ISOLATION OF MICROORGANISM FROM OIL PALM SAP

MUHAMMAD RIDZUAN BIN KAMARUZAMAN

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Faculty of Chemical & Natural Resources Engineering
University Malaysia Pahang

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ABSTRACT

Oil palm sap is obtained from squeeze the oil palm trunk. The sap is good medium because rich of nutrients for yeast growth. The objectives of this research are to isolate and identify the yeast species from oil palm sap and determine the growth kinetic of the mix culture of yeast. The isolation of yeast is using streaking method that streak on sabouraud dextrose agar and incubate for 30°C and 48 hours for development yeast colonies on the agar plate. The yeast colonies will re-streak to get pure colonies. The pure colonies are identified by using morphological and biochemical test to determine the species. The growth kinetics are based on Monod kinetics which using cell dried weight and substrate concentration taken every 4 hours until the reading constant or decline. The yeast species in the oil palm sap are *Candida spp., Hansenula spp., Fisobasidiella spp., Saccharomyces spp., Pichia spp., Sporobolomyces spp.* and *Trichosporon spp.* The growth kinetics values are 0.0288 (yield coefficient), 0.0058h^{-1} (decay coefficient), 0.3509h^{-1} (maximum growth rate) and 1850.711mg/l (half saturation constant). As conclusion, the yeast species can obtain from oil palm sap and can be used for further research.

ABSTRAK

Sap kelapa sawit diperoleh dengan cara memerah batang kelapa sawit. Sap ini merupakan medium yang baik kerana kaya dengan pelbagai nutrien untuk pertumbuhan yis. Objektif kajian ini adalah mendapatkan koloni-koloni yang hanya mempunyai satu spesis sahaja dan mengenalpasti spesis yis tersebut.Teknik goresan digunakan bagi mendapatkan koloni yis dengan menggunakan agar sabouraud dextrose sebagai medium dan dieramkan selama 48 jam pada suhu 30°C untuk pertumbuhan koloni yis diatas medium tersebut. Teknik ini akan diulang bagi mendapatkan koloni yang tulen (satu spesis sahaja). Koloni-koloni yis tersebut akan dikenalpasti spesisnya dengan mengenalpasti morfologi dan melakukan ujian bio kimia. Pertumbuhan kinetik adalah berdasarkan kinetik Monod dengan mengambil bacaan berat sel kering dan kepekatan substrat setiap 4 jam sehingga bacaan malar atau menurun. Spesis yis yang diperolehi dari sap kelapa sawit adalah *Candida, Hansenula, Fisobasidiella, Saccharomyces, Pichia, Sporobolomyces* dan *Trichosporon.* Nilai kinetik adalah 0.0288 bagi hasil pekali, 0.00588per jam (pekali kerosakan), 0.3509 per jam (kadar pertumbuhan maksimum) dan 1850.711 mg/l (pemalar kepekatan separa). Kesimpulanya, yis spesis boleh didapati dari sap kelapa sawit dan boleh digunakan untuk kajian seterusnya.

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LIST OF ABBREVIATIONS/SYMBOLS

g/l	-	gram per litre
h	-	hour
Kd	-	Decay coefficient
Ks	-	Half saturation constant
mg/l	-	milligram per liter
NaCl	-	Sodium Chloride
RPM	-	Revolution per minutes
SPP.	-	Species
Y	-	Yield coefficient
μ_m	-	maximum growth rate
^oC	-	Degree Celsius
%	-	percentage

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CHAPTER 1

INTRODUCTION

1.1 Research Background

The Oil palm sap is obtained from the oil palm trunk by squeeze the trunk. The oil palm trunk contains a large amount of moisture which is the sap. Only the bark part of the trunk is makes the plywood. The inner stem of trunk contains three parts which are core, middle and outer and the moisture contents are 83.4%, 75.8% and 68.5% respectively (Mori Utaka et al., 2007). This sap is rich with nutrients for growth of microbe such as carbohydrate, organic acid, protein and vitamin C (Olawale et al., 2010). The sap is very suitable for natural microbial flora to growth and resulting the clean, sweet and colourless sap rapidly turn to milky white due to increase microbial suspension in the sap (Ogbulie et al., 2007).

The sap is providing the good condition for microbe to growth especially yeast (Bechem et al., 2007). The *Saccharomyces* constituents about 70% of total population yeast in the sap (Ayogu, 1998). The isolation by streaking technique is to separate the mixed population and pure cultures from the oil palm sap (Michelle Furlong, 2005). The streaking also to get the discrete colonies of yeast that isolated from sap (Marilyn and Lawrence, 1960). The pure colonies have to

identify the species by using morphology characteristics (Muhammad Mushtaq et al., 2004).

The development of growth kinetic of mix culture of yeast is important because can predict the metabolic pathway prevailing at any time during process in carrying out the entire production which mainly to produce bio ethanol (Di Serio et al., 2001). The kinetics of mix culture is important for optimization of operational condition (Hamza et al., 2009).

1.2 Problem Statement

The oil palm trunks that have been chopping down are only use to make plywood but only the bark part only. The high moisture part which contains sap will leave behind. This sap contains microorganisms which more concerned of yeast (Olawale et al., 2010). Yeast has many uses in fermentation and baking. This sap can be source of yeast rather than being waste. The oil palm sap also being make wine because the sap is rich in sugar which 10 to 16.5% w/v mainly sucrose. This wine is consumed with the microbe alive (Olawale et al., 2010). This is essential to know the yeast species in the sap because there might have potential pathogenic species.

1.3 Objective of Study

1. To isolate and identify yeast species from the oil palm sap.
2. To determine the growth kinetic value of the mix culture of yeast from oil palm sap.

1.4 Scope of Study

The research scopes are identified in order to achieve the objectives of this study. Firstly, the isolation of the yeast species is used the streaking technique and used morphological features and biochemical test to identify the yeast species in the oil palm sap. Secondly, the kinetic values that need to determine are maximum growth rate, decay coefficient, yield coefficient and substrate coefficient. The growth kinetic is calculated by measured the substrate concentration and biomass weight.

1.5 Rationale and Significance of Study

Over the past seven years Malaysian scientists have been researching the use of oil palm trunk for plywood as every year 10 million oil palms are chopped down as most trees have a life span of only 25 years. The felled trees are either burnt or left to rot. Oil palm has 300 more moisture so it is heavy. Drying it was also a problem said Forest Research Institute of Malaysia FRIM senior research officer Dr Wan Tarmeze Wan Ariffin (Priya Menon, 2010). The moisture part of the trunk has not many research done on that, so it only been thrown away. The moisture or sap can be the source of yeast. The yeast is very needed in industry especially in baking and fermentation.

CHAPTER 2

LITERATURE REVIEW

2.1 OIL PALM SAP

2.1.1 Oil palm sap content

The oil palm contain a large amount of sap which the moisture in the trunk. The inner part of oil palm trunk contains three parts which are the core, middle and outer. The moisture contains are 83.4%, 75.8% and 68.5% respectively (Mori Utaka et al., 2007). The sap contains sugar and fermented to ethanol and others by mixture of wild yeast and bacteria. The sap contains carbohydrate, organic acid, protein, vitamin C and ash (Olawale et al., 2010). The unfermented sap is clean, sweet and colorless syrup containing about 10 – 12 % sugar which mainly sucrose. The sap will goes fermentation by the natural microbial flora, the sugar level decreases rapidly as it is converted to alcohol and other products whereas, the sap becomes milky-white due to the increased microbial suspension resulting from the prolific growth of the fermenting organism (Ogbulie et al., 2007).

The palm sap is obtained from either the immature male inflorescence and from stem by tapping. The process of tapping involves first felling or cutting down the tree and leaves the tree for 2 weeks for the sap to concentrate, followed by tapping for up to 8 weeks (Stringini et al., 2009). Other method to obtain sap is by squeezing the oil palm trunk (Mori Utaka et al., 2007).

The sap constitutes a good growth medium for numerous microorganisms especially for yeast, lactic and acetic acid bacteria (Bechem et al., 2007). These microorganisms are not originated from the sap but from several sources which includes tapping equipment, containers and environment (Ogbulie et al., 2007). The oil palm sap has *Saccharomyces cerevisiae* that constitutes about 70% of the total population in palm wine (Ayogu, 1998).

2.2MEDIUM

The media is for growth and isolate of the mix culture of yeast from the oil palm sap. The medium are broth medium and agar medium.

2.2.1 Agar

2.2.1.1 Sabouraud dextrose agar

Sabouraud dextrose agar is the mixture of peptone, glucose and agar (Ailsa, 1995). The PH of this agar is 5.6±2 for growth of yeast and suppresses

the growth of the bacteria. The high content of glucose also helps to inhibit the development of the bacteria (Kurtzman and Jack, 1998). The peptone is the protein hydrolyse which protein that cleaved by acids or enzymes into amino acids and peptides that provide carbon and nitrogen (Washington and Elmer, 2006; Messias Miranda Júnior et al., 2008), and for cell multiplication (True and Stier, 1941). The glucose is the carbon source for the energy source and serve as a substrate in biochemical reaction for identification unknown organisms (Hamedi et al., 2007; Washington and Elmer 2006). The agar which made from marine red algae is for solidifying agent which contain agarose and agaropectin which readily to from gel when mixed with high amount of water. This gel will melt at about 100°C and solidify at about 45°C (Sathyanarayana and Dalia, 2007).

2.2.1.2 Yeast malt agar

Yeast malt agar contains yeast extract, malt extract, peptone, glucose and agar (Kei Yamagata et al., 1980). The yeast extracts support the cell growth and providing amino acids, vitamins, trace elements and nucleotides (Ulrika Eriksson and Lena Häggström, 2005). The malt extract is for nitrogen sources for the cultures (Srinubabu et al., 2006). The PH of this agar is 3.7-3.8 (Somyot Tuntiwongwanich and Borwonsak Leenanon, 2009).

2.2.1.3 Potato dextrose agar

Potato dextrose agar consists of potato infusion, glucose and agar. The potato infusion provide the nitrogen and vitamin sources. Potato infusion need to prepared by autoclaved the potato and hold in cheesecloth to get the infusion before mix with glucose and agar (Sholberg, 1980)

2.2.2 Broths

The broths are medium that not contain agar as solidifying agent. The agar not supported the growth of microorganism (Dabai and Muhammad, 2005).

i. Nutrient Broth

The nutrient broth compose beef extract, peptone and yeast extract. The broth supports the growth of yeast (James, 2001). The beef extract supply vitamins, mineral and others nutrients (Jeffrey, 2009).

ii. Sabouraud dextrose broth

The ingredient to make sabouraud dextrose broth is same as sabouraud dextrose agar except it not contain agar.

iii. Yeast malt broth

The ingredient to make yeast malt broth is same as yeast malt broth agar except it not contain agar.

iv. Potato dextrose broth

The ingredient to make potato dextrose broth is same as potato dextrose agar except it not contain agar.

2.2.3 Selection of medium

The medium for isolation of mix culture from oil palm sap is Sabouraud dextrose agar. Material that used is glucose, peptone and agar only. The PH of the media is suitable for isolation of yeast species because if too small like yeast malt agar, some species cannot growth on the media such as *Schizosaccharomyces* (Kurtzman and Jack, 1998). The PH below 4.5, the agar does not gel (Sathyanarayana and Delia, 2007) and agar does not solidify after being cool down. The potato dextrose agar is need to added inhibitor of the bacteria or acidified to inhibit the growth of bacteria from the oil palm sap.

The medium for growth of mix culture of yeast to determine the kinetic value is nutrient broth. The sabouraud dextrose broth, yeast malt broth and potato dextrose broth are not being used because the media is not supplied enough nutrient for yeasts culture to growth. The nutrient is affecting the growth kinetics such as the different types and concentration of carbon and nitrogen source will give different values (Ooi et al., 2008).

2.3 ISOLATION

Isolation is the technique to get the single and pure colony.

2.3.1 Streaking technique

Streaking technique is the technique that to separate mixed population in culture media (Michelle Furlong, 2005). This streaking allowed the microbes to growth on the semi solid surface to produce the discrete colonies. These discrete colonies will be easier to purify the strain from contaminations and identify. The quadrant streak technique is used because to obtain well-isolated discrete colonies. The original sample is diluted by streaking it over successive quadrants. The third or fourth quadrant will have a few organisms that transferred on the inoculating loop (Teresa Thiel, 1999).

2.3.2 Pour plate

Pour plate is usually method for counting the number of colony forming. The preparation of pour plate technique is time consuming. Pour plate also will kill the microbe that heat sensitive (Van Soestbergen and Ching Ho Lee 1969). This pour plate also can restrict the mould growth but the colony morphology is obscured (Hagler et al., 1995).

2.3.3 Spread plate

Spread plate is method to obtain pure culture from the mix culture of the sample. The sample must be dilute before use spread plate technique. The sample is serially dilute in sterile media. The small volume of dilute sample will transferred to sterile culture plates and spread with sterile bend glass rod. The dilution is to reduce the number of organism in the sample and get isolated colonies. This technique can determine the population of microbe in sample (Michelle Furlong, 2005).

2.3.4 Selection of isolation

The isolation technique that used to isolate yeast species from the oil palm sap is streaking method. This method obtains the well isolated discrete colonies that easy to identify. The colony also showed the morphology character. The pour plate will kill certain type of yeast that cannot live in high temperature. The spread plate need to do dilution to reduce the number of microorganism same as streaking technique which reduce the number of microorganism over the successive quadrants.

2.4 IDENTIFICATION

2.4.1 Morphology

Morphology is the size, shape and the internal structure of the yeast (George, 1955). The morphology of the microorganism can be observe microscopically on slide culture (Marilyn et al., 1960)

i. Ascopore

Ascospores are regarded as haploid cells which are produced by reduction or meiotic division within an ascus. The ascospores have different shape like round, elongate, kidney and others (Andries Sechaba Bareetseng, 2004)

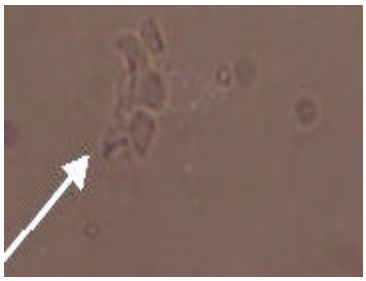

Figure 1.0: Ascospores (Andries Sechaba Bareetseng, 2004)

ii. Chlamydospore

Chlamydospore is an asexual thick-walled resting spore which originated as a vegetative cell (Michael and Anthony, 1971)

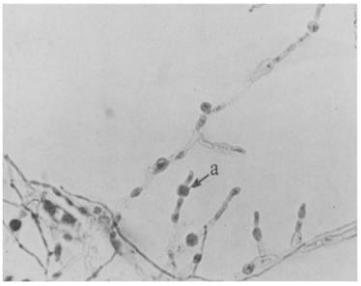

Figure 1.1 : Chlamydospore (Michael and Anthony, 1971)

iii. Conidia

A spore which genetically identical to the vegetative parent cell.

a. Arthroconidia

Arthroconidia or artrospore is an asexual spore resulting from fragmentation of a hypha (King and Jong, 1976; Michael and Anthony., 1971)

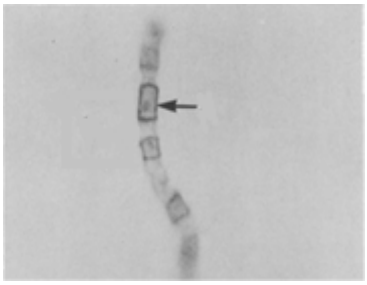

Figure 1.2: Arthroconidia (Michael and Anthony, 1971)

b. Ballistoconidia

Ballistoconidia are formed on short sterigmata and are asymmetric, ellipsoidal to reniform (Jian-Hua Zhao, 2003)

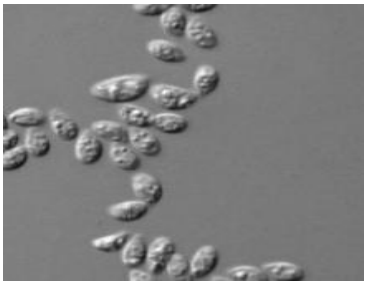

Figure 1.3: Ballistoconidia (Jian-Hua Zhao, 2003)

iv. Basidiospores

Basidiospore is the sexual spore that produced by mushroom, bracket fungi, puffballs and basidiomycetes (Estelle Levetin, 1990)

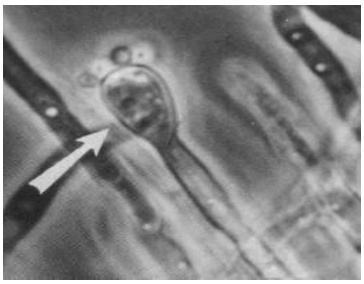

Figure 1.4: Basidiospores (Keith, 1976)

v. Hypae/pseudohyphae

A series of elongate blastospores which remain attached to each other forming a chain (Michael and Anthony, 1971)

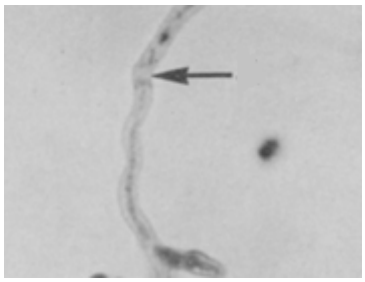

Figure 1.5: Hyphae/pseudohypae (Michael and Anthony, 1971)

vi. Sporangia

An asexual spore produced spore produced within a sporangium which a structure in the internal content is converted into asexual spores (Michael and Anthony, 1971)

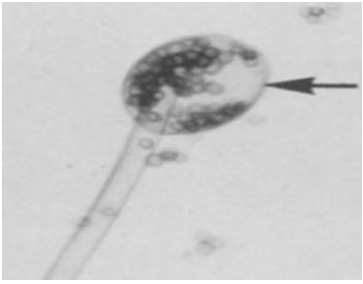

Figure 1.6: Sporangia (Michael and Anthony, 1971)

vii. Blastoconidia

An asexual spore produced by budding from pre existing cell or spores (Michael and Anthony, 1971).

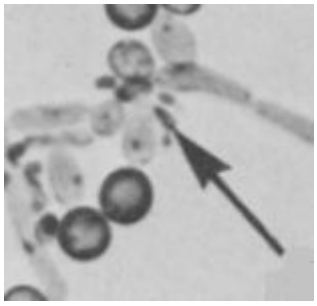

Figure 1.7: Blastoconidia (Michael and Anthony, 1971)

vii. Satellite colonies

Satellite colonies are the daughter cell that forms around the cell (Radford et al., 1997).

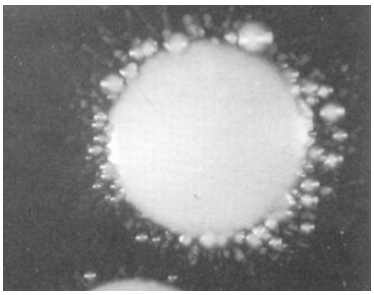

Figure 1.8: Satellite colonies (Radford et al., 1997)

2.4.2 Biochemical

Nitrate utilization is important to indentify genera of *Cryptococcus, Rhodotorula and Trichosporon*. This nitrate utilization is to determine nitrate assimilation of the yeast. The intact yeast will react by reaction of nitrate reductase (Hopkins and Land, 1977). Germ tube test is to determine the *Candida albicans*. The germ tube test perform by incubate the isolates yeast in serum for a few hours and observe under microscope (Kyoung et al., 1999). Fermentation of sugar is tested by using the Durham tube. The positive result is indicated by the accumulation of gas in the Durham tube. The assimilation of carbon and nitrogen is to determine the species can assimilate the compounds. The positive result when the solution turbidity is increase. The urea hydrolysis is incubated the isolated species in the urea and positive result will show the change colour of slant/broth into pink colour (Nahvi and Moeini, 2004)

2.4.3 Molecular test

The molecular test is used the DNA of the yeast to identify the species of yeast. The DNA of the yeast must be extract before it analysis using restriction analysis, randomly amplified polymorphic DNA (RAPD) and gel electrophoresis. The cell mass and the reaction buffer is vortexes, boil and transferred to ice. Chemical which are TRIS-HCl, EDTA, SDS and potassium acetate are added and incubate for 30 min. Then centrifuge the mixture. The supernatant is added ice cold Iso Propanol and the precipitate (nucleic acid) that form will collected. The will wash, dried and re-suspended in buffer before the DNA template is incubate and store at 20°C. The analysis should within 2 weeks (Marzia Stringini et al., 2008; Sule Sense-Ergul et al., 2006)

2.4.4 Selection of identification method

The identification of yeast genera can be achieves by only morphological test and supplement with a few physiological test (Graeme, 2000). The molecular test is complicated compare to morphological and physiological test.

2.5 GROWTH KINETICS

2.5.1 Yield and decay coefficient

The specific growth rate is first order reaction according to the first principle

$$r_g = \mu x \qquad (1)$$

In Monod Kinetics,

$$\mu = \frac{\mu_{max}\, S}{K_s + S} \qquad (2)$$

Substituting equation 2 into 1 to obtain

$$r_g = \frac{\mu_m x S}{K_s + S} \qquad (3)$$

In the equation (3), can be considered mathematical model for microbial growth rate and consider substrate concentration as (S). This equation cannot used to describe the complete growth rate because it not gives the net growth rate. The net growth rate must consider others parameter that affect the growth rate that include temperature, PH and endogenous decay. Since the temperature and PH do not give significant effect on the growth rate and relatively constant. The endogenous decay need to consider because it energy required for cell maintenance. The other factor like death rate and predation must be considered too. All these factors are lump together and represented by r_d.

$$r_d = -K_d x - \mu \qquad (4)$$

So, the net rate microbial growth r^1_g is the sum of two growths.

$$r_g{}^1 = r_g + r_d \qquad (5)$$

Substituting equation (4) into (5)

$$r_g{}^1 = r_s + (-K_d x)$$

$$r_g{}^1 = r_s - K_d x \qquad (6)$$

Substituting equation (3) into (6)

$$r_g^1 = \frac{\mu_m x S}{K_s + S} - K_d x \qquad (7)$$

Dividing equation (7) by X