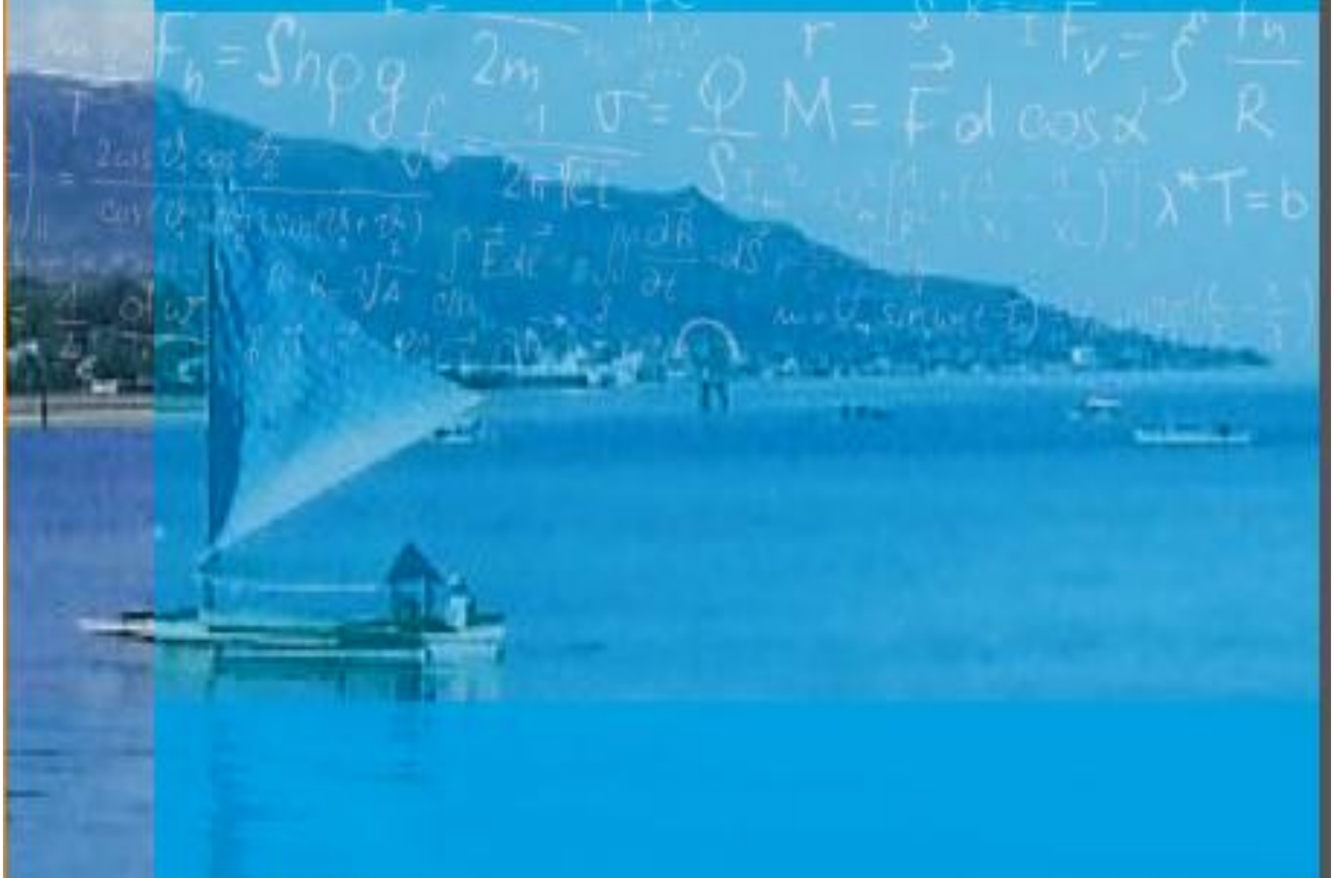
Basic Science
for Sustainable Marine Development

PROCEEDING
INTERNATIONAL SEMINAR 2015

Ambon, 3-4 June 2015



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Pattimura University

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October 2015

Welcoming Address by The Organizing Committee

The honorable, the rector of Pattimura University

The honorable, the vice rector of academic affair, Pattimura University

The honorable, the vice rector of administration and financial affair, Pattimura University

The honorable, the vice rector of planning, cooperation and information affair, Pattimura University

The honorable, all the deans in Pattimura University

The honorable, the key note speakers and other guests.

We have to thank The Almighty God for the blessings that allow this International seminar can be held today. This is the first seminar about MIPA Science in which the Faculty of MIPA Pattimura University becomes the host. The seminar under the title Basic Science for Sustainable Marine Development will be carried out on 3 June 2015 at Rectorate Building, the second floor. There are 250 participants from lecturers, research institute, students, and also there are 34 papers will be presented.

This International seminar is supported by the amazing people who always give financial as well as moral supports. My special thanks refer to the rector of Pattimura University, Prof. Dr. Thomas Pentury, M.Si, and the Dean of MIPA Faculty, Prof. Dr. Pieter Kakissina, M. Si. I also would like to express my deepest gratitude to Dr. Kotaro Ichikawa, the director of CSEAS Kyoto University, Prof. Bohari M. Yamin, University of Kebangsaan Malaysia, Prof. Dr. Budi Nurani Ruchjana (Prisident of Indonesian Mathematical Society/Indo-MS), Dr. Ir. A. Syailatua, M.Sc (Director of LIPI Ambon), and Hendry Ishak Elim, PhD as the key note speakers. We expect that this international seminar can give valuable information and contribution especially in developing basic science for sustainable marine development in the future.

Last but not least, we realize that as human we have weaknesses in holding this seminar, but personally I believe that there are pearls behind this seminar. Thank you very much.

Chairman

Dr. Netty Siahaya, M.Si.

Opening Remarks
By Dean of Mathematic and Natural Science Faculty

I express my deepest gratitude to The Almighty God for every single blessing He provides us especially in the process of holding the seminar until publishing the proceeding of International Seminar in celebrating the 17th anniversary of MIPA Faculty, Pattimura University. The theme of the anniversary is under the title Basic Science for Sustainable Marine Development. The reason of choosing this theme is that Maluku is one of five areas in Techno Park Marine in Indonesia. Furthermore, it is expected that this development can be means where the process of innovation, it is the conversion of science and technology into economic value can be worthwhile for public welfare especially coastal communities.

Having the second big variety of biological resources in the world, Indonesia is rich of its marine flora and fauna. These potential resources can be treated as high value products that demand by international market. Basic science of MIPA plays important role in developing the management of sustainable marine biological resources.

The scientific articles in this proceeding are the results of research and they are analyzed scientifically. It is expected that this proceeding can be valuable information in terms of developing science and technology for public welfare, especially people in Maluku.

My special thanks refer to all researchers and reviewers for your brilliant ideas in completing and publishing this proceeding. I also would like to express my gratefulness to the dies committee-anniversary of MIPA Faculty for your creativity and hard working in finishing this proceeding, God Bless you all.

Dean of Mathematic and Natural Science Faculty

Prof. Dr. Pieter Kakisina, M.Si.

Contents

The Use of Fish as Carbon Sources for The Production of Riboflavin (Vitamin B2) Using Eremothecium Gossypii

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ABSTRACT

Riboflavin or vitamin B2 is a vitamin which used as a nutritional, therapeutic, and livestock feed supplements.In techno economy, the cost of riboflavin production by fermentation were cheaper, less waste generated, and lower the energy required compared to the chemical and fermentation-chemical processes.In addition, the fermentation process also uses substrate from renewable sources.This study aims to produce riboflavin using fish as a substrate.Fish that contain fatty acids and proteins are expected to be a source of carbon for the production of riboflavin by fermentation using *Eremothecium gossypii*.The fermentation process was observed at 0-120 hours to obtain an optimal time and substrate for the production of riboflavin.Riboflavin was tested using a spectrophotometer at a wavelength of 445 nm.The results showed that the optimal time for the production of riboflavin was 72 hours.Glucose as a carbon source, optimal at 10 g/L with a number of riboflavin obtained amounted to 40.5 mg/L.The use of fish as a source of carbon, optimal at 10 g/L with the amount of riboflavin was 24.8 mg/L.The use of a mixture of glucose and fish as carbon sources, optimal at 10 g/L with the amount of riboflavin was 51.2 mg/L.

Keywords: Riboflavin, vitamin B2, *Eremothecium gossypii*, fermentation

INTRODUCTION

Riboflavin or more popularly known as vitamin B2, is used as a nutritional, therapeutic and also as a livestock feed supplement. Humans who lack this vitamin will experience hair loss, skin inflammation, and growth failure. This vitamin has also been successful as the treatment of migraine disease and malaria (Shrikant, 2006). Riboflavin is also required for the metabolism of tryptophan, an amino acid that is essential for growth in childhood. Riboflavin plays an important role in the transfer of electrons and it is the precursor of coenzyme flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), that is needed for the enzymatic oxidation-reduction reaction (Sybesma, et.al., 2004).

Every year, an estimated 3,000 tons of riboflavin are produced worldwide, 2500 tons are produced through fermentation (Choe, et al, 2005). In general, the production of riboflavin can be categorized into three methods, namely chemical synthesis, fermentation, and through biotransformation of glucose into D-ribose which is a combination of fermentation and chemical synthesis. Techno-economical environmental studies showed that riboflavin production through fermentation processes continues to increase due to lower production costs, less waste produced and lower energy requirement (Shrikant, 2006).

Several studies have shown that the two fungi from the classes ascomycetes, namely *Eremothecium ashbyii* and *Eremothecium (Ashbya) gossypii,*can produce more riboflavin through the fermentation process than other microorganisms such as *Saccharomyces cerevisiae, Candida famata*, or *Bacillus subtilis* (Kato et al., 2012; Suzuki et al., 2012). *Eremothecium ashbyii* and *Eremothecium (Ashbya) gossypii*can produce riboflavin as much as 20 g/L by using molasses and glucose as the carbon source. Thus, the number is much larger when compared with *Bacillus subtilis* (0.1 g/L), *Candida flareri* (0.6 g/L) and *Candida guilliermondii* (0.2 g/L) (Alosta, 2007). More interestingly, a sewage Active Bleaching Earth (ABE), the results of bleaching CPO (crude palm oil), can be used as a carbon source for the production of riboflavin by fermentation using *Ashbya gossypii* without having to waste the ABE (Park, et al, 2006).

The use of soybean oil as carbon source was able to increase the production of riboflavin 1.6-fold compared with no use of it. Several other carbon sources used for the production of riboflavin namely vegetable oil, whey and peanut seed cake (Lim, et al, 2003). Fish that contain fatty acids is expected to be a good source of carbon for the production of riboflavin by fermentation, given *Eremothecium (Ashbya) gossypii*is capable of cleaving lipid (fat) as a carbon source through the flow of fatty acids and store them as well as the formation of the riboflavin through the flow of glucose (Lim, et.al., 2001). This research was conducted for the production of riboflavin (vitamin B2) with a substrate of fish for consumption as a carbon source. Riboflavin fermentation was done by using*Eremothecium gossypii*.

RESEARCH METHODS

Microorganism

Eremothecium gossypii (Ashby et Nowell) Kurtzman (ATCC 10 895) was grown on potato dextrose agar (PDA)medium. 19.53 g of PDA were dissolved in 500 mL of sterile aquabidest to get a PDA medium. The pH was made to 6.8 before autoclave session. Then, the medium is poured into a 5 pieces of4 mL glass tube respectively and the rest of the medium was poured in the sterilized reagent bottle from autoclave at a temperature of 121°C, 103 kPa for 30 minutes. *Eremothecium gossypii* grown on medium, then incubated for 5 days at 37 ° C and stored at 4°C for two months of use.

Composition of Medium Production

Medium production was consisted of peptone 5.0 g/L; yeast extract 5.0 g/L; malt extract 5.0 g/L; potassium hydrogen phosphate (K_2HPO_4) 0.2 g/L; and magnesium sulphate ($MgSO_4.7H_2O$) 0.2 g/L (Ozbas and Kutsal, 1986). Glucose as the carbon source used at a concentration of 10.0 - 20.0 g/L. Simultaneously, fish meat will be added as many as -20.0 10.0 g/L in the production process.

Production of Riboflavin

The determination of the growth curve was performed to observe the mid-log phase of*Eremothecium gossypii*. A total of 30 mL *Eremothecium gossypii*culture was inoculated into 270 ml medium in 1 L erlenmeyer. The carbon source of glucose, then incubated at 30 ° C in an incubator which rotated at 120 rpm. A total of 5 mL samples were analyzed every 24 hours for biomass monitoring.

Riboflavin production was using the same growth medium with added glucose and fish meat. For comparison, the riboflavin production uses also glucose only medium and then only use fish meat as well. Biomass will be analyzed after a constant interval at 24 hours using gravimetric method.

Analysis of Riboflavin

To determine the concentration of riboflavin, 0.5 ml of the culture broth was mixed with 4.5 ml of distilled water and centrifuged at 1000 g for 10 minutes. Supernatant was separated and mixed with 0.8 ml of 1N NaOH and 5 ml of 50 mM phosphate buffer (pH 7.0) and then centrifuged at 9000 g for 5 minutes. Supernatant was separated, and tested using a spectrophotometer at a wavelength of 445 nm.

RESULTS AND DISCUSSION

Growth curve ofEremothecium gossypii

*Eremothecium gossypii*growth was done with biomass as early as 0.06 g/L with a growth rate of a maximum of 0.03 mg/L. Calculation of the growth rate was based on a formula:

$$\mu = \frac{2.3\,(\log X - \log Xo)}{t} \qquad (1)$$

With μ is the growth rate, X is the biomass at a given time, Xo is the initial biomass, and t is the determination time of the biomass.

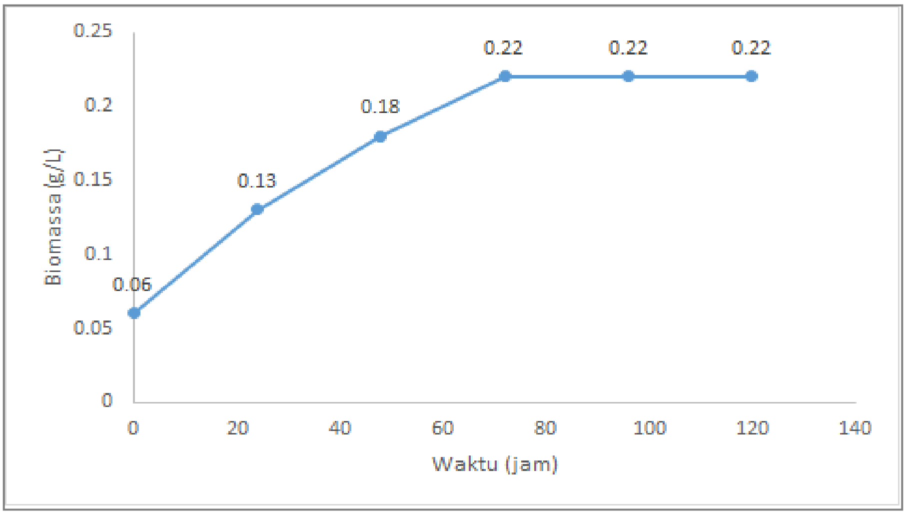

Figure 1. Graph of *Eremothecium gossypii*growth for 120 hours

Growth chart (Figure 1) shows that the lag or starters phase are not on the curve, as the chart patterns of growth in general. The absence of lag phase is due to the occurrence of *Eremothecium gossypii*adaptation with the medium, so that it immediately follow a process of growing, given the utilized medium is specific for growth of*Eremothecium gossypii*. Stationary phase are in the 72 hours up to 120 hours.This time frame is used as a reference for the production of riboflavin in the range of 3-5 days. From the data it is known that the growth charts mid-log phase between the initial phase of growth until the end of the log phase is 36 hours. This time is used as a reference for manufacturingthe inoculum that is used for the production of riboflavin. The best growing conditions is in the mid growth duration, because the enzymes that play a role in growth has been producing primary metabolites that are needed to grow the microbes.

The standard curve of Riboflavin

Riboflavin analysis was performed with spectrophotometer at a wavelength of 445 nm. the wavelength was obtained from the standard riboflavin at a concentration of 50 mg/L. Standard curve (Figure 2) is made to measure the concentration of fermented riboflavin, obtained from the linearity calculation with R^2 value of 0.9989 which indicates a very high level of curve accuracy. Riboflavin concentration value calculation was done using the formula:

$$y = mx + c \quad (2)$$

With y as absorbance of riboflavin, m is the coefficient of riboflavin concentration of 0.0394, x is the concentration of riboflavin and c is a constant with a value of 0.0177.

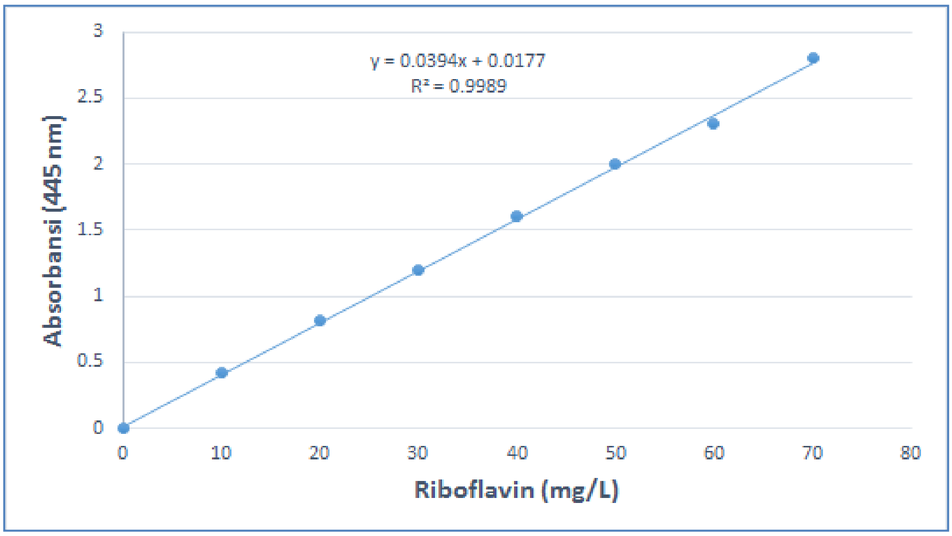

Figure 2. Standard curves of riboflavin in 0.02 M acetic acid with R^2=0.9989 which will be used to calculate the concentration of fermented riboflavin.

Production of Riboflavin Using Glucose

Riboflavin production with carbon sources of glucose can be seen in Figure 3. At the beginning of the fermentation there is just a little bit concentration of riboflvin, this can be due to the utilized inoculum was in the mid-log phase so the *Eremothecium gossypii* has produced secondary metabolites.

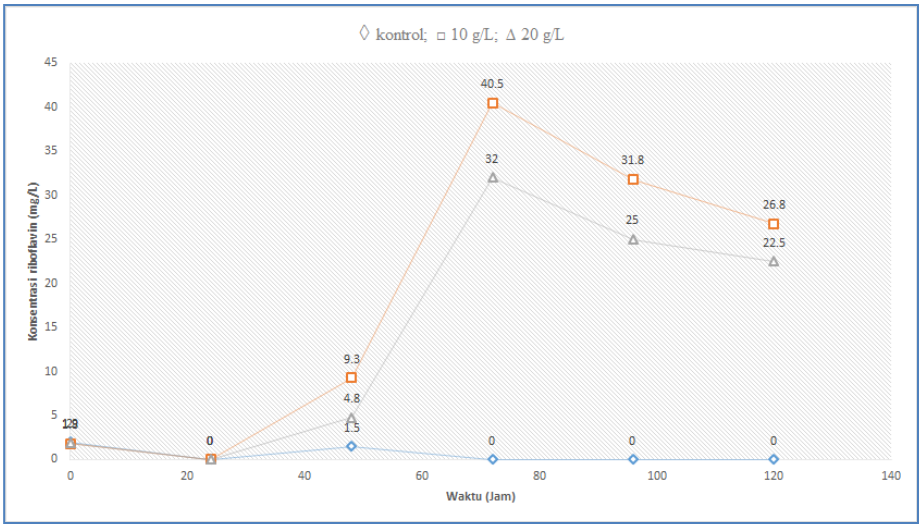

Figure 3. Production of riboflavin by *Eremothecium gossypii* using a carbon source of glucose with different concentrations. ◊ without glucose control; □ glucose 10 g/L; Δ glucose 20 g/L.

At 24 hours fermentation time,the riboflavin was not found.It is because riboflavin wassince 0 hours reused by *Eremothecium gossypii* to grow. At 48 hours fermentation time , it began to find little bit of riboflavin concentration. The optimum time of production of riboflavin is found at 72 hours which has the highest concentration of 40.5 mg/L with the use of 10 g/L glucose. Thus, the use of 20 g/L glucose only reached 32 mg/L, and glucose control was seen without visible signs of riboflavin. The high production of riboflavin at 10 g/L of 20 g/L glucose is too excessive so as the time to achieve stationary conditions for producing secondary metabolites will become longer. At the controls without glucose, riboflavin production will be re-used to be a source of carbon when riboflavin is not found.

At 96 hours and 120 hours fermentation time, it appears that riboflavin production begins to decline and this is because riboflavin was produced from reused by *Eremothecium gossypii* as an additional source of carbon as the glucose fermentation depleted over time.

Production of Riboflavin Using Fish

Riboflavin production with carbon source of fish at the start of fermentation to 24 hours appears that there was a small amount of riboflavin and none at all (Figure 4). At 48 hours riboflavin production control looks no different from the controls on the production of riboflavin with glucose as the carbon source. Visible difference to the number of fish substrate was between 10 g/L and 20 g/L. Thus, the production of riboflavin is much smaller due the use of glucose and this is because the process of adaptation to break the fish into a carbon source. The process of decomposition of fish can occur because *Eremothecium gossypii* has protease and lipase enzymes to degrade proteins and fats.

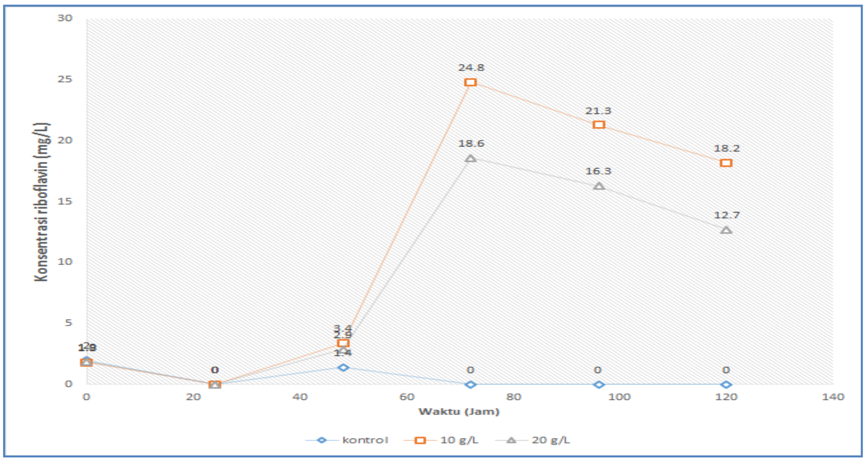

Figure 4. Production of riboflavin by *Eremothecium gossypii* using carbon sources of fish with different concentrations. ◊ control without fish; □ fish 10 g/L; Δ fish 20 g/L

The optimum fermentation time is at 72 hours with a greater amount of riboflavin at 10 g/L of fish amounted to 24.8 mg/L, whereas at 20 g/L of fish reached 18.6 mg/L. At 96 hours and 120 hours fermentation time, it appears that the production of riboflavin becomes less and this is because of riboflavin production by *Eremothecium gossypii*was reused as an additional source of carbon considering carbon source of fish began to diminish over time of fermentation.

Production of Riboflavin Using Glucose and Fish

The use of carbon sources of glucose and fish together turned out to produce riboflavin more than the utilization of glucose and fish separately. As shown in Figure 5, the optimum time is at 72 hours fermentation time with the amount of riboflavin produced amounted to 51.2 mg/L for the number of fish were added by 10 g/L. This result is greater than the result of the addition of the fish at 5 g/L, 15 g/L and 20 g/L. This is due to parse the fish as a source of carbon necessary for protease and lipase enzyme which of course would be optimal at 10 g/L of fish.

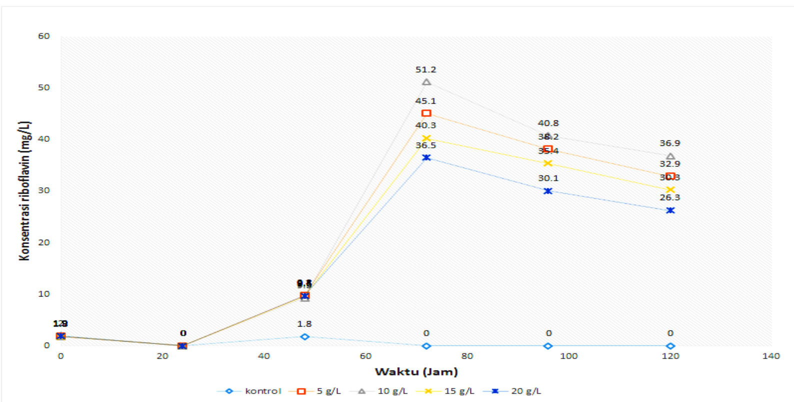

Figure 5. Production of riboflavin by *Eremothecium gossypii* using carbon sources glucose 10 g/L and fish with different concentrations. ◊ control; □ fish 5 g/L; Δ fish 10 g/L; x fish 15 g/L; x fish 20g/L

At 96 hours and 120 hours fermentation time it appears that riboflavin production begins to decline and this is because riboflavin is produced from by *Eremothecium gossypii* as an additional source of carbon considering carbon sources of glucose and fermented fish depleted over time.

When viewed from the pathway by *Eremothecium gossypii* riboflavin formation, there are two lines of riboflavin formation (Figure 6), the first track of the pathways of glucose to be converted into 3-phosphoglycerate (3PG) and then through a pathway in mitochondrial pyruvate is converted into guanine triphosphat (GTP) which is a starter of riboflavin formation. The second path is from the path of triglycerides (fats) by a lipase that will be converted into fatty acids, through the cytoplasm of the fat will be taken to peraxisome converted into malate and then becomes guanine triphosphat.

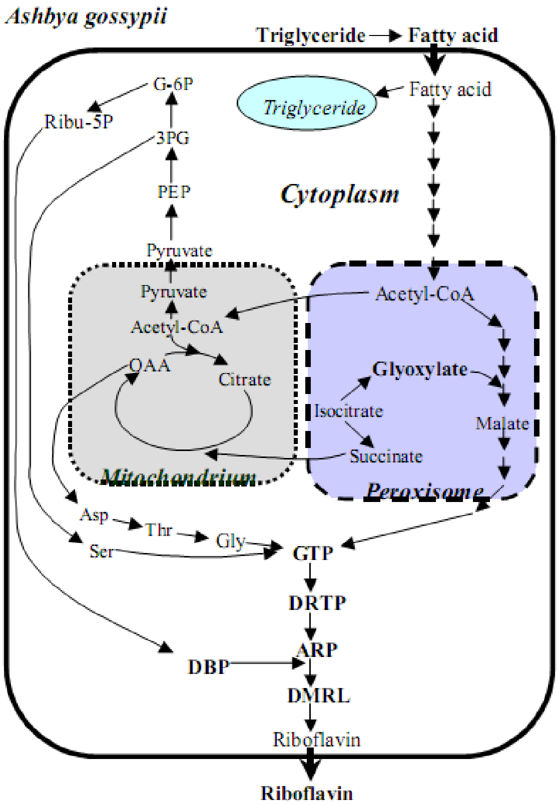

Figure 5. The model of metabolism riboflavin production using *Ashbya gossypii.*Abbreviations: G-6P, Glucose-6-phosphate; 3PG, 3-phosphoglycerate; PEP, Phosphoenolpyruvate; Ribu-5P, Ribulose-5-phosphate; OAA, Oxaloactate; Asp, Aspatate; Thr, Threonine; Gly, Glycine; Ser, Serine; GTP, Guanosine triphosphate; GMP, Guanosine monophosphate; XMP, Xanthine monophosphate; IMP, Inosine monophosphate; DRTP, 2, 5-diamino-6-ribosylamino-4 (3H)-pyrimidinedione 5-phosphate; ARP, 5-amino-6-ribitylamino-2, 4 (1H, 3H)-pyrimidine; DBP, L-3, 4-dihydroxy-2-butanone-4-phosphate; DMRL, 6, 7-dimethyl-8-ribityllumazine(Lim, et al, 2001)

These two pathway of riboflavin production can be improved by mixing the glucose and fish substrates, so it appears that the optimum time of 72 hours of use of the glucose and fish substrate will produce riboflavin (51.2 mg/L) greater than the optimum time of 72 hours with the substrate glucose (40.5 mg/L) and fish (24.8 mg/L).

CONCLUSIONS AND SUGGESTIONS

Conclusion

The use of fish substrates as a carbon source for the production of riboflavin can be done through a fermentation process using *Eremothecium gossypii*. Optimal time needed for the production of riboflavin is at 72 hours. The use of glucose as a source of carbon substrates was optimally at 10 g/L with a number of riboflavin obtained at 40.5 mg/L. The use of fish as the optimal carbon source at 10 g/L with the amount of riboflavin 24.8 mg/L. The use of a mixture of glucose and fish substrates as carbon source optimally at 10 g/L with the amount of riboflavin produced amounted to 51.2 mg/L.

Suggestions

Riboflavin production is necessary to be scaled for greater capacity to see the time and the optimum substrate to approach industrial conditions. To increase the production of riboflavin, itneeds to conduct*Eremothecium gossypii* mutations to optimize and stabilize enzymes that play a role in the production of riboflavin.

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