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THE TIME VARIATION OF *Saccharomyces cerevisiae* INOCULATION IN SIMULTANEOUS SACCHARIFICATION AND FERMENTATION OF COCOA (*Theobroma cacao* L.) POD FOR BIOETHANOL PRODUCTION

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ABSTRACT

*Lignocellulosic cocoa (Theobroma cacao*L.*)podswereagricultural waste product. That materialwas rarely usedtoproducebioethanolas alternativebiofuelsthat were environmentally friendly. The process ofbioethanol productionfromcocoapodscould bedone throughsimultaneoussaccharification andfermentation SSF). The objectiveof this studywas todetermine the effect oftime variation of Saccharomyces cerevisiaeinoculationtosimultaneoussaccharification andfermentation ofcocoapodsbiomassforbioethanolproduction. The resultsindicate thatcocoapodscould producebioethanolwiththe highestcontent of bioethanol (4.33%w/w)inthe 6th day of S. cerevisiaeinoculation.*

Keywords: Bioethanol, Cocoa pod, *Saccharomycescerevisiae*, Simultaneous saccharification and Fermentation.

INTRODUCTION

Increasing costs of fossil fuels and their greenhouse gases emission effects are creating a dire need to explore cheaper and environment friendly biofuels as a strategy for reducing global warming (Iqbal and Kamal, 2012). In addition, the unfettered use of fossil fuels showsnegative impacts on the environment because of emissionof greenhouse gases (CO2, CH4 and CO) resulting inglobal warming and pollution.Hence, the overwhelming scientific evidence was that theunfettered use of fossil fuels has caused the world'sclimate to change, with potential disastrous effect (Ganesh *et al.*, 2012).

Rising concern over depleting fossil fuel andgreenhouse gas limits has resulted in a high level of interest innon-conventional fuel originating from biorenewable sources including sugars, starches and lignocellulosic materials (Limayema *et al.*, 2012). Lignocellulosic biomass provides a noteworthy solution in respect to the direct competition with food stuff, therefore, should be the

favored as a raw material for liquid biofuels of future (Iqbal and Kamal, 2012). Lignocellulosic biomass is primarily composed ofcellulose, hemicellulose, and lignin. Cellulose and hemicelluloseare the main substrates used for ethanol production, but lignin is composed of aromatic lignols that need to beseparated and removed before enzymatic hydrolysis (Jessen and Orlygsson, 2012).

During the last decade, the production of ethanol from biomassmaterials received moreattention in the worldwide (Limayema *et al.*, 2012). One potential method for the lowcost fermentative production of ethanol is to utilize lignocellulosic or agroindustrial waste materials (e.g. wood, straw, switch grass, banana waste, wheat straw, rice straw, corn Stover, corn cobs, sugar cane bagasse, apple pomace, orange peel, and paper waste) because they contain carbohydrates that must be first converted into simple sugars (glucose) and then fermented into ethanol (Iqbal and Kamal, 2012). Bioethanol from lignocellulosic biomass is one of the important alternatives being considered due to theeasy adaptability of this fuel to existing engines and because thisis a cleaner fuel with higher octane rating than gasoline. Lignocellulosic biomass is considered asthe only foreseeable feasible and sustainable resource for renewablefuel (Sukumaran *et al.*, 2010). Ethanol contains 35 per cent oxygen that helps completecombustion of fuel and thus reduces particulate emissionthat pose health hazard to living beings (Raji *et al.*, 2008). The production of fuel ethanol from biomass involves prehydrolysis, hydrolysis, fermentation,and distillation (Nigam, 2002).

The biological conversion of bioethanol from lignocellulosic biomass can be achieved by simultaneous saccharification and fermentation (SSF) process. SSF is a good strategy for increasing the overall rate of cellulose to bioethanol conversion. In SSFprocess both cellulose hydrolysis and fermentation ofglucose are carried out in presence of fermentative microorganisms in a single step and the processoptimally operates at 37 to 38°C. This technique reduces the number of steps in the process, and is a promisingway for converting lignocellulose to bioethanol(Joshi *et al.*, 2011).

For microbial hydrolysis, *Trichodermaviride* can produce cellulolytic enzyme such as cellulose and hemicellulose. One of the most extensively studied of cellulolytic microorganism which is also industrially used for enzyme production (Thanapimmetha *et al.*, 2011). Meanwhile*Saccharomyces cerevisiae* is well known yeast for its fermentation capacity and hence canbe employed for alcohol production from various sugarcontaining materials.

The bulk of waste from cocoa processing industriesespecially the pod that is usually discarded after the fruithas been removed. A cocoa fruit on the average contains about 20 to 60 seeds (usually called cacao beans) whichare embedded in the white pulp. The cocoa pod makes up about 75% of the total weight of the fruit andbecomes an agricultural waste, and a health hazard forthe healthy immature cocoa pods, as it harbors cocoa stem borers (Adeleke *et al.*, 2012).

Time offermentationis one of thefactors that influenceoutcomesandcontent ofbioethanolproduced at the SSF. The result of study, Anindyawati (2009) showed the highest bioethanol productionthroughSSFis2.709g.L^{-1}or 4.7% permass ofbagassefor72-96hours. According Komarayati and Gusmailina (2010) in his study, bunches of empty fruit of oil palmsubstratefor 72hours with theconversion ofsugars intoethanolat47.32%. The results (Sunardi., 2010) showed thattheoptimumfermentation timeis 7daysafterdistillationat a temperature of80^{o}Cderivedbioethanolwith10%contentofwaste product of tofu.

Based on the above explanation, cacao pods can be used as raw material for the production of bioethanol. Making bioethanol from cacao pods can be done through simultaneous saccharification and fermentation with utilize *Trichoderma*sp into account as a source of cellulolytic enzymes and *Saccharomyces cerevisiae* as an appliancefor alcohol fermentation from saccharified liquor extracted.

MATERIALS AND METHOD

The Raw Material

The material used wascacao pods from gardenin HutaLomang village, Padangsidimpuan, South Tapanuli, North Sumatera,pure culture of *Trichodermaharzianum* and *Saccharomyces cerevisiae* from the microbiology laboratory Bandung Institute of Technology, PDA (Potato Dextrose Agar), distilled water, alcohol, buffered phosphate pH 5.5, PDB (Potato Dextrose Broth), HCl, NaOH, 1% NaOCl, NaOH 15%, NPK, ZA.

The Experimental Design

The study was conducted from August to September 2012, in Biology Laboratory of Science and Technology Faculty of State Islamic University, Bandung, Indonesia.The design of experiments in this study was using completely randomized design with variations in timing inoculation of *Saccharomyces cerevisiae*consistingtimesofinoculationi.e. day 1 (H1), 2 (H2), 3(H3), 4 (H4), 5(H5), 6(H6), 7(H7) and 8 (H8). Every treatment replicate 3 times so the number of experimental units are a total of 24 units.

Preparation of Cacao Pods

Cacao pods washed, cut into pieces and dried in the sun then smoothed using a blender. The substrate of cacao pods in delignification using 1% NaOCl for 5 hours at 28ºC. Substrates that have been washed, filtered and dried and then soaked in 15% NaOH for 24 hours at 28 º C. Next the substrate was dried at a temperature of 50 º C for 48 hours, so that the resulting substrate cacao pods.

Simultaneous Saccharification and Fermentation (SSF)

A total of 7 g substrate cacao pods have delignification plus 5.5 g substrate cacao pod without delignification added 40 ml phosphate buffer pH 5.5 and 40 ml nutrient (PDB). Substrate pH

regulated in 7.00, using HCl and NaOH after it entered into the container. Furthermore sterilized at 121^{o}C for 15 minutes. Suspension *Trichodermaharzianum* as much as 10% (v / v) were inoculated into SSF media and incubated at room temperature for 3 days. After 3 days, SSF media added with NPK and ZA fertilizers 0.04 g and 0.15 g respectively. 10% of the total volume of *Saccharomyces cerevisiae* included in the SSF under semianaerobic condition for 8 days.

Analytical Method

The results of filtrate and solid was measured. Further distillation of filtrate was done to separate the ethanol from other materials. Distillate must be clear and not contain other essential oils. Specific gravity distillation using a Pycnometer. Then calculating the specific gravity of the liquid with the formula (Horwitz *et al.*, 1970):

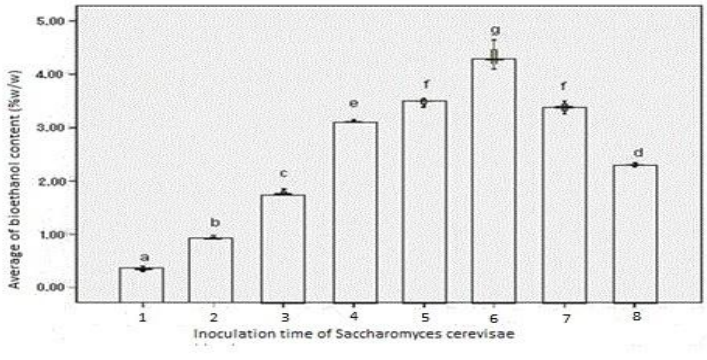
Description: B = Weight of empty Pycnometer, B1 = Weight of Pycnometer + distilled water, B2 = Weight of Pycnometer+ Sample.

The volume of fluid was measured using a measuredtube and weighed with an analytical balance. Data analysis used the analysis of variance, and if there was a real difference of treatment then tested further by Duncan Multiple Range Test (Gomez and Gomez, 1995).

RESULTS AND DISCUSSION

The resultsof thebioethanolcontentobtainedfromcacao podsthrough simultaneous accharification andfermentation(SSF)processusing *Trichodermaharzianum*and*Saccharomycescerevisiae*demonstrates the value ofwhich variesbased onthe dataanalysisof variance.

Figure-1. Effect oftime ofinoculation of*S.cerevisiae*tobioethanolcontent (Bars indicated as means and followed by same letter are not significantly different atp < 0.05ofDuncan'sMultipleRangeTest)



Shown in Figure1, thetimeof inoculationof *S.cerevisiae*producesincreased ethanolcontentafter a fewdaysthe fermentation, whereasinoculationat day0 yieldsthe smallestcontent ofbioethanol. According Anindyawati (2009) the concentration ofethanolproducedis strongly influencedby temperature, pH, carbon source, nitrogensourceandincubation timeof each of

themicrobesduringfermentation. This is because thesimultaneoussaccharification and fermentation(SSF) processinflouncefungalgrowth and metabolismof *T.harzianum*and*S.cerevisiae*.

Averagethe highestcontent ofethanol(4.33%w/w)on day6 of *S.cerevisiae*inoculation showed thatSSFlastedoptimum. The lowestcontent of ethanol (0.35%w/w)at day1of *S.cerevisiae*inoculation. According (Raji *et al.*, 2008) SSFlastsfor 7dayswhiletheoptimumfermentationlasts 3days andtolerancelimits*S.cerevisiae*fermentsugarfor 6 daysdependingon thenutrients available. Allowinga changeof metabolitesofethanol were convert to acetic acidandothercompounds.InSSF, the material left behindduringsaccharificationstill allowingit fermentssimple sugarsformationby*T.harzianum*thoughalreadyentered the stage offermentation ofsugar intoethanolby*S.cerevisiae*. In addition,mold*T.harzianum*livein a state ofsemi-aerobic and anaerobic

On day1of *S.cerevisiae*inoculationindicatesthe growth ratewas quiteslow andevenhampered, because the anaerobic condition ofsugarfermentation led to decrease growthof*T.harzianum*andjustsupportedbythe rest ofthe availableairin thefermentationcontainer. In this regard, the optimum work of *T.harzianum*seenwhenitentered thepeakgrowth.

Inanaerobic condition*T.harzianum*could stillmetabolizeremaining lignocelluloseon the growth ofday2untillday5of *S.cerevisiae*inoculationwhere nutrientsand environmental conditionsas well as the ongoingfermentation time successivelyincrease content ofbioethanolproducedassaccharificationprocesswas longerintreatmentso gaveeffect toproduction of bioethanol content.

Meanwhile,on day7 and8of *S.cerevisiae*inoculationshowed decreased content ofbioethanolthesemightfermentation processthat lastsfora very shortat thetime of optimumsaccharificationwas taken place. This led to the result ofsaccharification cannot be fermented entirely intobioethanol. Bioethanolproducedoptimumwhensimultaneoussaccharification and fermentationprocesstakes placein a timely manner, resulting in the production ofglucoseinsaccharificationprocessesin linewith the result ofthe fermentation of sugarsto formethanolthusobtainedmaximum results.

CONCLUSION

The substrate from cacao pods could be used as ethanol production through simultaneous saccharification and fermentation process. Based on analysis of variance showed that the variation oftime of *Saccharomyces cerevisiae*inoculationgave real effect on bioethanol content. The highest content of bioethanol produced by treatment of the 6th day of *S.cerevisiae* inoculation with an average of 4.33% w/w, while the lowest content of bio ethanol produced by treatment of the 1th day of *S.cerevisiae* inoculation with an average of 0.35% w/w.

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