoamyl esters of short chain fatty acid. It is an organic compound which is ester which can be formed through reaction between isoamyl alcohols with acetic acid or reaction between isoamyl alcohol and acetic anhydride. It is slightly soluble in water and very soluble in most organic solvent. The molecular formula of isoamyl acetate is $C_7H_{14}O_2$. This ester gives a strong fruity smell which is closely similar to the aroma of banana and pear . Isoamyl acetate is also known as "banana oil" (Fahlbusch et al., 2003). Currently, this ester is widely used in food industry for flavouring purposes. They are also used as intermediates in the

manufacture of perfumes, pharmaceuticals, synthetic flavourings, cleaners and other organic compound . The physical properties and chemical properties are shown in the table below:

Table 2.1 Properties of isoamyl acetate (OSHA, 2011)

| Properties | Values |
|---|---|
| Chemical Structure |  |
| Molecular formula | $C_7H_{14}O_2$ |
| Molar mass | 130.19 g/mol |
| Density | 0.876 g/cm$^2$ |
| Melting point | -78°C |
| Boiling point | 142°C |
| Flash point | 25°C |
| Auto ignition temperature | 379°C |
| Vapour pressure | 4 mm Hg |
| Solubility | Very soluble in organic solvents but Slightly soluble in water |

## 2.4 Production of Isoamyl Acetate

Esters are chemical compound which gives fruity smell. Esters are widely used in food industry for flavouring purposes. Among the flavouring esters, esters with low molecular weight have higher demand in industry. In such case, isoamyl acetate is said to be the most sought after flavour in food industry. This is because isoamyl acetate possesses strong banana flavour and it is widely being in food such as honey, butterscotch, coffee and beverages. Fermented alcoholic beverages also use isoamyl acetate to enhance its taste. For instance, sake, beer and wines (Torres et al., 2010). Synthesis of isoamyl acetate can be done naturally and also chemically. Traditionally, production of isoamyl acetate was done naturally by extracting it from banana. However, shortage of supply was a major problem because very less amount only can

be extracted from banana. According to a research, it has been stated that only 20% of yearly banana production is being exported to foreign countries. The rest is being consumed by the local people as fresh fruit. Moreover, since banana is categorised under climacteric fruit, the flavours and fragrance can only be extracted after the maturation stage of the fruit (Salmon et al., 1996). Furthermore, it is always expensive to extract favours naturally. For example, the most common flavour which is being used in food industry is vanillin. Artificial vanillin is cheaper which cost about 11-20 USD per kg. However, naturally extracted vanillin can cost up to 1400 USD per kg (Torres et al., 2010) .Due to the higher price, industries couldn't afford the naturally extracted flavours. The limited amount of production and the longer time taken to extract the flavours causes the esters extracted from plants are often too scarce or expensive for commercial use (Ghamgui et al., 2006). Thus, various studies were conducted to find alternative ways to synthesis banana flavouring in greater amount as well as in shorter time.

Researchers develop another route to produce isoamyl acetate chemically through Fischer esterification mechanism. Fischer esterification is an equilibrium reaction whereas other esterification routes do not involve equilibrium. One of the reactants is used in excess depends on the cost and also availability to favour the production of esters. The mechanism starts with protonation of carboxyl group, attack by the nucleophilic hydroxyl, a proton transfer and loss of water followed by loss of catalysing proton to give ester.

For example,

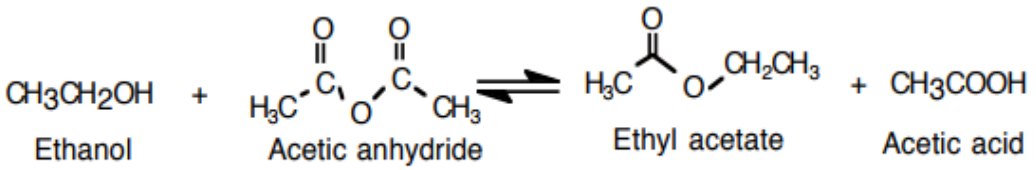



Figure 2.2 Molecular equation of isoamyl acetate esterification (Aslam et al., 1993)

In this reaction between ethanol and acetic anhydride, ethyl acetate is produced together with acetic acid. This acetic acid will react with ethanol to form ethyl acetate. Chemically synthesised product gives higher yield compared to naturally extracted product. However, it had several disadvantages. Firstly, consumers prefers foods that is labelled natural and food that uses chemically synthesized flavouring were not allowed to label as natural (Schrader et al., 2004). Next, chemical reaction can produce undesired product which is environmentally unfriendly (Longo and Sanromán, 2006). Separation of desired product from undesired product and unreacted reactant is money consuming. Furthermore, sulphuric acid is used as acid catalyst in this method. Sulphuric acid needed to be handled with care, easy to separate from reactants and can be used repeatedly (Huirong and Yingde, 1998). However, heterogeneous catalysts also had several drawbacks such as corrosive in nature, high cost, unstable in nature. Due to these drawbacks, the solid acid catalysts were not able to use efficiently in production of esters (Joseph et al., 2005). Due to the high risk, certain industries were looking for other better alternatives which are much more safer and cost saving to apply in industry.

One of the alternative processes was microbial fermentation process. Microorganisms have played a key role in the synthesis of flavours for the usage of food industries for ages. For example, isoamyl acetate is found in the fermented drink which determines the flavour quality of beer wine and sake. Production of isoamyl acetate through fermentation is conducted by preparing a culture medium and grows the fungus in order to prepare inoculums. The duration for fermentation process is about 7 days and finally the ester is obtained by extraction process. In order to obtain higher yield and higher quality of final product, several microbial studies were carried out. Among the research conducted on various microorganisms, it has been found that wild type of microorganisms has ability to produce isoamyl acetate with higher yield. There are several types of wild-type microorganisms. The examples are as below (Torres et al., 2010):

Table 2.2 Different microorganisms used by various researchers to synthesis isoamyl acetate

| **Strain** | **Enzyme / Culture Medium** | **Yield (%)** | **References** |
|---|---|---|---|
| *Saccharomyces cerevisiae* | AATase / grape must | 5.1 | (Erten and Tanguler, 2010) |
| *S. cerevisiae* + *Williopsis saturnus* NCYC22 | AATase / grape must | 5.7 | |
| *B. licheniformis* S-86 | Type II esterase / Synthetic medium + 0.6% isoamyl alcohol | 1.1 | (Torres et al., 2009b) |
| Sake yeast strain 2NF | AATase / YPD medium | 1.1 | (Hirooka et al., 2005) |
| *Acetobacter sp.* N23 | Esterase / YPGE medium + 0.05% isoamyl alcohol | 3.7 | (Kashima et al., 2000) |

Yet again, the longer duration to synthesis isoamyl acetate from microorganism was a major issue. It was not economical for industry to produce isoamyl acetate for a very long duration. Thus, more researches were carried out to opt for better option in producing isoamyl acetate efficiently as well as economically.

Over the time, another method was developed to produce isoamyl acetate which is use of biotechnology process. This alternative was considered better than the previous methods especially chemical synthesis method. This is because biocatalyst provides high enantioselectivity to obtain pure compounds, stable in thermal condition, stable in organic solvent and no need cofactors (Pohar et al., 2009). Many reactions were carried out with carboxylesterases and lipases.

Table 2.3 Different enzymes used by various researchers to synthesis isoamyl acetate

| Enzyme | Reaction conditions | Yield (%) | Reference |
|---|---|---|---|
| Type II esterase *B. licheniformis* S-86 | *n*-hexane, *p*NP-acetate, 28°C, enzyme immobilized en DEAE sepharose CL- 6B | 42.8 | (Torres et al., 2009a) |
| Novozym 435 *Candida antarctica* | *n*-hexane, acetic anhydride, 40°C, enzyme immobilized in acrylic resin. | > 100.0 | (Romero et al., 2007) |
| Lipase *Geotrichum sp.* | Water, acetic acid, 60°C, free enzyme | 24.0 | (Macedo et al., 2003) |
| Lipozyme IM-20 *Rhizomucor miehei* | *n*-heptane, acetic acid, 39ºC, enzyme immobilized in anionic exchange resin Duolite. | 40.0 | (Krishna et al., 2000) |

Esterification is usually performed with immobilized enzyme reactors. This criterion increases the stability and enables to reuse the enzyme when organic solvent is used. According to research, the best and highest yield for isoamyl acetate was obtained, in n-hexane using acetic anhydride and immobilized Candida Antarctica

lipase B (CAL-B)(Romero et al., 2005a). Conventionally, it is used in anhydrous organic medium together with activated zeolite (Pohar et al., 2009), enzymatic catalysis can be done in organic solvent as well . Some organic solvent is proven to be best media for bio catalytic process because organic solvents aids in enzyme activity and also stability, increases the enantioselectivity of the enzymes and so on. However, due to higher cost of organic solvent, it is not usually used in large scale industry. CALB show high thermal and operational stability in organic solvents (Pohar et al., 2009).

There are many organic solvents used in the production of isoamyl acetate. The most common organic solvent used is n-hexane (Romero et al., 2005a) and n-heptane (Pohar et al., 2009). The advantage of using organic solvent is it gives higher yield. However, there are several drawbacks. Firstly, organic solvents are toxic for humans. Due to its toxicity, it was highly restricted to use in food industry. Too much exposure to organic solvents can cause eye irritation, headache, peripheral neuropathy, numb extremities, weakened muscles, dermatitis, dizziness, chemical pneumonitis (aspiration liquid) and so on (NIOSH, 2016). Next, the use of organic solvent makes the process complicated because of the need of downstream process to obtain pure product (Güvenç et al., 2002). Furthermore, organic solvents are also very expensive for the use of large scale industry (Ghamgui et al., 2006).

To overcome the drawbacks due to organic solvents, solvent free system was introduced. Though the yield obtained from solvent free system is not up to the yield from organic solvent, there are several advantages of using solvent free system. For instance, solvent free system is cost saving because the cost for organic solvent has been cut down (Yahya et al., 1998). Next, the process becomes much simpler with the

elimination of downstream process to separate organic solvent from product (Azudin et al., 2013a). The isoamyl acetate produced through solvent free system is safe to be consumed because there's no toxicity due to organic solvents (Siong et al., 2014).

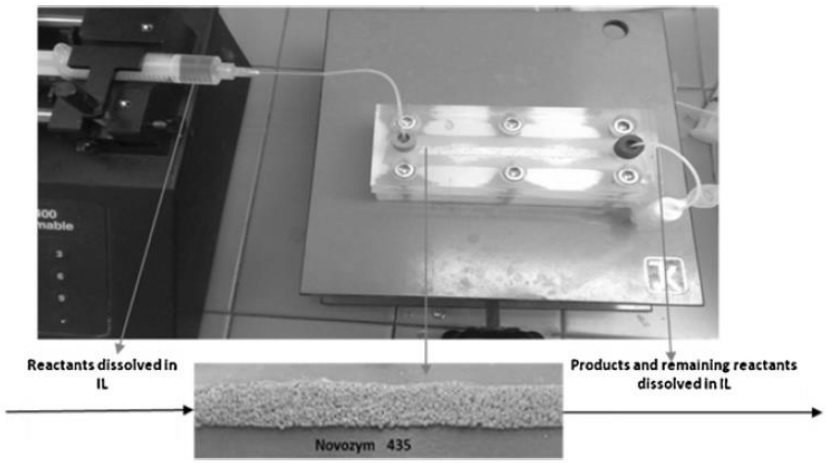



Figure 2.3 miniaturised intensified reactor used by researchers (Cvjetko et al., 2012) to synthesis ester

Recently, there are many researches is being conducted to study the production of isoamyl acetate in miniaturised intensified reactor. miniaturised intensified reactor is gaining popularity because it offer better selectivity, improves yields over shorter period of time, improves process control, more safe to use, flexible production and so on (Mills et al., 2007). For production of isoamyl acetate, continuously operating catalytic packed bed miniaturised intensified reactor is being used. This is because, production of isoamyl acetate gives greater yield with the usage of enzyme CALB. Thus, the reactor mentioned above gives several advantages such as extended operational life of biocatalyst, easier product recovery, reduce possibility of reverse reaction and gives higher productivity(Woodcock et al., 2008, Kulkarni et al., 2007). The studies shows that conducting experiment in micro reactor is highly beneficial compared to conducting

experiment in batch scale process. For example, for Novozym 435 biocatalyst immobilisation technique, the reaction rate is higher, the biocatalyst stability is extended, higher productivity and many more.

Miniaturised intensified reactor has high surface area to volume ratio and also effective heat and mass transfer. It is also easier to control the process parameters by numbering up instead of scaling up. There are studies uses organic solvent in miniaturised intensified reactor as well (Jähnisch et al., 2004). Conducting experiment with organic solvent in miniaturised intensified reactor, minimises the organic solvent consumption, reduces environmental pollution and safer to use.

Velocity profiles of the reactants are needed to simulate steady state concentration profiles inside the channels of miniaturised intensified reactor. The fluid flow inside the channel is typically laminar. Thus, the 2 reactants flow side by side parallel to each other. Mass transfer occurs by diffusion between these 2 reactant streams. The interphase of the 2 reactants depends on the ratio of flow rates of the reactants (Žnidaršič-Plazl and Plazl, 2007, Pohar and Plazl, 2008). A low flow rate gives ample contact time for the reactant to mix and conduct mass transfer by diffusion. Length of the tubing also plays major role in the mixing behaviour of reactants.

## 2.5 Application of enzymes

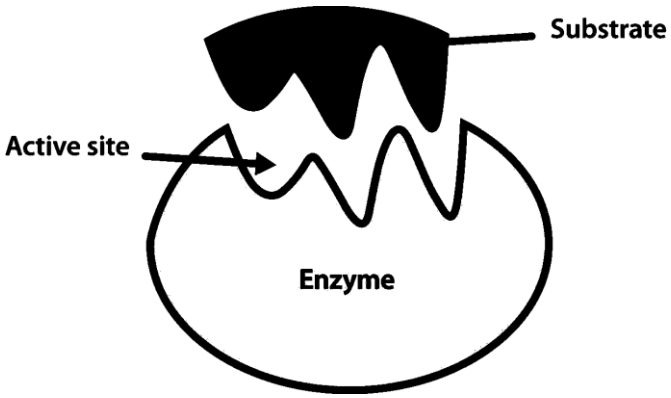



Figure 2.4 Enzyme molecules binding to substrate

Enzymes are macromolecular biological catalyst which increases the rate of reaction. Enzymes work in a way that when substrate molecules bind to the active site of the enzyme, it will be catalysed into products. Enzymes are highly specific because not all substrate can bind to the active site of the enzymes. Only certain substrates which are compatible to the function of enzyme are allowed to bind to the active site of the enzyme. In general, enzymes are globular protein. They are 3D in structure and the sequence of amino acid determines the function and type of the enzyme(Anfinsen, 1973). At high temperatures, the enzymes can denature. When denaturation occurs, enzymes loss it's biological structure. It can't convert substrates into products.

As it has been said earlier, enzymes increases the rate of reaction in several ways by lowering the activation energy. For example, the reaction rate is accelerated by stabilising the transition state, providing an alternative reaction pathway and destabilising the substrate ground state.

Some enzymes need cofactors to convert substrates into products. Cofactors can be metals or enzymes which primary function is to help the enzyme to carry out enzymatic activity. These enzymes which need cofactor are unable to function without the presence of cofactors (Voet et al., 2016). Inhibition is a process whereby enzymatic activity can be reduced due to the presence of enzyme inhibitors. There are several types of inhibition such as competitive inhibition, non-competitive inhibition, and uncompetitive inhibition.

Firstly, competitive inhibition occurs when inhibitor binds to the active site where a substrate supposes to bind. Substrate cannot be converted into products if it cannot bind to the active site of the enzyme. This can be avoided with substrate concentration.

Non-competitive inhibition occurs when non-competitive inhibitor binds to a site other than active site. Substrate may still bind to the active site of the enzyme. However, the enzymatic efficiency is reduced by the inhibitor. This type of inhibition cannot be overcome with high substrate concentration unlike competitive inhibition.

Next is uncompetitive inhibition. This type of inhibition occurs when the inhibitor binds to the enzyme-substrate complex. They cannot bind to the free enzyme. This makes the enzyme-substrate complex inactive. Thus, the substrates cannot be converted into products.

Food industry were keen to use enzymes after discovering flavouring through chemical synthesis gives poor results, causes undesirable reaction, low yield, pollution, high manufacturing cost and constraints in labelling the product as natural. The use of

right enzyme increase the enantioselectivity, increases process efficiency and reduces downstream costs. There are many enzymes were used in food industry to produce flavours. Among them, the most promising enzyme is lipase. Lipase is widely used to catalyse many substrates. The use of enzyme lipase allows the manufacturers to label the product as natural. Lipase can be obtained from many microorganisms to catalyse different substrates.

Table 2.4 Enzyme Lipase that is obtained from different microorganisms

| Substrate | Microorganism | Product | References |
|---|---|---|---|
| Olive cake and sugar cane bagasse | -*Rhizopus rhizopodiformis*<br>-*Rhizomucor pusillus* | Lipase | (Cordova et al., 1998) |
| Coconut oil cake | -*Candida rugosa* | Lipase | (Benjamin and Pandey, 1997) |
| Soy cake | -*Penicillium simplicissimum* | Lipase | (Di Luccio et al., 2004) |
| Barley bran, triturated nut | -*Yarrowia lipolytica* | Lipase | (Dominguez et al., 2003) |
| Peanut press-cake | -*Neurospora sitophila*<br>-*Rhizopus oligosporus* | Lipase | (Beuchat, 1982) |

There are several lipases has been used specifically in isoamyl acetate production. For instance, *Candida Antartica(Azudin et al., 2013a), Rhizomucor miehei* (Krishna et al., 2001), *Mucor miehei* (Krishna et al., 2000), *Staphylococcus simulans* (Ghamgui et al., 2006*)* and so on. However, lipase from different microorganisms gives different conversions (Azudin et al., 2013b).