# ETHANOL PRODUCTION AND SUGARS CONSUMTION OF CO-CULTURE *Saccharomyces cerevisiae* FNCC 3012 WITH *Candida tropicalis* FNCC 3033 IN MEDIA CONTAINING INHIBITORS FERMENTATION

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Inhibitor fermentation is one of the problems that arise in the ethanol production from lignocellulose waste. This work examined ethanol yield and sugar consumption of mono and co-culture *S. cerevisiae* with *C. tropicalis* in media containing inhibitors fermentation. Furfural and phenol were used inhibitor fermentation in basal medium with concentration 2.0 and 5.0 %, respectively. The basal medium contained 20 $g.L^{-1}$ glucose, 20 $g.L^{-1}$ D (+) xylose, 20 $g.L^{-1}$ arabinose, 4 $g.L^{-1}$ urea, 3 $g.L^{-1}$ $NaNO_3$, 3 $g.L^{-1}$ $NH_4NO_3$, 1 $g.L^{-1}$ $KH_3PO_4$ and 0.7 $g.L^{-1}$ $MgSO_4{\cdot}7H_2O$ and pH adjusted 5.5 with 1 $mol.L^{-1}$ HCl. After furfural or phenol addition separately, and inoculated by mono and co-culture *S. cerevisiae* FNCC 3012 with *C. tropicalis* FNCC 3033, all media incubated at 28–29°C, 50% r.h. in the dark for 5 days in a rotary incubator at 60 rev min. We found yeast colony count, sugar consumption, ethanol yialds and efficiency of fermentation by co-culture *S. cerevisiae* with *C. tropicalis* higher than mono-culture *S. cerevisiae* or *C.tropicalis* in the fermentation media with or without inhibitors. This work indicated that co-culture *S. cerevisiae* with *C. tropicalis* more tolerance to furfural and phenol. In basal medium, basal medium plus 2.5 or 5.0% furfural, ethanol yield 8.52%, 5.37% and 3.83%, respectively and efficiency fermentation 27.00%, 17.00% and 12.20%. In basal medium, basal medium plus 2.5 or 5.0% phenol, ethanol yields 8.13%, 5.62% and 3.19%, respectvely and efficiency fermentation 28.20%, 20.00% and 14.00%. Co-culture *S. cerevisiae* FNCC 3012 with *C. tropicalis* 3033 demonstrated potential as a fermentation process for ethanol production from lignocelulosic medium or media content inhibitors. The use of this co-culture effectively utilize hexose and pentose sugars in the substrate, increasing the yield and efficiency of fermentation for ethanol production.

Key words : co-culture, fermentation, ethanol, inhibitor, *S.cerevisiae*, and *C.tropicalis*

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## INTRODUCTION

The fossil fuels limitation and environmental condition have attention developing alternative energy sources that lower impact on the environment (**Abreu-Cavalheiro and Monteiro, 2013**). Ethanol is an alternative energy source has the potential to replace fossil energy sources and received high attention over the past few years (**Chen, 2011**). Ethanol can be produced from various agricultural raw materials including lignocellulose (**Balat, 2011; Tesfaw and Assefa, 2014**). Due to renewability, large quantities, relatively low prices than grain or sugar, and potential environmental benefits, lignocellulosic biomass has been considered as possible raw material for ethanol production (**Cardona and Sa´nchez, 2007; Kumar *et al*., 2008; Lee and Huang, 2000; Mielenz, 2001; Service, 2007; Zaldivar *et al*,. 2001**). Preferably lignocellulosic biomass as raw material for ethanol production compared with sugar or starch derived from crops products because it does not compete as food needs and concern the utilization of agricultural residue (**Gutiérrez-Rivera *et al*,. 2012; Ishola *et al*,. 2014**).

Efficient fermentation of ethanol production from lignocellulosic biomass is affected by the consumption of glucose and xylose which is the main product of lignocellulosic hydrolysates (**Lee and Huang, 2000; Service, 2007, Eiteman *et al*., 2008**). However, the lack of a microorganism able to ferment efficiently all sugars released by hydrolysis from lignocellulosic materials has been one of the main factors preventing utilization of lignocellulose (**Zaldivar *et al*., 2001**). *Saccharomyces cerevisiae* is the dominant yeast used for ethanol production can not metabolize xylose and convert it into ethanol (**Jeffries and Jin, 2004; Lin and Tanakan, 2006**).

In addition, others problem associated with efficient conversion of cellulose and hemicellulose sugars to ethanol is that during dilute acid hydrolysis a broad range of compounds which inhibit the fermenting microorganism are liberated or formed along with the sugars (**Larsson *et al*., 2001**). The presence of inhibiting compounds, such as weak acids, furans and phenolic compounds that formed or released during thermo-chemical pre-treatment step such as acid and steam explosion can decreased ethanol yield and productivity of fermentation lignocellulosic (**Parawira and Tekere, 2011**). Reducing in the ethanol yield and productivity by

inhibiting components can be caused influence on the performance of microorganisms during fermentation step (**Almeida *et al*., 2007**).

The choice of the fermenting microorganism, complete substrate utilization, inhibitor tolerance and ethanol productivity are important aspects in ethanol production from lignocellulose (**Bettiga *et al*., 2009**). Microorganisms that consume sugars such as glucose and xylose sequentially must have lower productivities for the generation of a product than if the organism were to consume the sugars simultaneously (**Zaldivar *et al*., 2001**). For economical bioethanol production from lignocellulosic materials, The microorganism should use efficiently all glucose and xylose in the lignocellulose hydrolysate and the microorganism should have high tolerance to the inhibitors present in the lignocellulose hydrolysate (**Cheng *et al*., 2014**).

Strategies of using a single microorganism to convert glucose and xylose simultaneously have limitations (**Eiteman *et al*., 2008**). The co-culture among microorganisms could potentially increase ethanol production and efficiency of fermentation from hydrolyzate lignocellulosic hydrolysate. Co-culture of *S. cerevisiae* and other microorganism reduced inhibitory compounds in lignocellulosic hydrolysates (**Taherzadeh *et al*., 2013; Wan *et al*., 2012**), increased ethanol yield and production rate (**Singh et al., 2014; Wan 2012**), shortens fermentation time, and reduced process cost (**Hickert et al., 2013; Tesfaw and Assefa, 2014**). Co-culture *S.cerevisiae* with *C. tropicalis* have ability to generate and convert fermentable sugars from a waste stream rice husk to ethanol (**Sopandi and Wardah, 2015**). This work examined ethanol yield and sugar consumption of mono and co-culture *S. cerevisiae* with *C. tropicalis* in medium containing inhibitors fermentation.

## MATERIAL AND METHODS

### Culture microorganism

*Saccharomyces cerevisiae* FNCC 3012 and *Candida tropicalis* FNCC 3033 were obtained from Microbiology Laboratories, PPAU Gadjah Mada University, Yogyakarta, Indonesia. Saubroad agar (Oxoid) was used to maintain the strain *S. cerevisiae* and *C. tropicalis*. Working stock cultures were prepared from stock, 7 days, 28$^o$C SA plate cultures subcultures from the master stock. Colonies were aseptically sampled by scraping the top with an inoculating loop and transfer to 10 ml sterile water. Inoculums stock suspension were prepared from working stock and diluted to 1.7 x $10^6$ cell.$mL^{-1}$, as enumerated using a haemocytometer.

### Fermentation

The batch fermentation experiments were carried out in 250 mL Erlenmeyer flask with working volumes of 100 mL. The basal medium contained 20.0 g.$L^{-1}$ glucose, 20.0 g.$L^{-1}$ D (+) xylose, 20.0 g.$L^{-1}$ arabinose, 4.0 g.$L^{-1}$ urea, 3.0 g.$L^{-1}$ $NaNO_3$, 3.0 g.$L^{-1}$ $NH_4NO_3$, 1.0 g.$L^{-1}$ $KH_3PO_4$ and 0.7 g.$L^{-1}$ $MgSO_4{\cdot}7H_2O$ and pH adjusted 5.5 with 1.0 mol.$L^{-1}$ HCl. Liquid basal medium (9.0 l) were mixed thoroughly and 100 ml individually dispensed into 250-mL Erlenmeyer flasks, autoclaved, cooled to room temperature. Media in Erlenmeyer was divided into 2 groups, furfural was added to individual flasks and final concentrations of 0.0, 2.5 and 5.0% into ones group and another group, phenol was added to individual flasks and final concentration of 0.0, 2.5, and 5.0%. Aseptically dispensed into individual Erlenmeyer flasks, 1.0 mL inoculum stock suspension of *S. cerevisiae* and 1.0 mL *C. tropicalis* for mono culture, respectively and 0.5 mL *S. cerevisiae* with 0.5 mL *C. tropicalis* for co-culture added into the flasks and incubated at 28–29°C, 50% r.h. in the dark for 5 d in a rotary incubator at 60 rev min. This inoculation and incubation method was used for all cultivation in this study.

### Yeast count

To observed yeast count, 10 mL media before and after fermentation (5 d) was pipetted and serial dilution $10^{10}$ in sterile water. Each serial dilution (0.1 mL) inoculated and spread onto Saubroad agar (Oxoid), incubated at 28–29°C, 50% r.h. in the dark for 3 d. Colony counter was used to counting colony on media.

### Determination of ethanol

Ethanol were measured using a gas chromatograph Carbomax t70-10-0 column, FID t220 detector, helium as carrier gas with flow rate 40.3 mL.$min^{-1}$, tin column Porapack Q, detector temperature at 160$^o$C and column temperature at 180$^o$C with injection volume 1.0 µL. Fermented media were filtered through Whatmann No.1 paper.

### Determination of sugar

Glucose, D(+) xylose and arabinose were determined using HPLC (Shimadzu, Kyoto, Japan) at 85$^o$C, a Metacharb 87C column, $H_2O$ as eluent, flow rate 0.6 mL/min and RID detector. After fermentation, medium was mixture and aseptically filtered through

Whatmann No.1 paper. Filtrate were centrifuged at 12,000 rpm for 15 min, refiltered through millex 0.45 µm and 25 µL of sample injected to HPLC. Standard was used glucose, D(+) xylose and arabinose (Merck) with concentration 62.5, 125, 250 and 500 ppm, respectively, as a standard.

**Efficiency fermentation**

To determinate of efficiency fermentation ethanol production by mono and co-culture *S. cerevisiae* and *C. tropicalis*, we used formula:

$$EF_{biomass}(\%) = \frac{\text{Ethanol yields (L)}}{\text{Amount sugar before fermentation (g) x 0,511}} \times 100$$

**Statistical analysis**

Tukey's honestly significant difference multiple comparison test and paired sample t-test were used to segregate significantly different treatment using SPSS 16 software. Analysis variance (ANOVA) was performed to determine significant differences between experiments ($P<0.05$).

**RESULTS AND DISCUSSION**

**Yeast count**

Addition of furfural (Fig 1A) to the growth medium significantly ($p<0.05$) decreased yeast colony count in mono or co-culture *S. cerevisiae* with *C. tropicalis*. This work indicated furfural inhibits growth of yeast in mono-culture and co-culture *S. cerevisea* with *C. tropicalis*. Some investigators have reported the effect of furfural on the growth of microorganisms. **Palmqvist and Hahn-Ha¨gerdal (2000)** reported furfural inhibited on the specific growth and fermentation rate of yeasts. **Agbogbo *et al.* (2007)** reported concentration of furfural 1.5 $g.L^{-1}$ could interfere respiration and growth of microorganisms. **Hristozova *et al.* (2000)** reported concentration furfural 0.04% inhibited glutamate dehydrogenase and ion ammonia assimilation at alanine metabolism of *C.blankii* 35 and *C. pseudotropicalis* 11. **Kelly *et al.* (2008)** reported concentration furfural 1 $g.L^{-1}$ or higher inhibited growth of *C. guilliermondii*. **Jones (1989)** and **Almeida *et al.* (2009)** suggested furfural and 5-hydroxymethyl furfural (HMF) can consume by *S. cerevisiae* with consequence ATP sole. **Mattam *et al.* (2016)** reported increasing of the level of furfural, HMF and acetic acid in growth media led to gradual decrease in *C. tropicalis* biomass.

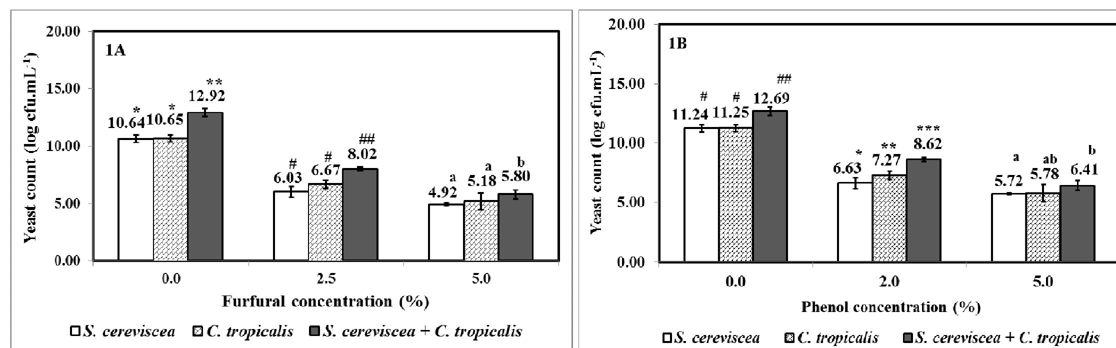


Figure 1 Yeast count in medium plus different concentration of furfural (1A) and phenol (1B) after five days fermentation by mono and co-culture *S. cerevisiae* with *C. tropicalis,* the values are means of 5 independent observations in same furfural concentration different superscripts ($^{a}$ and $^{b}$, * and ** or $^{\#}$ and $^{\#\#}$ ) differ significantly ($p<0.05$).

However, this work show yeast count colony in co-culture *S. cerevisiea* with *C. tropicalis* significantly ($P<0.05$) higher than mono-culture *S. cerevisiae* or *C. tropicalis* in the medium growth with or without furfural. We hypothesized there are synergistic mechanism to stimulate growth yeast through simultaneous utilization of fermentable sugars such as glucose and xylose and degrade furfural in growth medium by co-culture *S. cerevisiae* with *C. tropicalis.* Some investigators have been reported consumption of fermentable sugars (glucose and xylose) simultaneously by co-culture fermentation. **Hikert *et al.* (2013)** reported co-culture *C. shehatae* HM 52.2 with *S. cerevisiae* ICV D254 can simultaneously consumed glucose and xylose in synthetic medium and rice hull hydrolysate. **Fu and Peiris (2008)** reported co-culture *Zymomonas mobilis* with *Pachysolen tannophilus* fully consumed in a mixture of glucose and xylose media. Some investigators have been reported the degradation and conversion of furfural by yeast.

**Taherzadeh *et al.* (1999)** reported *S. cerevisiae* converted furfural to furfuryl alcohol at exponentially growing cells. **Cheng *et al.* (2014)** reported *C. tropicalis* W103 degraded furfural and 5-hydromethylfurfural under aerobic conditions after 60 h aerobic incubation. Some investigators reported co-culture *S. cerevisiae* with other microbes can reduces inhibitory compounds in lignocellulosic hydrolysates. **Tomás *et al.* (2013)** reported co-culture *Thermoanaerobacter pentosaceus* with *S. cerevisiae* was able to growth and metabolize furfural up to 0.5 g.L$^{-1}$ in the liquid fraction of alkaline-peroxide pretreated rapeseed straw. **Wan *et al.* (2012)** reported co-cultures of *S. cerevisiae* Y5 with *Pichia stipitis* CBS6054 can growth and metabolized furfural and HMF in medium of xylose and glucose mixture.

Addition of phenol (Fig 1B) to the growth medium significantly ($p<0.05$) decreased yeast colony count in mono or co-culture *S. cerevisiae* with *C. tropicalis.* This work indicated phenol inhibits growth of yeast in mono-culture and co-culture *S. cerevisiae* with *C. tropicalis*. Some investigators have reported the inhibitory effect of phenol on the growth of microorganisms. **Heipieper *et al.* (1994)** reported phenol component can degrade cell membrane integrity and decrease membrane affection as buffer selective. **Ding *et al.* (2011)** sugested acetic acid, furfural and phenol are main inhibitor on growth, fermentation and some yeast metabolite. Some inhibitors fermentation such as HMF and phenol component can inhibited yeast metabolism (**Sluiter *et al.*, 2010; Palmqvist and Hahn-Hägerdal, 2000; Almeida *et al.*, 2009; Larsson *et al.*, 1999**). However, **Paca *et al.* (2002)** suggested *C. tropicalis* can use phenol as carbon source and energy. **Adeboye *et al.* (2014)** reported phenolic compounds can exhibit lag phase elongation and decreased maximum specific growth rate of *S. cerevisiae.* **Pizzolitto *et al.* (2015)** reported inhibitory effect of phenol on growth parameter of *Aspergillus parasiticus* depend on the compound assayed and its concentration in the medium. Similar furfural, this work show yeast count colony in co-culture *S. cerevisea* with *C. tropicalis* significantly ($P<0.05$) higher than mono-culture *S. cerevisea* or *C. tropicalis* in the medium with or without phenol. We hypothesized there are synergistic mechanism to stimulate growth yeast through simultaneous utilization of fermentable sugars and degraded phenol in the growth medium by co-culture *S. cerevisiae* with *C. tropicalis.* Some investigators have reported *C. tropicalis* can degrade phenol component **(Krug *et al.*, 1985; Komarkova *et al.*, 2003; Ahuatzi-Chacon *et al.*, 2004; Wang *et al.*, 2012)**. **Jönsson *et al.* (2013)** and **Larsson *et al.* (2000)** reported *S. cerevisiae* can convert some inhibitory phenolics to less toxic compounds such as coniferyl aldehyde is reduced to coniferyl alcohol and dihydroconiferyl alcohol. **Kuntiya *et al.* (2013)** reported phenol can be degraded and used as a source of carbon energy by *C. tropicalis.*

**Residue and sugars consumption**

Addition of furfural to the growth medium significantly ($p<0.05$) influenced on glucose residue (Fig 3) and xylose (Fig 4), but no significantly ($p>0.05$) influenced on arabinose residue (Fig 5A). Addition of furfural to the growth medium also significantly ($p<0.05$) decreased glucose (Fig 3B) and xylose (Fig 4B) consumption, but no significantly ($p>0.05$) influenced on arabinose consumption (Fig 5B). Glucose, fructose, and mannose are fermented via the Embden-Meyerhof pathway of glycolysis, and galactose requires the Leloir pathway (**Wendland *et al.*, 2009**). Effect inhibitory furfural and phenol on glucose consumption were reported by several researchers (**Lin *et al.*, 2015; Sluiter *et al.*, 2010; Palmqvist *et al.*, 2000, Almeida *et al.*, 2009: Larsson *et al.*, 1999**). **Wikandari *et al.* (2010)** reported consumption glucose by *S. cerevisiae* isolate of Bekonang only 34.94 and 1.93% in the medium containing 1.0 and 1.5 g.L$^{-1}$ of furfural, respectively.

However, this work show glucose consumption by co-culture *S. cerevisiae* with *C. tropicalis* significantly ($P<0.05$) higher than mono-culture *S. cerevisiae* or *C. tropicalis* in the medium with or without furfural. Higher glucose consumption by co-culture than mono-culture in this study suspected due to degradation of furfural by each yeast in the mixture fermentation. Under anaerobic conditions, *S. cerevisiae* can convert furfural to furfuryl alcohol (**Diaz de Villegas *et al.*, 1992; *Sárvári Horváth et al., 2003*)** *and r*eduction of furfural has been linked to the co-factor NADH (**Wahlbom *et al.*, 2002**).

In addition, higher sugar consumption by co-culture than mono-culture in this study also suspected due to the contribution of glucose consumption by *C. tropicalis* in the substrate mixture of glucose and xylose. In this work, *C. tropicalis* can consume glucose from media although lower than *S. cerevisiae*. This observation similar with **Panchal *et al.* (1988)** and **du Preez *et al.* (1986)** who reported a diauxic (sequential) consumption of D-glucose and D-xylose in the same order by *C. shehatae* and *P. stipites* when using mixtures of these sugars in the culture medium. **Laplace *et al.* (1993)** reported co-culture *C. shehatae* with *S. cerevisiae* completely consumed D-glucose from mixture medium containing 70% of D-glucose and 30% of D-xylose after 14 h fermentation,

while D-xylose, in practice, not been consumed. These researchers suggest xylose consumption by *C. shehatae* can be inhibited in the presence of glucose.

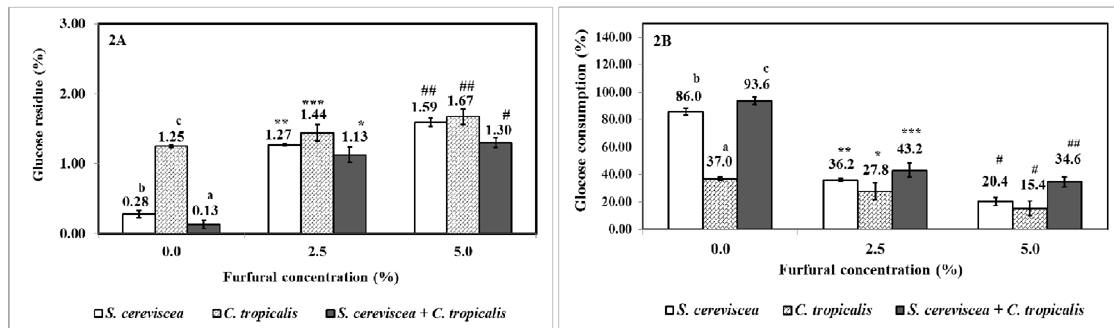


Figure 2 Glucose residue (2A) and consumption of glucose (2B) after five days fermentation by mono and co-culture *S. cerevisiae* with *C. tropicalis* in the media plus different furfural concentration, the values are means of 5 independent observations in same furfural concentration different superscripts ([a] and [b], * and ** or [#] , [##] and [###]) differ significantly ($p<0.05$).

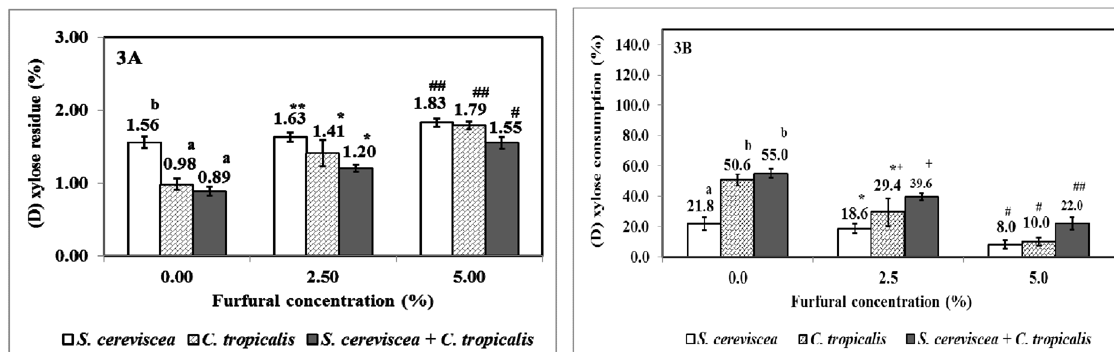


Figure 3 (D) xylose residue (3A) and consumption of xylose (3B) after five days fermentation by mono and co-culture *S. cerevisiae* with *C. tropicalis* in media plus different furfural concentration, the values are means of 5 independent observations in same furfural concentration different superscripts ([a] and [b], * and ** or [#] , [##] and [###]) differ significantly ($p<0.05$).

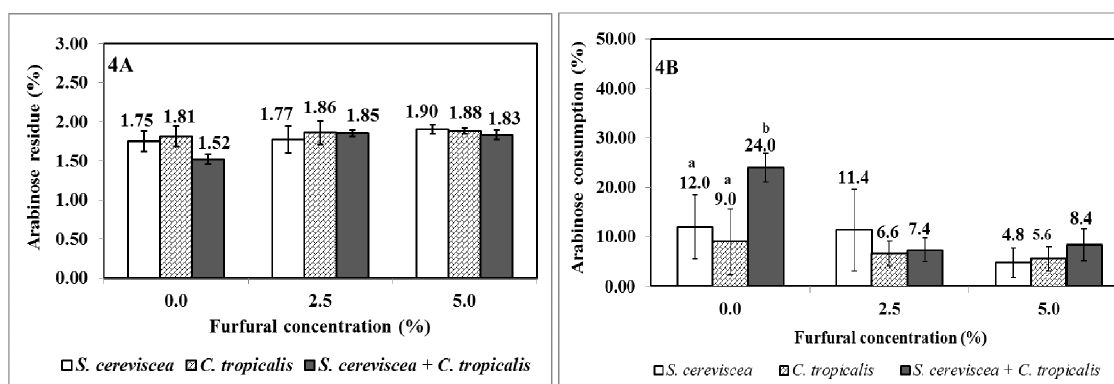


Figure 4 Arabinose residue (4A) and consumption of arabinose (4B) after five days fermentation by mono and co-culture *S. cerevisiae* with *C. tropicalis* in media plus different furfural concentration, the values are means of 5 independent observations in same furfural concentration different superscripts ([a] and [b]) differ significantly ($p<0.05$).

In this work, D(+) xylose consumed by mono-culture *C. tropicalis* and co-culture *S. cerevisiae* with *C. tropicalis* higher than *C. tropicalis*. **Cheng *et al.* ( 2014)** reported *Candida tropicalis* W103 was able to use xylose as the carbon source for cell growth under aerobic or anaerobic conditions and when glucose was used as the carbon source, ethanol was produced under aerobic or anaerobic

conditions, but *C. tropicalis* grew slightly slower under anaerobic conditions than under aerobic conditions and displayed sequential sugar consumption, first utilizing glucose and then xylose. Higher D(+) xylose consumption by co-culture than mono-culture in this study besides allegedly due to inhibitors degradation by each yeast in the mixture fermentation as describe before, as well as the contribution *S. cerevisiae* to consume D(+) xylose. Native *S. cerevisiae* does not metabolize xylose (**Jeffries and Jin, 2004; Lin and Tanakan, 2006**) and nearly all reported xylose isomerase-based pathways in *S. cerevisiae*suffer from poor ethanol productivity, low xylose consumption rates, and poor cell growth compared with an oxidoreductase pathway and, additionally, often require adaptive strain evolution **(Lee *et al.*, 2012).** As all yeasts of the genus *Saccharomyces* lack the gene that produces the enzyme xylose isomerase (**Van Maris *et al.*, 2006**), conversion of xylose to xylulose is necessary for carbon uptake (**Chiang *et al.*, 1981; Gong *et al.*, 1981**). Although low (21.80%), this work indicates that *S. cerevisiae* can consume D(+) xylose, allegedly due to lack of glucose in culture medium, as a mechanism of adaptation to nutritional deficiencies or our *S. cerevisiae* has undergone mutations in fermentation conditions. Figure 5 show the glucose, D(+) xylose and arabinose residues in the media after fermented by *S. cerevisiae*. **Shin *et al.* (2015)** suggested *S. cerevisiae* is able to ferment xylose but first utilizes D-glucose before the D-xylose can be transported and metabolized.

Addition of furfural to the growth medium significantly ($p<0.05$) decreased arabinose consumption by mono or co-culture *S. cerevisiae* with *C. tropicalis* in the fermentation media. Co-culture *S. cerevisiae* with *C. tropicalis* significantly ($p<0.05$) increased arabinose consumption in the fermentation media. **Schimer-Michel *et al.* (2008)** argued that arabinose be metabolize in a later phase, when both glucose and xylose were exhaused. Generally, in this work *S. cerevisiae* and *C. tropicalis* can consume arabinose in the media very low with or without furfural . We found arabinose consumption depends on the availability of glucose and xylose in the media.

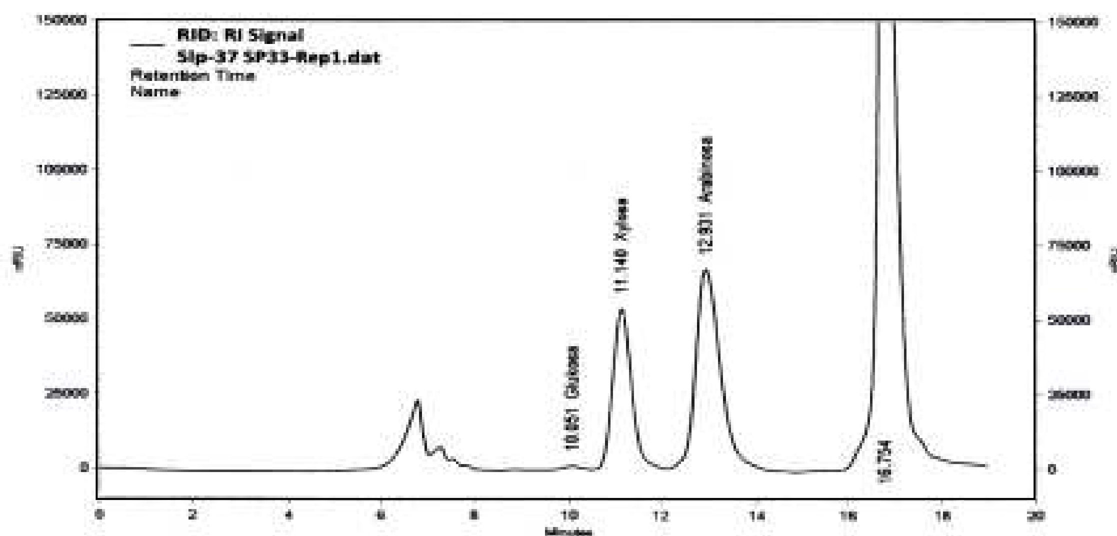


Figure 5. An HPLC chromatogram of residue glucose, D(+) xylose and arabinose in medium after five days fermentation by *S.cerevisiae*.

Addition of phenol to the growth medium significantly ($p<0.05$) influnced glucose (Fig 6A) and xylose (Fig 7A) residue, but no significantly ($p>0.05$) influenced on arabinose residue (Fig 8A). Addition of phenol to the growth medium also significantly ($p<0.05$) decreased glucose (Fig 6B) and xylose (Fig 7B) consumption, but no significantly ($p>0.05$) effect on arabinose consumption (Fig 8B). The toxicity of phenolics is very variable as it depends on the functional groups **(Ando *et al.*, 1986; Jonsson *et al.*, 2013; Adeboye *et al.*, 2014)**, more methoxy groups are related to high hydrophobicity and toxicity **(Klinke *et al.*, 2004)**. Yeast *S. cerevisiae* can assimilate many of phenolics which can be part of the detoxification process occurring during fermentation **(Mills *et al.*, 1971; Delgenes *et al.*, 1996).** Phenolic compounds mainly interfere with proteins function and trigger changes in the protein to lipid ratio **(Keweloh *et al.*, 1990)**. Hence, these compounds affect cellular functions like sorting and signaling, as well as cause membrane swelling **(Caspeta *et al.* 2015)**.

**Richard *et al.* (2003)** have describe the fungal pathways L-arabinose and D-xylose, to convert L-arabinose and D-xylose to D-xylulose 5-phosphate go through oxidation and reduction reactions before they are phosphorylated by xylulokinase. D-Xylose is first reduced to xylitol by an reduced nicotinamide adenine dinucleotide phosphate (NADPH)-consuming reaction. Xylitol is then oxidised by an NADþ-consuming reaction to form D-xylulose. In fungi, L-arabinose goes through four redox reactions. Two oxidations are coupled to NADþ consumption and two reductions to NADPH consumption. Futhermore, **Richard *et al.*** (2003) reported *S. cerevisiae* enables growth on L-arabinose and under anaerobic conditions ethanol is produced from L-arabinose, but at a very low rate. Similar furfural, in this work *S. cerevisiae* and *C. tropicalis* can consume arabinose in the media very low with or without phenol. We found arabinose consumption depends on the availability of glucose and xylose in the media.

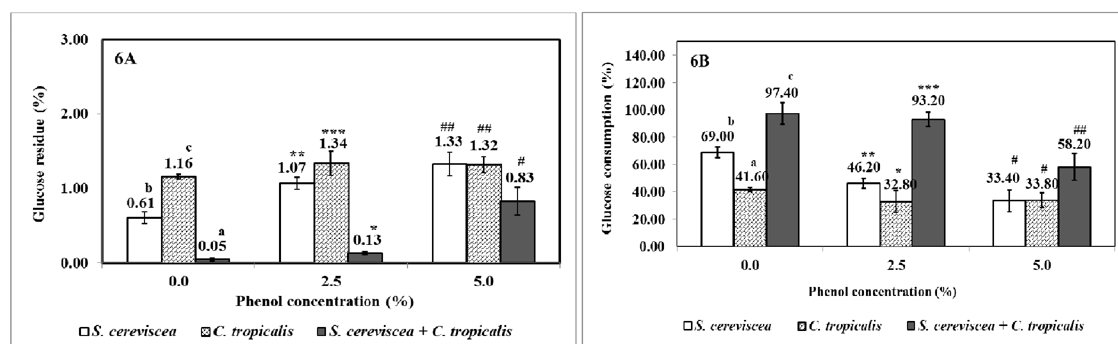


Figure 6 Glucose residue (6A) and consumption of glucose (6B) after five days fermentation by mono and co-culture *S. cerevisiae* with *C. tropicalis* in media plus different phenol concentration, the values are means of 5 independent observations in same phenol concentration different superscripts ([a] and [b], * and ** or [#], [##] and [###]) differ significantly ($p<0.05$).

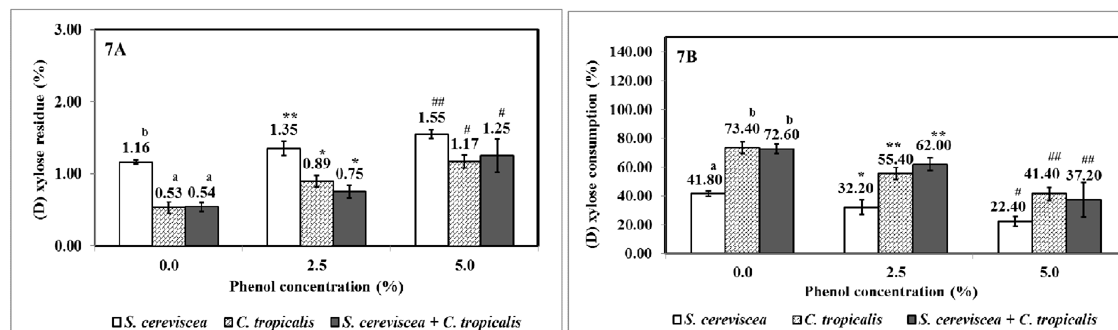


Figure 7 Xylose residue (7A) and consumption of xylose (7B) after five days fermentation by mono and co-culture *S. cerevisiae* with *C. tropicalis* in media plus different phenol concentration, the values are means of 5 independent observations in same phenol concentration different superscripts ([a] and [b], * and ** or [#], [##] and [###]) differ significantly ($p<0.05$).

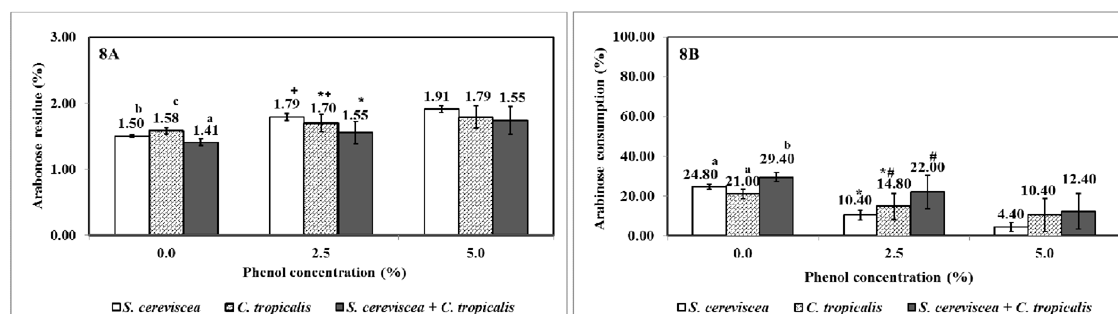

Figure 8 Arabinose residue (8A) and consumption of arabinose (8B) after five days fermentation by mono and co-culture *S. cerevisiae* with *C. tropicalis* in media plus different phenol concentration, the values are means of 5 independent observations in same phenol concentration different superscripts ($^{a}$ and $^{b}$, or *, $*^{+}$ , and $^{+}$) differ significantly (p<0.05).

**Ethanol production**

*Saccharomyces cerevisiae* ferments certain sugars very efficiently into ethanol, even under aerobic conditions. Addition of furfural (Fig 9A) or phenol to the growth medium significantly (p<0.05) decreased ethanol yields. This work indicated furfural inhibits sugar conversion to ethanol in mono-culture and co-culture *S. cerevisea* with *C. tropicatlis*. Some investigators have been reported inhibitory effect of furfural on yeast growth and ethanol production. **Zaldivar *et al.* (1999)** reported furfural and HMF to compromise membrane integrity leading to extensive membrane disruption/leakage, which eventually will cause reduction in cell replication rate, ATP production, and consequently lower ethanol production. **Agbogbo and Wenger, (2007)** reported furfural concentration 1.5 g.L$^{-1}$ can inhibit respiration and growth of microorganism and leading reduced ethanol production 90.4% and productivity 85.1%. **Ylitervo *et al.* (2013)** reported furfural at the lower concentration (0.8 and 1.5 g L−1) decreases ethanol yields less than 10% and at the higher concentration decrease ethanol yield up to around 20% and 60%.

Phenolic compounds are known to partition into biological membranes altering the permeability and lipid/protein ratio, which thus increases cell fluidity, leading to cell membrane disruption, dissipation of proton/ion gradients and compromising the ability of cellular membranes to act as selective barriers (**Heipieper *et al*., 1994**). **Kuntiya *et al*. (2013)** reported isolate *C. tropicalis* No 10 fully able degradation phenol concentration 100 mg.L$^{-1}$ at 20-42$^{o}$C, but this degradation inhibited by decreasing concentration of oxygen in media.

However, this work show ethanol yields in the co-culture *S. cerevisea* with *C. tropicalis* significantly (P<0.05) higher than mono-culture *S. cerevisea* or *C. tropicalis* with or without inhibitors furfural or phenol in the medium growth. We hypothesized there are synergistic mechanism to stimulate ethanol production through simultaneous utilization of fermentable sugars such as glucose and xylose and degrade furfural or phenol in the growth medium by co-culture *S. cerevisea* with *C. tropicalis.* **Chen (2011)** suggested that co-culture fermentation is a strategy for efficient conversion of glucose and xylose to ethanol and increase ethanol yield and production rate. **Thurnheer *et al*. (1988)** and **Shim *et al*. (2002)** suggested that a co-culture as a mimic of natural environment has been used for biodegradation of aromatic compounds **Bader *et al*. (2010)** suggested that in cocultures, degradation and metabolization of substrates occur by the combined metabolic activity of the known microbial strains under aseptic conditions.Some investigators have been reported ethanol production by co-culture microorganism in the medium containing furfural. **Wan *et al.* (2012)** reported co-culture *S. cerevisiae* Y5 and *P.stipitis* CBS6054 effectively converting glucose and xylose to ethanol, as well as effectively degrading inhibitors in the hydrolysate. Furthermore, **Wan *et al*. (2012)** reported co-culture *S. cerevisiae* Y5 and *P.stipitis* CBS6054 used up and completely metabolized glucose, furfural and 5-HMF within 12 h; xylose was used up in 96 h at 80 rpm with ethanol concentration and yield of 27.4 g L$^{−1}$ and 0.43 g ethanol/g sugar without detoxification of the hydrolysate, respectively. **Komarkova *et al*. (2003)** reported *C. tropicalis* can use phenol concentration 500 mg.L$^{-1}$ as carbon source and energy.

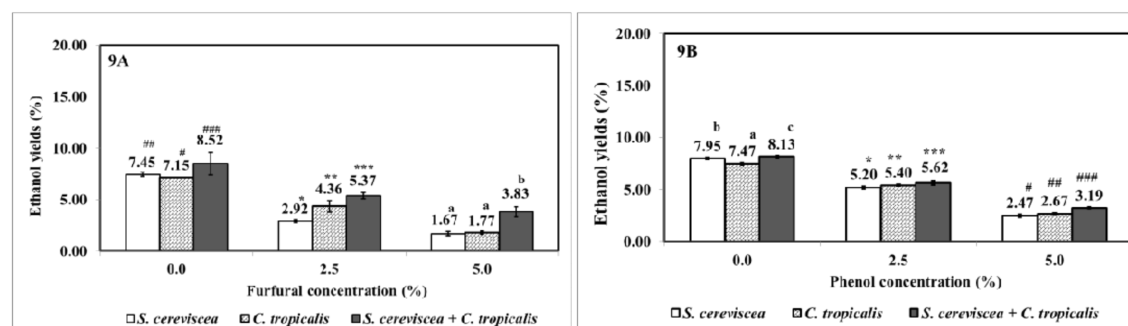


Figure 9 Ethanol yields in media plus different furfural (9A) and phenol (9B) concentration after five days fermentation by mono and co-culture *S. cerevisiae* with *C. tropicalis,* the values are means of 5 independent observations in same furfural or concentration different superscripts ($^{a}$ and $^{b}$, *,** and *** or $^{\#}$ , $^{\#\#}$ and $^{\#\#\#}$) differ significantly (p<0.05).

**Efficiency fermentation**

Addition of furfural (Fig 10A) and phenol (Fig 10B) to the growth medium significantly ($p<0.05$) decreased efficiency fermentation of ethanol production by mono and co-culture *S. cerevisiae* with *C. tropicalis*. However, co-culture *S. cerevisiae* with *C. tropicalis* significantly ($p<0.05$) higher efficiency fermentation of ethanol production than mono-culture *S. cerevisiae* or *C. tropicalis* from medium with or without furfural and phenol. Although *S. cerevisiae* efficiently convert hexoses into ethanol, but this native yeast is not able to metabolize pentose sugars present in lignocellulosic hydrolyzate. This work indicates that co-culture of *S. cerevisiae* and *C. tropicalis* more efficient use of sugar in media to be converted into ethanol. Co-culture *S. cerevisiae* with *C. tropicalis* higher consume glucose and D(+) xylose than *C. tropicalis* and *S. cerevisiae* alone. **Wang *et al.* (2012)** suggested that the co-culture between the two microorganisms in a single process is an alternative to reduce the effects of inhibitors present in the media. **Hickert *et al.* (2012)** reported co-culture *C. shehatae* HM 52.2 and *S. cerevisiae* ICV D254 can produce ethanol 0.42 from synthetic media and 0.51 from rice husk hydrolysate. **N'Guessan *et al.* (2010)** reported that ethanol production from sorghum by co-culture *C. tropicalis* and *S. cerevisiae* with a 2: 1 ratio is higher than pure cultures of *S. cerevisiae*. Co-cultures of *S. cerevisiae* is more preferred hexose sugars with yeast more preferred pentose to produce ethanol efficiently is one alternative for optimizing the production of ethanol in the hydrolyzate containing xylose (**Gutiérrez-Rivera *et al.*, 2012; Karagoz and Ozkan, 2014; Licht, 2006**). **Gutiérrez-Rivera *et al.* (2012)** reported ethanol production by co-culture *S. cerevisiae* ITV-01 and *Pichia stipitis* NRRL Y-7124, 5 times higher than ethanol production by mono-culture *S. cerevisiae* ITV-01 and mono-culture *P. stipitis* NRRL Y-7124. Increased ethanol productivity can be caused enrichment substrates that can be utilized as *S. cerevisiae* use six carbon sugars (glucose) and *P. stipitis* using 5-carbon sugar to produce ethanol **(Tesfaw and Assefa, 2014)**. Co-culture fermentation *S. cerevisiae* MTCC 174 with *Scheffersomyces stipitis* NCIM No. 3497 can produce maximum ethanol (20.8 $g.l^{-1}$) higher than ethanol production by mono-culture *S. cerevisiae* MTCC 174 (14.0 $g.l^{-1}$) or *S. stipitis* NCIM No. 3497 (12.2 $g.l^{-1}$) **(Singh *et al.*, 2014)**. Ethanol production by co-culture *S. cerevisiae* ATCC 26602 and *P. stipitis* DSM 3651 (7.36 $g.l^{-1}$) higher than mono-culture *S. cerevisiae* (6.68 $g.l^{-1}$) from wheat straw media with pre-treatment $H_2O_2$ and enzyme hydrolysis **(Karagoz and Ozkan, 2014)**. Tolerance microorganisms including co-culture fermentation to inhibitors and ethanol is one of the problems the production of ethanol from lignocellulosic waste. **Gutiérrez- Rivera *et al.* (2012)** reported *P. stipitis* NRRL Y-7124 has a low tolerance to ethanol that produced by *S. cerevisiae* ITV-01 and prevent further ethanol production by *P. stipitis* NRRL Y-7124. This work shown co-culture *S. cerevisiae* and *C. tropicalis* have high tolerance to inhibitors and higher ethanol yield than mono-culture *S. cerevisiae* or mono-culture *C. tropicalis* in basal medium and basal medium plus furfural or phenol. Co-culture *S. cerevisiae* and *C. tropicalis* also showed higher fermentation efficiency than mono-culture in basal media and basal media plus furfural or phenol. Increased ethanol production and efficiency of co-culture fermentation was allegedly due to the contribution of *C. tropicalis* to convert xylose into ethanol. **Karagoz and Ozkan (2014)** suggested that increased ethanol production by co-culture *S. cerevisiae* and *P. stipitis* due to the contribution of *P. stipitis* to convert xylose into ethanol. **Hickert *et al.* (2013)** reported co-culture *C. shehatae* HM 52 with *S. cerevisiae* ICV D254 in synthetic medium and rice hull hydrolyzate effectively convert glucose and xylose simultaneously, maximizing the utilization rate of the substrate, increasing the yield and rate of ethanol production.

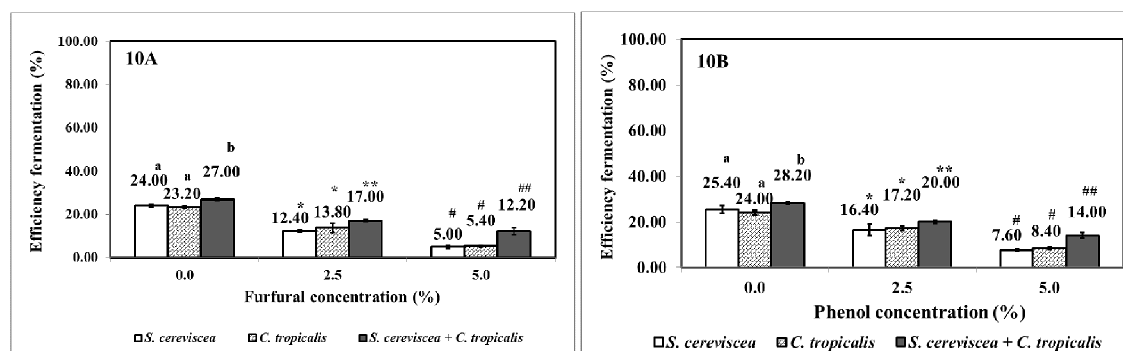


Figure 10 Efficiency fermentation ethanol production by mono and co-culture *S. cerevisiae* with *C. tropicalis* from basal media plus furfural (10A) and phenol (10B) with different concentration.

**CONCLUSIONS**

Sugar consumption and ethanol production by co-culture *S. cerevisiae* with *C.tropicalis* in the media fermentation with or without inhibitors have higher than mono *S. cerevisiae* or *C.tropicalis*. Co-culture *S. cerevisiae* with *C.tropicalis* demonstrated high tolerance to inhibitors fermentation than mono-cultures for ethanol production. Glucose and xylose consumption by *S. cerevisiae* and *C.tropicalis* contribute to the improvement and efficiency of ethanol production by culture fermentation from mixture substrate. Co-culture *S. cerevisiae* FNCC 3012 with *C.tropicalis* FNCC 3033 demonstrated potential as a fermentation process for ethanol production from lignocelulosic medium or media content inhibitors. The use of this co-culture effectively utilize hexose and pentose sugars in the substrate, increasing the yield and efficiency of fermentation ethanol production.

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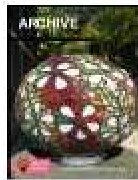


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# IMPROVED ETHANOL PRODUCTION BY CO-CULTURE FERMENTATION *Saccharomyces cerevisiae* WITH *Candida tropicalis* FROM RICE HUSK HYDROLYSATE MEDIA USING SUPLEMENTATION VARIOUS INORGANIC NITROGEN SOURCES AND MOLASSES

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ABSTRACT

The use of agricultural by-product as feed stock and co-culture fermentation is a good strategy to improve efficiency and ethanol production. Almost rice husk have low protein and nitrogen content and need to be supplemented with nitrogen for fermentation process. This research sought to determine optimal supplementation nitrogen sources and molasses to rice husk stream–based fermentation medium, initial pH and incubation time to maximize ethanol production by co-culture *S. cerevisiae* with *C. tropicalis*. Urea, sodium nitrate and ammonium nitrate were used nitrogen sources and molasses was used carbon sources. Co-culture *S. cerevisiae* with *C. tropicalis* can use different nitrogen sources and molasses for growth and ethanol production. Molasses supplementation to the rice husk hydrolysate medium, initial pH and incubation period significant influenced on ethanol yield and content of nitrogen and carbon DDG. Maximum ethanol yield (20.32± 0.42%) with nitrogen (4.40± 0.11%) and carbon (9.20± 1.01%) content of DDG were obtained in the rice husk hydrolysate medium contain 16.0 g/l urea, 12.0 g/l $NaNO_3$, 12.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses, 1.0 g/l $KH_2PO_4$ and 0.7 g/l $MgSO_4 \cdot 7H_2O$ with initial pH 5.5 and 6 days incubation period at 28-29$^o$C, 50% relative humidity in the dark for 5 d in a rotary incubator at 60 rpm.

Key words: rice husk, *Saccharomyces cerevisiae,* *Candida tropicalis,* co-culture, ethanol yield, nitrogen and molasses

## INTRODUCTION

The use of agricultural by-product as feed stock and co-culture fermentation is a good strategy to improve efficiency and ethanol production. Lignocellulosic biomass include agricultural by-product has been considered as possible raw material for ethanol

production due to its renewability, large quantities, relatively low prices than grain or sugar, potential environmental benefits and does not compete with food (Cardona and Sa´nchez, 2007; Kumar et al., 2008; Lee and Huang, 2000; Mielenz, 2001; Service, 2007; Zaldivar et al,. 2001; Ishola and Taherzadeeh, 2014). The lack of a microorganism able to ferment efficiently all sugars released by hydrolysis from lignocellulosic materials has been one of the main factors preventing utilization of lignocellulose (Zaldivar et al., 2001). *Saccharomyces cerevisiae*, which is by far the dominant yeast used for ethanol production, naturally converts glucose to ethanol but does not metabolize xylose (Jeffries and Jin, 2004; Lin and Tanakan, 2006).

Although it varies, almost rice husk have low protein and nitrogen content and need to be supplemented with nitrogen for fermentation process. Crude protein and nitrogen rice husk respectively 4.38% and 0.7% with the C / N ratio 57.93 (Ofoefule et al., 2011). Nitrogen sources such as ammonium (Jones et al., 1994 and Srichuwong et al., 2009) and urea (Jones and Ingledew, 1994; Yue et al., 2010) are widely used to increase yeast growth, rate of sugar utilize and reduce fermentation time (Chniti et al., 2015). Urea not only promoted the specific growth rate and ability of ethanol tolerance, but also increased the ethanol yield and reduced the formation of by product (Yue et al., 2010). However, several investigators have been reported negative effects of using ammonium and urea as nitrogen supplements in ethanol fermentation (Laopaiboon et al., 2009; Wang et al., 2003; Beltran et al., 2005; Chniti et al., 2015).

Elements such as carbon and nitrogen are both required in yeast metabolism. The type and concentration of carbon and nitrogen sources, as well as the C/N ratio of the medium for *S. cerevisiae* cultivation, influence cellular growth and metabolites biosynthesis (Thomas et al., 1996). Molasses is a waste of the sugar industry can be used as a substrate for ethanol production by *S.cerevisiae* (Fern´andez-L´opez et al., 2012; Sadik and Halema, 2014). Molasses contains readily utilizable carbohydrates available in the form of fermentable sugars and can be used by the alcohol producing yeasts without any pretreatment (Murtagh, 1999).

The co-culture is a potential bioprocess if there are no cross-interactions among microorganisms, and each microorganism metabolizing its substrate is unaffected by the presence of other microorganism (Park et al., 2012). Co-culture of *S. cerevisiae* and

other microorganism increased ethanol productivity might be due to enhanced substrate utilization (Tesfaw and Assefa, 2014). Co-culture *S. cerevisiae* with other microbes reduces inhibitory compounds in lignocellulosic hydrolysates (Tom´as et al., 2013; Taherzadeh and Karimi, 2011; Wan et al., 2012) increases ethanol yield and production rate (Singh et al., 2014 and Wan, 2012), shortens fermentation time, and reduces process cost (Hickert et al., 2013; Tesfaw and Assefa, 2014). Co-culture *S.cerevisiae* with *C. tropicalis* have ability to generate and convert fermentable sugars from a waste stream rice husk to ethanol (Sopandi and Wardah, 2015). This work explored supplementation of inorganic nitrogen sources and molasses to improve ethanol production by co-culture *S. cerevisiae* with *C. tropicalis* from rice husk hydrolisate.

## MATERIAL AND METHODS

### Culture microorganism

*Saccharomyces cerevisiae* FNCC 3012 and *Candida tropicalis* FNCC 3033 were obtained from Microbiology Laboratories, PPAU Gadjah Mada University, Yogyakarta, Indonesia. Saubroad agar (Oxoid) was used to maintain the strain *S. cerevisiae* and *C. tropicalis*. Working stock cultures were prepared from stock, 7 days, 28$^{o}$C SA plate cultures subcultures from the master stock. Colonies were aseptically sampled by scraping the top with an inoculating loop and transfer to 10 ml sterile water. Inoculums stock suspension were prepared from working stock and diluted to 1.7 x $10^{6}$ cell/ml, as enumerated using a haemocytometer.

### Rice husk hydrolysis

Locally farm-sourced rice husk from Sidoarjo, Indonesia, were air-dried then ground to approximately 2-mm diameter particles using a mill. The rice husk milled (900 g) were steamed at 130$^{o}$C for 3 h, cooled to room temperature, mixed with 15 l, 2.5% $H_2SO_4$ and autoclaved for 15 min at 121$^{o}$C. Hydrolysate was cooled and stored at 1-5$^{o}$C in the dark until used.

### Inorganic nitrogen supplementation

The basal medium contained 1500 ml rice husk hydrolysate, 1.0 g/l $KH_2PO_4$, and 0.7 g/l $MgSO_4.7H_2O$ was dispensed into three 1000 ml Erlenmeyer flasks and added urea, sodium nitrate ($NaNO_3$) and ammonium nitrate ($NH_4NO_3$) to final individual

concentrations of 9.0 g/l, respectively. Media were mixed thoroughly, adjusted pH 5.5 with add HCl 1 N, autoclaved, cooled to room temperature. One hundred millilitre was then aseptically dispensed into individual Erlenmeyer flasks (250 ml) and individually inoculated with one ml of *S. cerevisiae* FNCC 3012 and one ml of *C. tropicalis* FNCC 3033 inoculum stock suspension and all flask incubated at 28-29$^oC$, 50% relative humidity in the dark for 5 d in a rotary incubator at 60 rpm.

**Molasses supplementation**

Rice husk hydrolysate basal medium (2500 ml) containing 3.0 g/l urea, 3.0 g/l $NaNO_3$, 3.0 g/l $NH_4NO$, 1.0 g/l $KH_2PO_4$ and 0.7 g/l $MgSO_4{\cdot}7H_2O$ was dispensed into five 1000 ml Erlenmeyer flasks, added molasses to final concentrations of 0.0,5.0, 10.0,15.0 and 20.0 ml/l. Media were mixed thoroughly, adjusted pH 5.5 with add HCl 1 N, autoclaved and cooled to room temperature. One hundred millilitre was then aseptically dispensed into individual Erlenmeyer flasks (250 ml), one ml of *S. cerevisiae* FNCC 3012 and one ml of *C.tropicalis* FNCC 3033 inoculum stock suspension and all flasks incubated as described above.

**Formulation rice husk hydrolysate**

Four formulation of rice husk hydolysate media were examined to improve ethanol production by co-culture *S. cerevisiae* FNCC 3012 with *C.tropicalis* FNCC 3033. Rice husk hydrolysate basal medium (2000 ml) containing 1.0 g/l $KH_2PO_4$ and 0.7 g/l $MgSO_4{\cdot}7H_2O$ was dispensed into four 1000 ml Erlenmeyer flasks. Individually was added 4.0 g/l urea, 3.0 g/l $NaNO_3$, 3.0 g/l $NH_4NO_3$, and 20 ml/l molasses ($F_1$), 8.0 g/l urea, 6.0 g/l $NaNO_3$, 6.0 g/l $NH_4NO_3$, and 20 ml/l molasses ($F_2$), 12.0 g/l urea, 9.0 g/l $NaNO_3$, 9.0 g/l $NH_4NO_3$, and 20 ml/l molasses ($F_3$), and 16.0 g/l urea, 12.0 g/l $NaNO_3$, 12.0 g/l $NH_4NO_3$ and 20 ml/l molasses ($F_4$). Media were mixed thoroughly, adjusted pH 5.5 with add HCl 1 N, autoclaved and cooled to room temperature. One hundred millilitres was then aseptically dispensed into individual Erlenmeyer flasks (250 ml), one ml of *S. cerevisiae* FNCC 3012 and one ml of *C.tropicalis* FNCC 3033 inoculum stock suspension and all flasks incubated as described above.

**Initial medium pH**

To examine the effect of initial medium pH, 100 ml rice husk hydrolysate basal medium containing 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4{\cdot}7H_2O$, 16.0 g/l urea, 12.0 g/l $NaNO_3$, 12.0

g/l $NH_4NO_3$ and 20 ml/l molasses was aliquoted into 8, 250-ml Erlenmeyer flasks and the pH of each adjusted to 3.5, 4.0, 5.4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 prior to autoclaving. After cooling to room temperature, flasks were inoculated with 1-ml *S. cerevisiae* FNCC 3012 and 1- ml of *C.tropicalis* FNCC 3033 inoculum stock suspension and incubated as described above.

**Incubation period**

Effect of incubation period on ethanol yield, nitrogen and carbon content destilate residue was examined using a rice husk hydrolysate basal medium containing 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 16.0 g/l urea, 12.0 g/l $NaNO_3$, 12.0 g/l $NH_4NO_3$ and 20 ml/l molasses. Erlenmeyer flasks (250 ml) containing 100-ml sterile medium were inoculated with 1-ml *S. cerevisiae* FNCC 3012 and 1- ml of *C.tropicalis* FNCC 3033 inoculum stock suspension and incubated as described above. Ethanol yield, nitrogen and carbon content distillate residue analyses were carried out every day up to 9-days incubation. All data presented are means of four simultaneously incubated fermentation culture replicates.

**Determination of ethanol**

Ethanol were measured using a gas chromatograph Carbomax t70-10-0 column, FID t220 detector, helium as carrier gas with flow rate 40.3 mL/min, tin column Porapack Q, detector temperature at 160$^oC$ and column temperature at 180$^oC$ with injection volume 1.0 μL. Fermented media were filtered through Whatmann No.1 paper.

**Determination of distillers dried grains**

To determine distillers' dried grains, whole flask cultures were distillated at 78$^oC$ for 60 min and residue poured through predried (100°C) preweighed Whatman No.1 filter paper. Retained material was washed with distilled water then ethanol until colourless and dried at 100°C to constant weight (48 h).

**Determination of organic carbon**

Levels of total organic carbon (TOC) were determined using the wet oxidation method of Walkey and Black (1965). One hundred millilitre of liquid culture was evaporated at 100°C for approximately 2 h to obtain a dried powder, 0.5 g of which was used for TOC determination.

**Nitrogen determination**

Nitrogen (NH4-N) concentration was determined using the method of the American Society of Agronomy and Soil Science Society of America (1982). Ten-millilitre culture medium was evaporated at 100°C for approximately 2 h to obtain a dried powder. Samples (50 mg) were added to digestion tubes and 1-g selenium mixture (mashed 1.55 g $CuSO_4$, 96.9 g $Na_2SO_4$ and 1.55 g selenium) and 3-ml 97% $H_2SO_4$ added, mixed and digested at 350°C for 4 h to obtain a colourless extract, cooled to room temperature, diluted to 50 ml with distilled water, shaken vigorously and left to stand overnight. Two-millilitre of extract was placed, transferred to a new borosilicate glass test tube and 4-ml tartrate buffer (50 g NaOH and 50 g $KNaC_4H_4O_6$ in 1000 ml distillated water) and sodium phenate solution (100 g NaOH and 125 g phenol in 1000 ml distillated water) successively added, mixed and let stand for 10 min. Four-millilitre, 5% NaOCl was then added, the mixture shaken, let stand for 10 min and absorbance measured at 636 nm. $(NH4)_2SO_4$ was used to prepare N standards.

**Statistical analysis**

Tukey's honestly significant difference multiple comparison tests were used to segregate significantly different treatments using SPSS 16 software. Analysis of variance (ANOVA) was performed to determine significant differences between experiments ($P < 0.05$).

**RESULTS**

**Effect inorganic nitrogen supplementation**

No significant ($P>0.05$) difference in ethanol yield or nitrogen and carbon content of distillers' dried grains (DDG) was observed between nitrogen source type (Fig. 1). Not significantly ($P > 0.05$) different between ethanol yield from rice husk hydrolysate basal media with supplementation urea (5.15 ± 0.42%), $NaNO_3$ (4.94 ± 0.34%) and $NH_4NO_3$ (4.88± 0.28%). A similar trend was observed for nitrogen content in DDG, not significantly ($P > 0.05$) different between urea (2.21± 0.27%), $NaNO_3$ (2.41± 0.34%) and $NH_4NO_3$ (2.39± 0.30%). Addition of inorganic nitrogen to the rice husk hydrolysate basal medium also did significant ($P > 0.05$) on carbon content of DDG, not

significantly (P > 0.05) different between urea (13.57± 2.90%), $NaNO_3$ (10.53± 1.79%) and $NH_4NO_3$ (11.64± 2.83%).

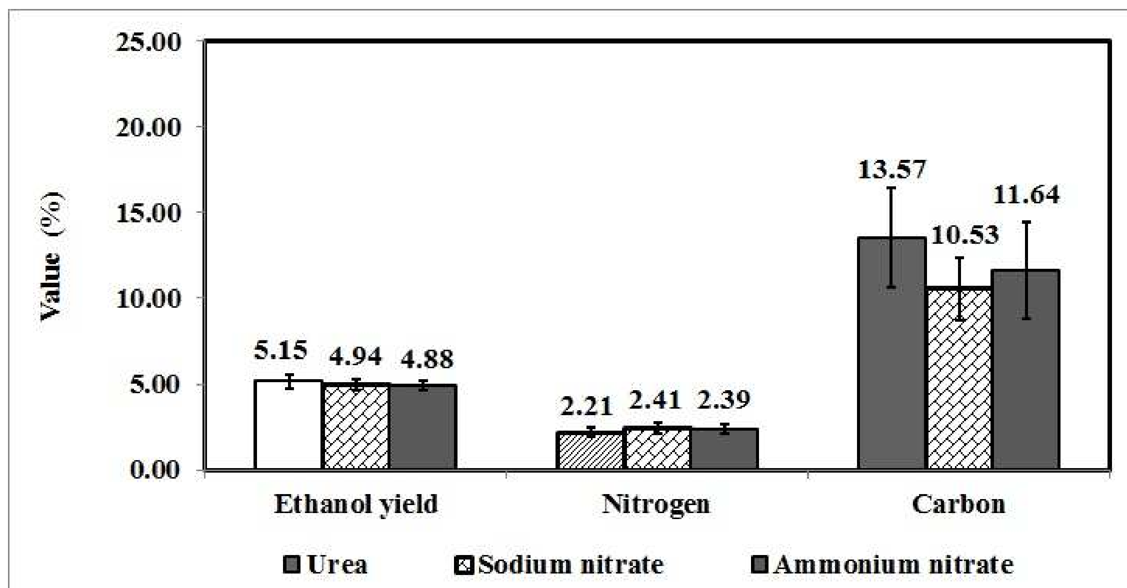



Figure 1. Ethanol yield, nitrogen and carbon content in DDG from rice husk hydrolysate medium supplemented inorganic nitrogen different that fermented by co-culture *S. cerevisiae* with *C. tropicalis*.

**Effect molasses supplementation**

Addition of 5–20 ml/l molasses to the rice husk hydrolysate basal medium significantly (P<0.05) increased ethanol yield, nitrogen and carbon content of DDG (Fig. 2). Increasing amounts of molasses (5, 10, 15 and 20 ml/l) in the medium progressively increased ethanol yield. Ethanol yield in the basal medium alone (5.38 ±0.28%) significantly (P<0.05) lower than that in the basal medium plus 5 ml/l (6.86± 0.52%), 10 ml/l (8.57± 0.31%), 15 ml/l (9.49± 0.29%), and 20 ml/l molasses (9.75± 0.14%), but no significant (P>0.05) different between 15 ml/l and 20 ml/l molasses. Nitrogen content of DDG from the rice husk hydrolysate basal medium was also significantly (P < 0.05) lower than that in the rice husk hydrolysate basal medium plus molasses (10, 15, and 20 ml/l). Nitrogen content of DDG in the basal medium (2.57± 0.12%) not significant (P>0.05) between in the basal medium plus 5 ml/l molasses ((2.84±0.07%), but significantly (P<0.05) lower than that in basal medium plus 10 ml/l (3.06±0.05%), 15 ml/l (3.45 ±0.28%) and 20 ml/l molasses (3.72±0.06%). However, no significant (P>0.05) different between 15 ml/l and 20 ml/l. This indicated a molasses-concentration

dependent stimulation growth of yeast and ethanol production. While mean, carbon content of DDG in the basal medium (7.90±0,73%) not significant ($P>0.05$) between in basal medium plus 5 ml/l molasses (9.76± 0.85%), but significantly ($P<0.05$) lower than that in the basal medium plus 10 ml/l (11.26± 0.60%), 15 ml/l (13.28± 0.99%) and 20 ml/l molasses (13.88± 1.52%). However, no significant ($P>0.05$) different between 15 ml/l and 20 ml/l.

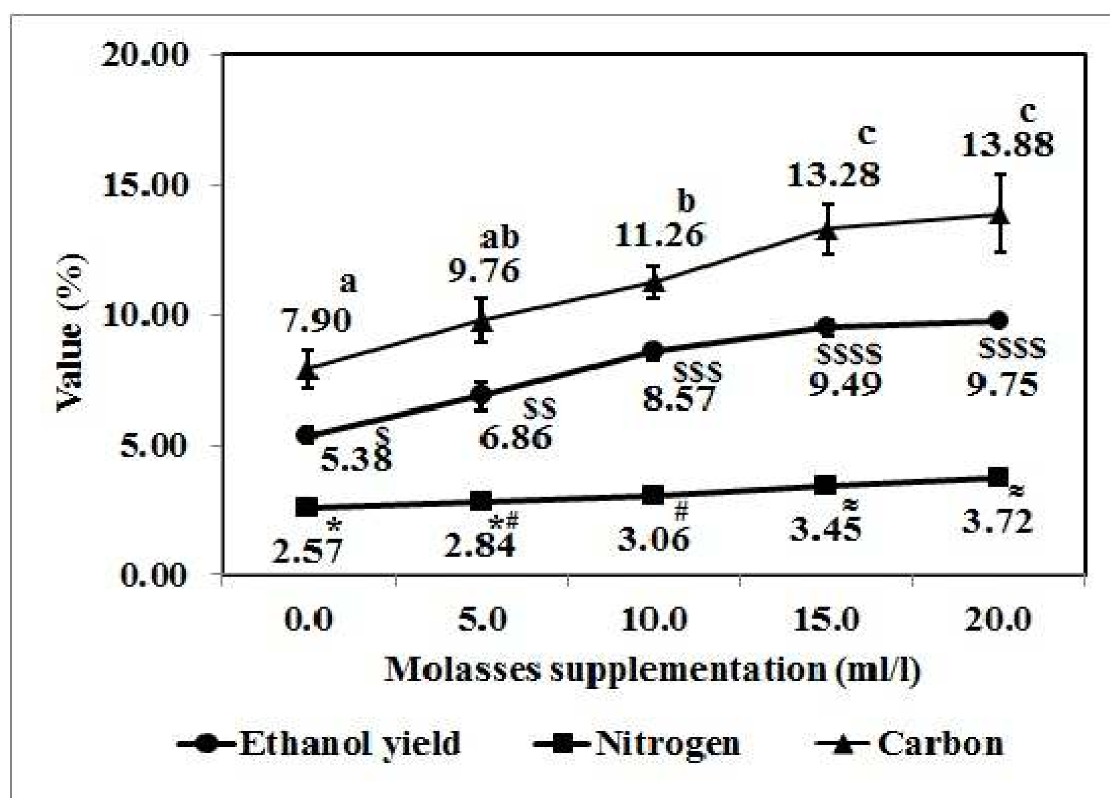
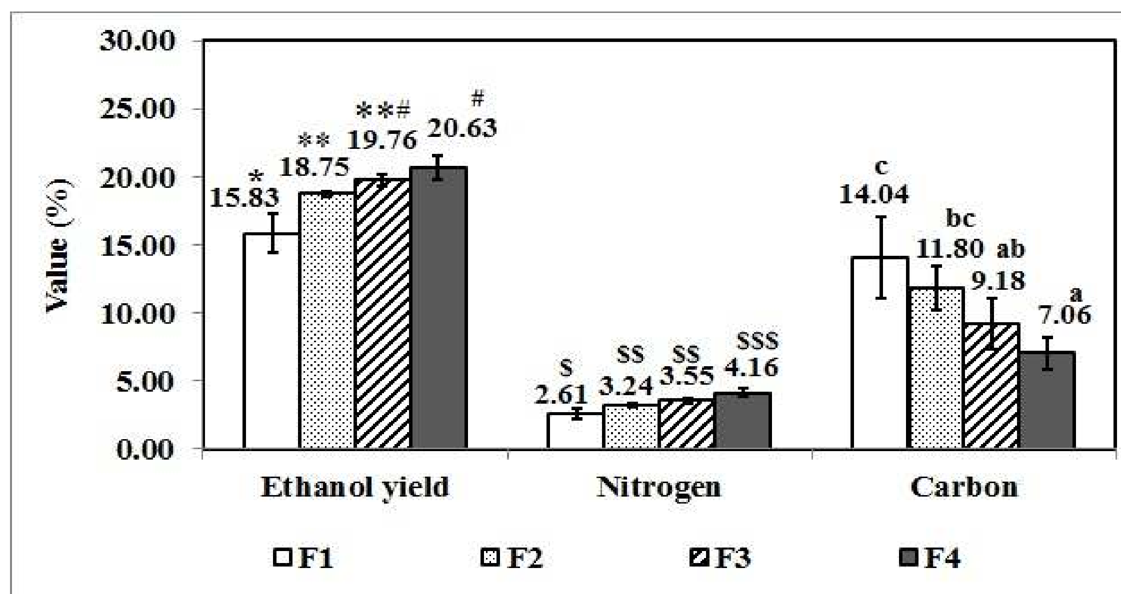



Figure 2 Effect of molasses addition on ethanol yield, nitrogen and carbon content of DDG rice husk hydrolysate medium that fermented by co-culture by co-culture *S. cerevisiae* with *C. tropicalis*.

**Formulation rice husk hydrolysate media**

Formulation of rice husk hydrolysate media supplemented inorganic nitrogen and molasses significantly ($P<0.05$) influenced on ethanol yield, nitrogen and carbon content of DDG (Fig.3). Maximum ethanol yield, nitrogen and carbon content of DDG were obtained in the rice husk hydrolysate medium supplemented with 16.0 g/l urea, 12.0 g/l $NaNO_3$, 12.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4{\cdot}7H_2O$, 20 ml/l molasses. Ethanol in F1 (the rice husk hydrolysate medium supplemented with 4.0 g/l

urea, 3.0 g/l $NaNO_3$, 3.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses (15.83± 1.43%) was significantly (P<0.05) lower than that in F2 (the rice husk hydrolysate medium supplemented with 8.0 g/l urea, 6.0 g/l $NaNO_3$, 6.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses (18,75± 0,23%), F3 (the rice husk hydrolysate medium supplemented with 12.0 g/l urea, 9.0 g/l $NaNO_3$, 9.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses (19.76±0.47%), and F4 (the rice husk hydrolysate medium supplemented with 16.0 g/l urea, 12.0 g/l $NaNO_3$, 12.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses (20.63± 0.89%), not significantly (P > 0.05) different between F2 and F2 and F3.




F1; 1000 ml rice husk hydrolysate, 4.0 g/l urea, 3.0 g/l $NaNO_3$, 3.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses; F2, 1000 ml rice husk hydrolysate, 8.0 g/l urea, 6.0 g/l $NaNO_3$, 6.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses; F3, 1000 ml rice husk hydrolysate 12.0 g/l urea, 9.0 g/l $NaNO_3$, 9.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses; F4, 1000 ml rice husk hydrolysate, 16.0 g/l urea, 12.0 g/l $NaNO_3$, 12.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses

Figure 3. Effect of different formulation of rice husk hydrolysate culture medium on ethanol yield, nitrogen and carbon content of DDG were fermented by co-culture *S. cerevisiae* with *C. tropicalis*.

While mean nitrogen content of DDG in the F1 medium (2.61±0.34%) significantly (P<0.05) lower than that in the F2 (3.24±0.15%), F3 (3.55±0.24%) and F4 medium (7.06± 1.21%), but no significant (P > 0.05) difference between F2 and F3 medium. Carbon content of DDG in the F1 medium (14.04± 2.97% was also significantly (P <

0.05) higher than that in the F3 (9.18± 1.89%) and F4 (7.06± 1.21%) medium, but no significant (P>0.05) different between F1 and F2 and no significant (P>0.05) different also was observed between F3 and F4 medium.

**Effect of initial medium pH**

Initial medium pH significantly (P<0.05) affected ethanol yield, nitrogen and carbon content of DDG (Fig.5). In this works shows *S. cerevisiae* and *C. tropicalis* grew and produced ethanol in co-culture over a broad pH range (3.0-7.0). An initial medium pH outside 5.5–6.5 decreased ethanol yield, nitrogen and carbon content of DDG. Ethanol yield at pH 5.5 (20.52± 0.60%) and 6.0 (19.66± 0.16%) was significantly (P<0.05) higher than that at pH 3.0 (1.63 ± 0.31%), 3.5 (2.37± 0.36%), 4.0 (8.25± 0.26%), 4.5 (9.23± 0.26%), 5.0 (13.20± 0.89%), 6.5 (19.10± 0.58%) or 7.0 (11.78±0.50%), with no significant (P > 0.05) difference were observed between pH 5.5 and 6.0 and between pH 6.0 and 6.5. Nitrogen content of DDG pH 5.5 (4.45±0.12%), 6.0 (4.42±0.13%) and 6.5

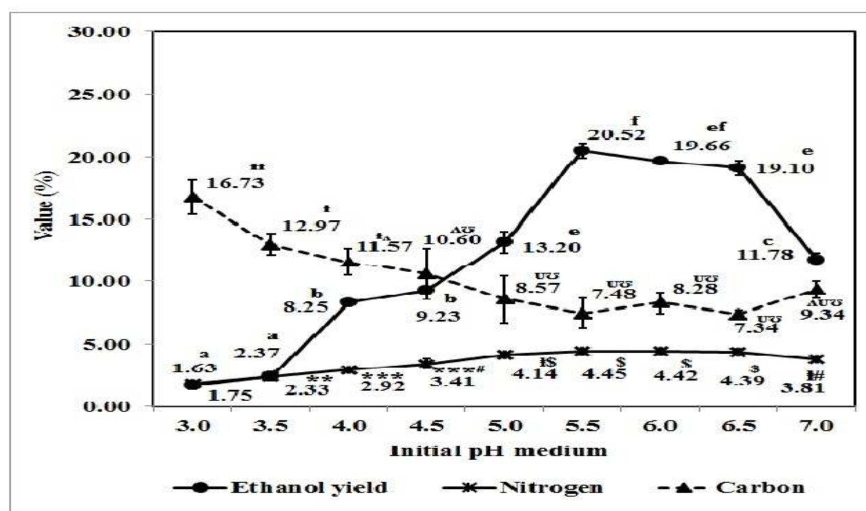



Figure 5. Effect of initial pH medium on ethanol yield, nitrogen and carbon content of DDG from rice husk hydrolysate culture medium with supplemented and fermented by co-culture *S. cerevisiae* and *C. tropicalis*

(4.39±0.26%) was significantly (P<0.05) higher than that at pH 3.5 (2.33± 0.26%), 4.0 (2.92±0.03%), 4.5 (3.41±0.42%), 5.0 (4.14±0.12%), or 7.0 (3.81±0.05%), with no significant (P > 0.05) difference were observed between pH 5.0, 5.5, 6.0 and 6.5. While mean carbon content of DDG at pH 3.0 (16.73± 1.38%), 3.5 (12.97± 0.84%), 4.0

(11.57± 1.11%), 4.5 (10.60± 2.07%), 5.0 (8.57± 1.86%), 5.5 (7.48± 1.20%), 6.0 (8.28± 0.84%), 6.5 (7.34± 0.33%), or 7.0 (9.34± 0.69%).

**Effect of incubation period**

Incubation period significantly ($P<0.05$) affected ethanol yield (Fig 6A), nitrogen and carbon content of DDG (Fig 6B). Ethanol yield increased between 3 and 5 days, then stagnant from 6 to 9 days total incubation. Ethanol yield 2 (0.37 ± 0.11%) and 3 days (0.94± 0.05%) were significantly ($P<0.05$) lower than that at 4 days (4.06±0.46%), with both 4 days ethanol yield significantly ($P < 0.05$) lower than that at 5 (19.71± 0.38%), 6 (20.32± 0.42%), 7 (20.59± 0.33%), 8 (20.65± 0.48%), and 9 days (20.70± 0.46). Differences in ethanol yield between 5 and 6 days incubation were not significant ($P > 0.05$). Ethanol yield at 5 days was also significantly ($P < 0.05$) lower than 7, 8 or 9 days, but no significant ($P < 0.05$) difference between 6, 7, 8 and 9 days ethanol yields. Nitrogen content of DDG increased between 1 and 7 days, then relatively stagnant from 7 to 9 days total incubation. Nitrogen content of DDG 1 day (0.32± 0.05%) 2 (0.71± 0.11%), 3 (1.81± 0.10%), 4 (3.18± 0.08%), 5 (3.58± 0.25%), 6 days (4.40± 0.11%) were significantly ($P<0.05$) lower than that at 7 (4.67± 0.08%), 8 (4.47±0.07%) and 9 days (4.35±0.20%). Differences in nitrogen content of DDG between 1, 2, 3, 4, 5 and 6 days incubation were insignificant ($P < 0.05$), but no significant ($P > 0.05$) difference were observed between 7, 8 and 9 days incubation. While mean nitrogen content of DDG decreased between 1 and 4 days, then relatively stagnant from 5 to 9 days total incubation. Carbon content of DDG 1 day (46.57±1.57%), 2 (43.16± 2.68%), 3 (23.80± 0.26%), and 4 (12.90± 0.41%) were significantly ($P<0.05$) higher than that at 5 (9.72± 0.05%), 6 (9.20± 1.01%), 7 (8.68± 0.25%), 8 (8.30± 0.07%) and 9 days (7.29± 0.47%). Differences in nitrogen content of DDG between 1, 2, 3, and 4 days incubation were insignificant ($P < 0.05$), but no significant ($P > 0.05$) difference were observed between 5, 6, 7, 8 and 9 days incubation.

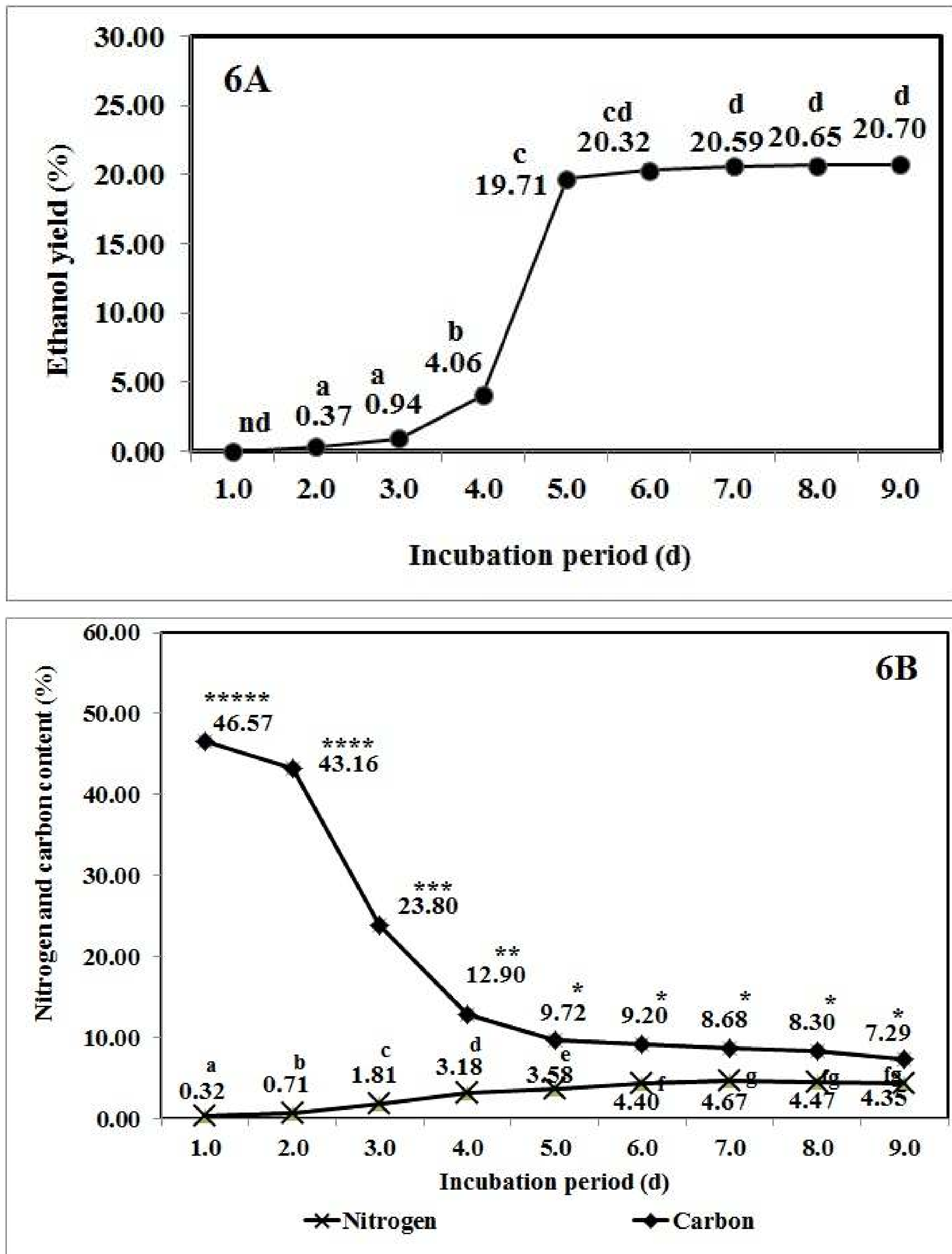



Figure 6 Effect of incubation period on ethanol yield (6A) and nitrogen and carbon content (6B) of distillate residue fermented rice husk hydrolysate medium by co-culture *S. cerevisiae* and *C. tropicalis*

## DISCUSSION

Nitrogen deficiency in the fermentation medium will leads to slow and stuck fermentation rate (Vilanova et al., 2007). Nitrogen sources are very crucial and strongly influence the yeast growth and metabolism during fermentation (Beltran et al., 2005). In this work shows no significant different of ethanol yield or nitrogen and carbon content of DDG at exogenous nitrogen sources supplementation ($NH_4NO_3$, urea and $NaNO_3$) to rice husk fermentation media which indicated the co-culture *S. cerevisiae* and *C. tropicalis* can utilize on the various sources of nitrogen for growth and stimulate ethanol production. Some investigators have reported varying effects of exogenous nitrogen sources supplementation to the lignocellulosic fermentation media on ethanol production by yeast. These results of this work are similar to several studies reported that supplementation of the various sources of nitrogen to fermentation media does not significantly effect on the production of ethanol. Chu et al. (2012) reported supplementation of $(NH_4)_2SO_4$ and yeast extract to the SSF of pretreated corn stover with dilute $H_2SO_4$ did not bring any change on ethanol yield using thermotolerant strain *S. cerevisiae* DQ1. Akaracharanya et al. (2011) reported $(NH_4)_2SO_4$ supplementation to the hydrolysate of cassava pulp (a waste from cassava starch production) did not enhance ethanol production by the yeast. Fern´andez-L´opez et al. (2012) reported the addition of yeast extract, ammonium sulfate, urea, and their combination to medium of sugar rich molasses that obtained during the second step of crystallization did not improve ethanol productivity significantly. Wang et al. (2012) reported at the integrated ethanol-methane fermentation system, ammonium and other component in the effluent promoted yeast growth and fermentation rate but did not increase the yield of ethanol. However, the results of this work differ from several studies reported that the supplementation of various sources nitrogen to fermentation media effected on ethanol production. P´erez-Carrillo et al. (2011) and Harde et al. (2014) reported the supplementation of exogenous nitrogen sources such as yeast extract, malt extract, peptone, and $(NH_4)_2SO_4$ to the natural growing media enhanced ethanol production in *S. cerevisiae.*

Initial sugar concentrations in the growth media can influence the specific rate of yeast growth and ethanol production (Tesfaw and Assefa, 2014). There are varieties of

yeast, which are used to convert molasses into ethanol and $CO_2$, like *saccharomyces services and Klyureomyces marxianus* (Parkash, 2015). This work shows supplementation of molasses in the growth media significantly ($P<0.05$) increases ethanol yield and nitrogen and carbon content in the media fermentation. Production of ethanol from molasses-based media by co-culture fermentation has been reported. Eiadpum et al. (2012) reported immobilized co-culture *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* can improve ethanol production from both sugarcane juice and blackstrap molasses when the operating temperature ranged between 33 °C and 45 °C and generated maximal ethanol concentrations of 81.4 and 77.3 g/l, respectively.

Carbohydrates and nitrogenous compounds are two major components affecting yeast performance in fermentation. A high of nitrogen sources significantly increased the efficiency of fermentation and yeast yield (Tyagi and Ghose, 1980). Increasing the nitrogen concentration in the fermentation medium can increase the rate of fermentation, decrease the duration and a lack of nitrogen triggers sluggish fermentations (Alexandre and Charpentier, 1998; Fleet and Heard, 1992; Varela et al., 2004). The ratio of nitrogen sources to carbon sources in the medium can influence on the yeast growth and metabolism of *Saccharomyces cerevisiae* (Larsson et al., 1993). Nitrogen deficiency with a high sugar transporter turnover rate, which resulted in a loss of sugar uptake capacity in the cells (Salmon, 1989 and Bisson, 1999). In this works, 4 formulation of rice husk hydrolysate media with different supplemented inorganic nitrogen and molasses significantly ($P<0.05$) influenced on ethanol yield, nitrogen and carbon content of DDG.

The specific rate of yeast growth and ethanol production were influenced by pH fermentation medium (Tesfaw and Assefa, 2014). In this work shows initial pH medium effected on ethanol yield and the content of nitrogen and carbon at DDG. A wide range of optimum pH (4.0–8.0) was reported for *S. cerevisiae* JZ1C isolated from rhizosphere of Jerusalem artichoke using inulin and Jerusalem artichoke tuber as substrate at 35°C (Hu et al., 2012). Optimum pH for *S. cerevisiae* BY4742 was in the range of 4.0–5.0, when the pH was lower than 4.0, the incubation period was prolonged though the ethanol concentration was not reduced significantly and when the pH was above 5.0, the concentration of ethanol diminished substantially (Lin et al., 2012). Some investigators

have been reported effect of incubation period on ethanol production from lignocellulosic medium by co-culture fermentation. Wright (1988) reported the maximum ethanol production of 4% (w/v) from the wheat straw medium after 48 h of incubation employing process of simultaneous saccharifi cation and fermentation using *T. reesei* cellulase and *Kluyveromyces fragilis*. Sharma (2000) reported maximum ethanol yield and fermentation efficiency of 0.397 $gg^{-1}$ and 77.84 per cent, respectively after 36 h of incubation at 30°C using mixed culture of *S. cerevisiae* and *P. tannophilus*. Verma *et al*. (2000) reported maximum ethanol concentration of 24.8 g $L^{-1}$ at 48 hours of incubation from starch medium in a single step process by co-culture of amylolytic yeasts and *S.cerevisiae*

## CONCLUSIONS

Inorganic nitrogen and molasses supplementation can increase the production of ethanol from rice husk hydrolysate medium by co-culture *S. cerevisiae* and *C. tropicalis*. The best formulation mediums to obtain maximum production of ethanol with pH 5.5 and incubation period of 6 days is 1000 ml rice husk hydrolysate, 16.0 g/l urea, 12.0 g/l $NaNO_3$, 12.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses.

## Conflict of Interests

The authors have not declared any conflict of interest.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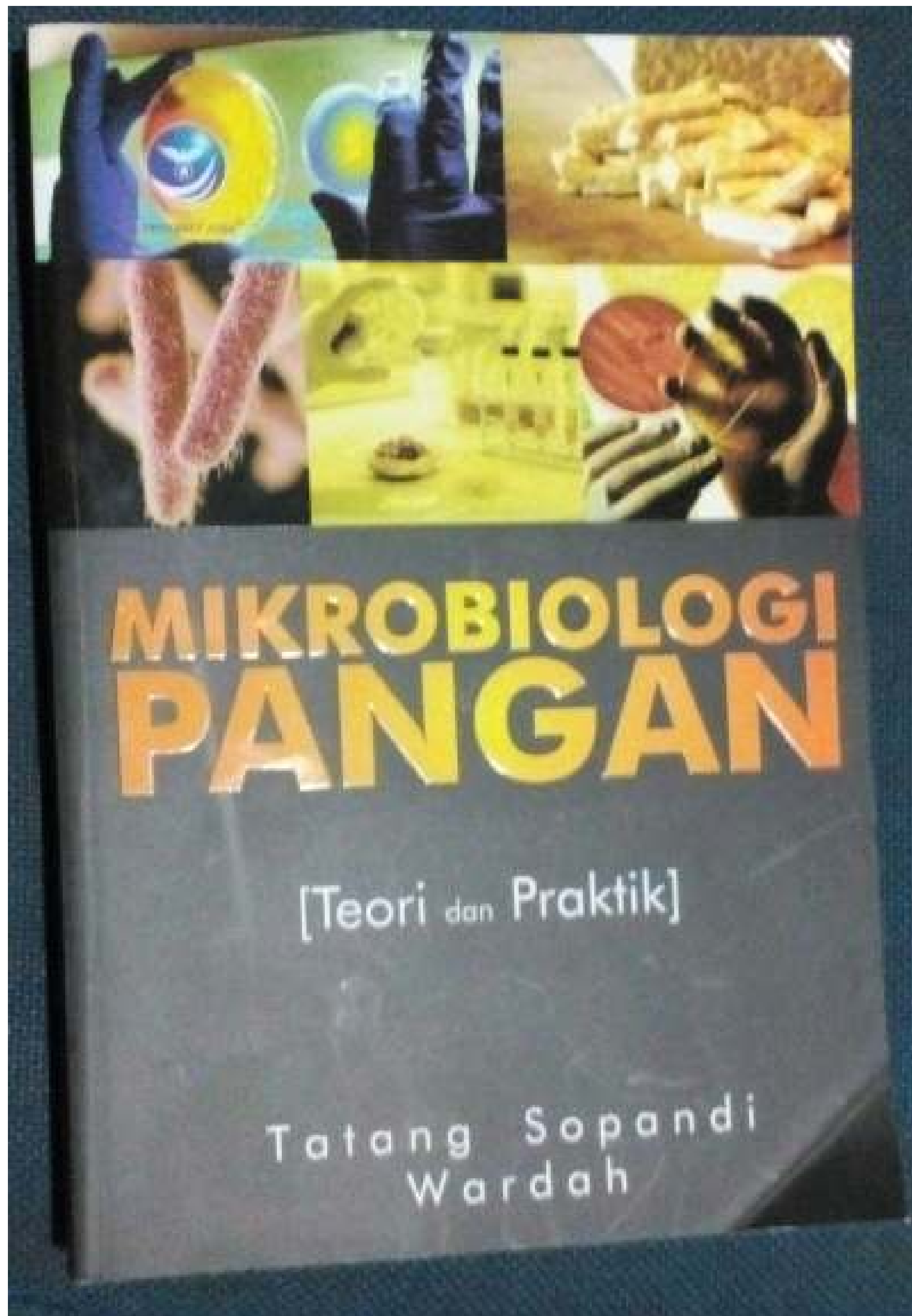
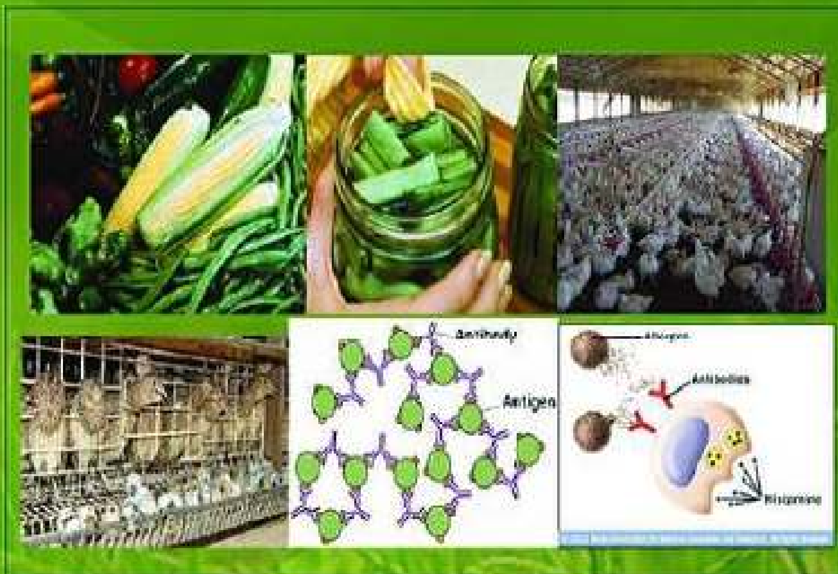
4. Buku ajar



5.Buku ajar teknologi hasil pertanian

TEKNOLOGI
HASIL PERTANIAN




Oleh

Dr. Ir. Wardah, MP., MM
Dr. Ir. Tatang Sopandi., MP

2. Seminar nasional

## ANALISIS DATA PENELITIAN

1. Efek sumber nitrogen

Data hasil pengamatan efek penambahan sumber nitrogen terhadap kadar etanol serta kadar nitrogen dan karbon dalam DDG

| Sumber Nitrogen | Ulangan | Kadar etanol (%) | Kadar Nitrogen DDG (%) | Kadar karbon DDG (%) |
|---|---|---|---|---|
| Urea | 1 | 4.52 | 2.48 | 9.76 |
| | 2 | 5.54 | 2.3 | 11.41 |
| | 3 | 5.43 | 2.14 | 16.61 |
| | 4 | 4.96 | 1.79 | 14.35 |
| | 5 | 5.32 | 2.35 | 15.7 |
| $NaNO_3$ | 1 | 4.67 | 2.70 | 9.25 |
| | 2 | 4.85 | 2.81 | 8.19 |
| | 3 | 4.93 | 2.36 | 10.82 |
| | 4 | 4.74 | 2.11 | 11.99 |
| | 5 | 5.53 | 2.05 | 12.38 |
| $NH_4NO_3$ | 1 | 4.96 | 2.42 | 9.75 |
| | 2 | 4.86 | 2.55 | 8.84 |
| | 3 | 4.89 | 2.37 | 10.29 |
| | 4 | 4.76 | 2.56 | 14.18 |
| | 5 | 4.93 | 2.07 | 15.15 |

Analisis statistika efek sumber nitrogen terhadap kadar etanol

**Descriptives**

Etanol

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1 | 5 | 5.1540 | .41603 | .18605 | 4.6374 | 5.6706 | 4.52 | 5.54 |
| 2 | 5 | 4.9440 | .34246 | .15315 | 4.5188 | 5.3692 | 4.67 | 5.53 |
| 3 | 5 | 4.8800 | .07714 | .03450 | 4.7842 | 4.9758 | 4.76 | 4.96 |
| Total | 15 | 4.9927 | .31517 | .08138 | 4.8181 | 5.1672 | 4.52 | 5.54 |

**ANOVA**

Etanol

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .205 | 2 | .103 | 1.040 | .383 |
| Within Groups | 1.185 | 12 | .099 | | |
| Total | 1.391 | 14 | | | |

Analisis statistika efek sumber nitrogen terhadap kadar nitrogen DDG

**Descriptives**

Nitrogen

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1 | 5 | 2.2120 | .26546 | .11872 | 1.8824 | 2.5416 | 1.79 | 2.48 |
| 2 | 5 | 2.4060 | .34136 | .15266 | 1.9821 | 2.8299 | 2.05 | 2.81 |
| 3 | 5 | 2.3940 | .19882 | .08892 | 2.1471 | 2.6409 | 2.07 | 2.56 |
| Total | 15 | 2.3373 | .27049 | .06984 | 2.1875 | 2.4871 | 1.79 | 2.81 |

**ANOVA**

Nitrogen

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .118 | 2 | .059 | .783 | .479 |
| Within Groups | .906 | 12 | .076 | | |
| Total | 1.024 | 14 | | | |

Analisis statistika efek sumber nitrogen terhadap kadar karbon DDG

**Descriptives**

Karbon

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1 | 5 | 13.5660 | 2.89692 | 1.29554 | 9.9690 | 17.1630 | 9.76 | 16.61 |
| 2 | 5 | 10.5260 | 1.78551 | .79850 | 8.3090 | 12.7430 | 8.19 | 12.38 |
| 3 | 5 | 11.6420 | 2.82870 | 1.26504 | 8.1297 | 15.1543 | 8.84 | 15.15 |
| Total | 15 | 11.9113 | 2.69888 | .69685 | 10.4167 | 13.4059 | 8.19 | 16.61 |

**ANOVA**

Karbon

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 23.648 | 2 | 11.824 | 1.811 | .205 |
| Within Groups | 78.327 | 12 | 6.527 | | |
| Total | 101.975 | 14 | | | |

## 2. Efek penambahan molases dalam media

Data hasil pengamatan efek penambahan molasses terhadap kadar etanol serta kadar nitrogen dan karbon dalam DDG

| Penambahan molasses (ml/l) | Ulangan | Bobot kering DDG (g) | Kadar etanol (%) | Kadar nitrogen (%) | Kadar karbon (%) |
|---|---|---|---|---|---|
| 0 | 1 | 4.45 | 5.47 | 2.40 | 8.34 |
| 0 | 2 | 4.87 | 4.91 | 2.59 | 7.75 |
| 0 | 3 | 4.62 | 5.34 | 2.62 | 6.68 |
| 0 | 4 | 4.57 | 5.55 | 2.66 | 8.38 |
| 0 | 5 | 4.67 | 5.28 | 2.56 | 8.34 |
| 5 | 1 | 5.15 | 7.45 | 2.77 | 8.93 |
| 5 | 2 | 4.91 | 6.07 | 2.80 | 9.02 |
| 5 | 3 | 4.96 | 6.67 | 2.81 | 9.56 |
| 5 | 4 | 5.48 | 7.09 | 2.88 | 10.61 |
| 5 | 5 | 5.26 | 7.03 | 2.93 | 10.69 |
| 10 | 1 | 5.27 | 8.69 | 3.00 | 11.92 |
| 10 | 2 | 6.08 | 8.67 | 3.01 | 11.66 |
| 10 | 3 | 5.66 | 8.76 | 3.05 | 11.39 |
| 10 | 4 | 5.92 | 8.70 | 3.11 | 10.40 |
| 10 | 5 | 5.36 | 8.01 | 3.11 | 10.95 |
| 15 | 1 | 5.92 | 9.47 | 3.28 | 14.70 |
| 15 | 2 | 6.34 | 9.69 | 3.32 | 12.17 |
| 15 | 3 | 6.25 | 9.60 | 3.36 | 13.59 |
| 15 | 4 | 6.84 | 9.00 | 3.94 | 13.40 |
| 15 | 5 | 6.22 | 9.68 | 3.35 | 12.56 |
| 20 | 1 | 7.63 | 9.91 | 3.66 | 15.71 |
| 20 | 2 | 7.42 | 9.58 | 3.78 | 12.23 |
| 20 | 3 | 7.53 | 9.80 | 3.72 | 13.51 |
| 20 | 4 | 7.38 | 9.84 | 3.78 | 15.21 |
| 20 | 5 | 7.07 | 9.64 | 3.65 | 12.75 |

Analisis statistika efek penambahan molasses terhadap bobot kering DDG

**Descriptives**

DDGS

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 0 | 5 | 4.6360 | .15421 | .06896 | 4.4445 | 4.8275 | 4.45 | 4.87 |
| 5 | 5 | 5.1520 | .23167 | .10361 | 4.8643 | 5.4397 | 4.91 | 5.48 |
| 10 | 5 | 5.6580 | .34860 | .15590 | 5.2252 | 6.0908 | 5.27 | 6.08 |
| 15 | 5 | 6.3140 | .33374 | .14925 | 5.8996 | 6.7284 | 5.92 | 6.84 |
| 20 | 5 | 7.4060 | .21173 | .09469 | 7.1431 | 7.6689 | 7.07 | 7.63 |
| Total | 25 | 5.8332 | 1.01212 | .20242 | 5.4154 | 6.2510 | 4.45 | 7.63 |

**ANOVA**

DDGS

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 23.164 | 4 | 5.791 | 81.524 | .000 |
| Within Groups | 1.421 | 20 | .071 | | |
| Total | 24.585 | 24 | | | |

**DDGS**

Tukey HSD

| Molases | N | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| 0 | 5 | 4.6360 | | | | |
| 5 | 5 | | 5.1520 | | | |
| 10 | 5 | | | 5.6580 | | |
| 15 | 5 | | | | 6.3140 | |
| 20 | 5 | | | | | 7.4060 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

Analisis statistika efek penambahan molasses terhadap kadar etanol

**Descriptives**

etanol

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 0 | 5 | 5.3100 | .24749 | .11068 | 5.0027 | 5.6173 | 4.91 | 5.55 |
| 5 | 5 | 6.8620 | .52203 | .23346 | 6.2138 | 7.5102 | 6.07 | 7.45 |
| 10 | 5 | 8.5660 | .31262 | .13981 | 8.1778 | 8.9542 | 8.01 | 8.76 |
| 15 | 5 | 9.4880 | .28665 | .12820 | 9.1321 | 9.8439 | 9.00 | 9.69 |
| 20 | 5 | 9.7540 | .13885 | .06210 | 9.5816 | 9.9264 | 9.58 | 9.91 |
| Total | 25 | 7.9960 | 1.74215 | .34843 | 7.2769 | 8.7151 | 4.91 | 9.91 |

**ANOVA**

etanol

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 70.710 | 4 | 17.678 | 165.847 | .000 |
| Within Groups | 2.132 | 20 | .107 | | |
| Total | 72.842 | 24 | | | |

**etanol**

Tukey HSD

| Molases | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| 0 | 5 | 5.3100 | | | |
| 5 | 5 | | 6.8620 | | |
| 10 | 5 | | | 8.5660 | |
| 15 | 5 | | | | 9.4880 |
| 20 | 5 | | | | 9.7540 |
| Sig. | | 1.000 | 1.000 | 1.000 | .701 |

Means for groups in homogeneous subsets are displayed.

Analisis statistika efek penambahan molasses terhadap kadar nitrogen DDG

**Descriptives**

nitrogen

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 0 | 5 | 2.5660 | .09990 | .04468 | 2.4420 | 2.6900 | 2.40 | 2.66 |
| 5 | 5 | 2.8380 | .06535 | .02922 | 2.7569 | 2.9191 | 2.77 | 2.93 |
| 10 | 5 | 3.0560 | .05273 | .02358 | 2.9905 | 3.1215 | 3.00 | 3.11 |
| 15 | 5 | 3.4500 | .27568 | .12329 | 3.1077 | 3.7923 | 3.28 | 3.94 |
| 20 | 5 | 3.7180 | .06261 | .02800 | 3.6403 | 3.7957 | 3.65 | 3.78 |
| Total | 25 | 3.1256 | .44122 | .08824 | 2.9435 | 3.3077 | 2.40 | 3.94 |

**ANOVA**

nitrogen

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 4.284 | 4 | 1.071 | 55.240 | .000 |
| Within Groups | .388 | 20 | .019 | | |
| Total | 4.672 | 24 | | | |

**nitrogen**

Tukey HSD

| Molases | N | Subset for alpha = 0.05 | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| 0 | 5 | 2.5660 | | | |
| 5 | 5 | | 2.8380 | | |
| 10 | 5 | | 3.0560 | | |
| 15 | 5 | | | 3.4500 | |
| 20 | 5 | | | | 3.7180 |
| Sig. | | 1.000 | .136 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.

Analisis statistika efek penambahan molasses terhadap kadar karbon DDG

**Descriptives**

karbon

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 0 | 5 | 7.8980 | .72947 | .32623 | 6.9922 | 8.8038 | 6.68 | 8.38 |
| 5 | 5 | 9.7620 | .84615 | .37841 | 8.7114 | 10.8126 | 8.93 | 10.69 |
| 10 | 5 | 11.2640 | .60169 | .26908 | 10.5169 | 12.0111 | 10.40 | 11.92 |
| 15 | 5 | 13.2840 | .98455 | .44030 | 12.0615 | 14.5065 | 12.17 | 14.70 |
| 20 | 5 | 13.8820 | 1.52103 | .68022 | 11.9934 | 15.7706 | 12.23 | 15.71 |
| Total | 25 | 11.2180 | 2.43345 | .48669 | 10.2135 | 12.2225 | 6.68 | 15.71 |

**ANOVA**

karbon

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 122.549 | 4 | 30.637 | 31.307 | .000 |
| Within Groups | 19.572 | 20 | .979 | | |
| Total | 142.120 | 24 | | | |

**karbon**

Tukey HSD

| Molases | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| 0 | 5 | 7.8980 | | |
| 5 | 5 | 9.7620 | 9.7620 | |
| 10 | 5 | | 11.2640 | |
| 15 | 5 | | | 13.2840 |
| 20 | 5 | | | 13.8820 |
| Sig. | | .051 | .156 | .871 |

Means for groups in homogeneous subsets are displayed.

3. Efek formulasi media hidolisat sekam padi

Data hasil pengamanatan efek formulasi media hidrolisat sekam padi

| Formulasi media | Ulangan | Kadar etanol (%) | Kadar nitrogen DDG (%) | Kadar karbon DDG (%) |
|---|---|---|---|---|
| F1 (1000 ml rice husk hydrolysate, 4.0 g/l urea, 3.0 g/l $NaNO_3$, 3.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses) $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses | 1 | 16.44 | 2.78 | 12.56 |
| | 2 | 14.51 | 2.00 | 18.75 |
| | 3 | 14.09 | 2.72 | 13.68 |
| | 4 | 17.09 | 2.81 | 10.80 |
| | 5 | 17.03 | 2.74 | 14.43 |
| F2 (1000 ml rice husk hydrolysate, 8.0 g/l urea, 6.0 g/l $NaNO_3$, 6.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses) | 1 | 19.05 | 3.36 | 9.65 |
| | 2 | 18.81 | 3.35 | 11.12 |
| | 3 | 18.42 | 3.17 | 13.08 |
| | 4 | 18.68 | 3.01 | 13.74 |
| | 5 | 18.80 | 3.29 | 11.44 |
| F3 (1000 ml rice husk hydrolysate 12.0 g/l urea, 9.0 g/l $NaNO_3$, 9.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses) | 1 | 19.49 | 3.65 | 8.32 |
| | 2 | 19.64 | 3.60 | 9.71 |
| | 3 | 19.34 | 3.61 | 12.14 |
| | 4 | 19.77 | 3.14 | 8.58 |
| | 5 | 20.54 | 3.74 | 7.17 |
| F4 (1000 ml rice husk hydrolysate, 16.0 g/l urea, 12.0 g/l $NaNO_3$, 12.0 g/l $NH_4NO_3$, 1.0 g/l $KH_2PO_4$, 0.7 g/l) | 1 | 20.72 | 4.50 | 6.20 |
| | 2 | 19.83 | 3.79 | 8.97 |
| | 3 | 21.90 | 4.48 | 7.55 |
| | 4 | 19.74 | 3.95 | 6.29 |
| | 5 | 20.97 | 4.07 | 6.27 |

Analisis statistika efek formulasi media hidrolisat sekam padi terhadap kadar etanol

**Descriptives**

etanol

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1 | 5 | 15.8320 | 1.42913 | .63913 | 14.0575 | 17.6065 | 14.09 | 17.09 |
| 2 | 5 | 18.7520 | .22906 | .10244 | 18.4676 | 19.0364 | 18.42 | 19.05 |
| 3 | 5 | 19.7560 | .46694 | .20882 | 19.1762 | 20.3358 | 19.34 | 20.54 |
| 4 | 5 | 20.6320 | .89004 | .39804 | 19.5269 | 21.7371 | 19.74 | 21.90 |
| Total | 20 | 18.7430 | 2.02306 | .45237 | 17.7962 | 19.6898 | 14.09 | 21.90 |

**ANOVA**

etanol

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 65.342 | 3 | 21.781 | 28.058 | .000 |
| Within Groups | 12.420 | 16 | .776 | | |
| Total | 77.763 | 19 | | | |

**etanol**

Tukey HSD

| Treat | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| 1 | 5 | 15.8320 | | |
| 2 | 5 | | 18.7520 | |
| 3 | 5 | | 19.7560 | 19.7560 |
| 4 | 5 | | | 20.6320 |
| Sig. | | 1.000 | .308 | .421 |

Means for groups in homogeneous subsets are displayed.

Analisis statistika efek formulasi media hidrolisat sekam padi terhadap kadar nitrogen DDG

**Descriptives**

nitrogen

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1 | 5 | 2.6100 | .34278 | .15330 | 2.1844 | 3.0356 | 2.00 | 2.81 |
| 2 | 5 | 3.2360 | .14724 | .06585 | 3.0532 | 3.4188 | 3.01 | 3.36 |
| 3 | 5 | 3.5480 | .23467 | .10495 | 3.2566 | 3.8394 | 3.14 | 3.74 |
| 4 | 5 | 4.1580 | .31901 | .14267 | 3.7619 | 4.5541 | 3.79 | 4.50 |
| Total | 20 | 3.3880 | .62486 | .13972 | 3.0956 | 3.6804 | 2.00 | 4.50 |

**ANOVA**

nitrogen

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 6.234 | 3 | 2.078 | 28.081 | .000 |
| Within Groups | 1.184 | 16 | .074 | | |
| Total | 7.419 | 19 | | | |

**nitrogen**

Tukey HSD

| Treat | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| 1 | 5 | 2.6100 | | |
| 2 | 5 | | 3.2360 | |
| 3 | 5 | | 3.5480 | |
| 4 | 5 | | | 4.1580 |
| Sig. | | 1.000 | .303 | 1.000 |

Means for groups in homogeneous subsets are displayed.

Analisis statistika efek formulasi media hidrolisat sekam padi terhadap kadar karbon DDG

**Descriptives**

karbon

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1 | 5 | 14.0440 | 2.96453 | 1.32578 | 10.3631 | 17.7249 | 10.80 | 18.75 |
| 2 | 5 | 11.8060 | 1.62913 | .72857 | 9.7832 | 13.8288 | 9.65 | 13.74 |
| 3 | 5 | 9.1840 | 1.88296 | .84208 | 6.8460 | 11.5220 | 7.17 | 12.14 |
| 4 | 5 | 7.0560 | 1.20879 | .54059 | 5.5551 | 8.5569 | 6.20 | 8.97 |
| Total | 20 | 10.5225 | 3.28537 | .73463 | 8.9849 | 12.0601 | 6.20 | 18.75 |

**ANOVA**

karbon

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 139.283 | 3 | 46.428 | 11.290 | .000 |
| Within Groups | 65.797 | 16 | 4.112 | | |
| Total | 205.080 | 19 | | | |

**karbon**

Tukey HSD

| Treat | N | Subset for alpha = 0.05 | | |
|---|---|---|---|---|
| | | 1 | 2 | 3 |
| 4 | 5 | 7.0560 | | |
| 3 | 5 | 9.1840 | 9.1840 | |
| 2 | 5 | | 11.8060 | 11.8060 |
| 1 | 5 | | | 14.0440 |
| Sig. | | .376 | .213 | .334 |

Means for groups in homogeneous subsets are displayed.

4. Efek pH awal media hidolisat sekam padi

Data hasil pengamanatan efek pH awal media hidrolisat sekam padi

| pH awal media | ulangan | Kadar etanol (%) | Kadar nitrogen DDG (%) | Kadar Karbon DDG (%) |
|---|---|---|---|---|
| 3.0 | 1 | 1.78 | 1.56 | 16.26 |
| | 2 | 1.95 | 1.67 | 15.67 |
| | 3 | 1.24 | 1.93 | 16.23 |
| | 4 | 1.53 | 1.84 | 18.75 |
| 3.5 | 1 | 2.86 | 2.04 | 12.87 |
| | 2 | 2.38 | 2.26 | 13.65 |
| | 3 | 2.05 | 2.34 | 13.54 |
| | 4 | 2.17 | 2.67 | 11.82 |
| 4.0 | 1 | 8.52 | 2.96 | 11.24 |
| | 2 | 7.89 | 2.89 | 10.63 |
| | 3 | 8.32 | 2.92 | 11.23 |
| | 4 | 8.27 | 2.89 | 13.18 |
| 4.5 | 1 | 9.23 | 2.78 | 13.14 |
| | 2 | 9.56 | 3.54 | 11.21 |
| | 3 | 9.19 | 3.65 | 8.32 |
| | 4 | 8.93 | 3.67 | 9.71 |
| 5.0 | 1 | 12.69 | 4.12 | 10.56 |
| | 2 | 12.23 | 4.23 | 9.53 |
| | 3 | 14.15 | 4.24 | 7.85 |
| | 4 | 13.71 | 3.98 | 6.35 |
| 5.5 | 1 | 20.23 | 4.34 | 8.97 |
| | 2 | 20.82 | 4.26 | 7.87 |
| | 3 | 19.83 | 4.53 | 6.79 |
| | 4 | 21.18 | 4.65 | 6.27 |
| 6.0 | 1 | 19.68 | 4.56 | 9.058 |
| | 2 | 19.44 | 4.49 | 8.83 |
| | 3 | 19.79 | 4.35 | 7.98 |
| | 4 | 19.74 | 4.27 | 7.23 |
| 6.5 | 1 | 19.97 | 4.23 | 7.76 |
| | 2 | 18.72 | 4.39 | 7.39 |
| | 3 | 18.88 | 4.75 | 7.23 |
| | 4 | 18.83 | 4.18 | 6.98 |
| 7.0 | 1 | 12.49 | 3.84 | 9.74 |
| | 2 | 11.75 | 3.79 | 9.43 |
| | 3 | 11.53 | 3.74 | 9.85 |
| | 4 | 11.35 | 3.86 | 8.35 |

Analisis statistika efek pH awal media hidrolisat sekam padi terhadap kadar etanol

**Descriptives**

Etanol

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 3 | 4 | 1.6250 | .30925 | .15462 | 1.1329 | 2.1171 | 1.24 | 1.95 |
| 3.5 | 4 | 2.3650 | .35707 | .17854 | 1.7968 | 2.9332 | 2.05 | 2.86 |
| 4 | 4 | 8.2500 | .26319 | .13159 | 7.8312 | 8.6688 | 7.89 | 8.52 |
| 4.5 | 4 | 9.2275 | .25851 | .12925 | 8.8162 | 9.6388 | 8.93 | 9.56 |
| 5 | 4 | 13.1950 | .88760 | .44380 | 11.7826 | 14.6074 | 12.23 | 14.15 |
| 5.5 | 4 | 20.5150 | .60158 | .30079 | 19.5577 | 21.4723 | 19.83 | 21.18 |
| 6 | 4 | 19.6625 | .15500 | .07750 | 19.4159 | 19.9091 | 19.44 | 19.79 |
| 6.5 | 4 | 19.1000 | .58384 | .29192 | 18.1710 | 20.0290 | 18.72 | 19.97 |
| 7 | 4 | 11.7800 | .50080 | .25040 | 10.9831 | 12.5769 | 11.35 | 12.49 |
| Total | 36 | 11.7467 | 6.81464 | 1.13577 | 9.4409 | 14.0524 | 1.24 | 21.18 |

**ANOVA**

Etanol

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 1619.004 | 8 | 202.376 | 857.261 | .000 |
| Within Groups | 6.374 | 27 | .236 | | |
| Total | 1625.378 | 35 | | | |

**Etanol**

Tukey HSD

| pH | N | Subset for alpha = 0.05 | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 3 | 4 | 1.6250 | | | | | |
| 3.5 | 4 | 2.3650 | | | | | |
| 4 | 4 | | 8.2500 | | | | |
| 4.5 | 4 | | 9.2275 | | | | |
| 7 | 4 | | | 11.7800 | | | |
| 5 | 4 | | | | 13.1950 | | |
| 6.5 | 4 | | | | | 19.1000 | |
| 6 | 4 | | | | | 19.6625 | 19.6625 |
| 5.5 | 4 | | | | | | 20.5150 |
| Sig. | | .461 | .148 | 1.000 | 1.000 | .777 | .284 |

Means for groups in homogeneous subsets are displayed.

Analisis statistika efek pH awal media hidrolisat sekam padi terhadap kadar nitrogen DDG

**Descriptives**

nitrogen

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 3 | 4 | 1.7500 | .16633 | .08317 | 1.4853 | 2.0147 | 1.56 | 1.93 |
| 3.5 | 4 | 2.3275 | .26120 | .13060 | 1.9119 | 2.7431 | 2.04 | 2.67 |
| 4 | 4 | 2.9150 | .03317 | .01658 | 2.8622 | 2.9678 | 2.89 | 2.96 |
| 4.5 | 4 | 3.4100 | .42387 | .21194 | 2.7355 | 4.0845 | 2.78 | 3.67 |
| 5 | 4 | 4.1425 | .12121 | .06060 | 3.9496 | 4.3354 | 3.98 | 4.24 |
| 5.5 | 4 | 4.4450 | .17748 | .08874 | 4.1626 | 4.7274 | 4.26 | 4.65 |
| 6 | 4 | 4.4175 | .13150 | .06575 | 4.2083 | 4.6267 | 4.27 | 4.56 |
| 6.5 | 4 | 4.3875 | .25773 | .12887 | 3.9774 | 4.7976 | 4.18 | 4.75 |
| 7 | 4 | 3.8075 | .05377 | .02689 | 3.7219 | 3.8931 | 3.74 | 3.86 |
| Total | 36 | 3.5114 | .96353 | .16059 | 3.1854 | 3.8374 | 1.56 | 4.75 |

**ANOVA**

nitrogen

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 31.265 | 8 | 3.908 | 85.902 | .000 |
| Within Groups | 1.228 | 27 | .045 | | |
| Total | 32.493 | 35 | | | |

**nitrogen**

Tukey HSD

| pH | N | Subset for alpha = 0.05 | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 |
| 3 | 4 | 1.7500 | | | | | |
| 3.5 | 4 | | 2.3275 | | | | |
| 4 | 4 | | | 2.9150 | | | |
| 4.5 | 4 | | | 3.4100 | 3.4100 | | |
| 7 | 4 | | | | 3.8075 | 3.8075 | |
| 5 | 4 | | | | | 4.1425 | 4.1425 |
| 6.5 | 4 | | | | | | 4.3875 |
| 6 | 4 | | | | | | 4.4175 |
| 5.5 | 4 | | | | | | 4.4450 |
| Sig. | | 1.000 | 1.000 | .060 | .218 | .421 | .553 |

Means for groups in homogeneous subsets are displayed.

Analisis statistika efek pH awal media hidrolisat sekam padi terhadap kadar karbon DDG

**Descriptives**

karbon

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 3 | 4 | 16.7275 | 1.37536 | .68768 | 14.5390 | 18.9160 | 15.67 | 18.75 |
| 3.5 | 4 | 12.9700 | .84060 | .42030 | 11.6324 | 14.3076 | 11.82 | 13.65 |
| 4 | 4 | 11.5700 | 1.11059 | .55529 | 9.8028 | 13.3372 | 10.63 | 13.18 |
| 4.5 | 4 | 10.5950 | 2.06673 | 1.03336 | 7.3064 | 13.8836 | 8.32 | 13.14 |
| 5 | 4 | 8.5725 | 1.85549 | .92774 | 5.6200 | 11.5250 | 6.35 | 10.56 |
| 5.5 | 4 | 7.4750 | 1.19893 | .59947 | 5.5672 | 9.3828 | 6.27 | 8.97 |
| 6 | 4 | 8.2745 | .83669 | .41835 | 6.9431 | 9.6059 | 7.23 | 9.06 |
| 6.5 | 4 | 7.3400 | .32690 | .16345 | 6.8198 | 7.8602 | 6.98 | 7.76 |
| 7 | 4 | 9.3425 | .68515 | .34257 | 8.2523 | 10.4327 | 8.35 | 9.85 |
| Total | 36 | 10.3186 | 3.12737 | .52123 | 9.2604 | 11.3767 | 6.27 | 18.75 |

**ANOVA**

karbon

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 299.538 | 8 | 37.442 | 23.632 | .000 |
| Within Groups | 42.779 | 27 | 1.584 | | |
| Total | 342.316 | 35 | | | |

**karbon**

Tukey HSD

| pH | N | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| 6.5 | 4 | 7.3400 | | | | |
| 5.5 | 4 | 7.4750 | | | | |
| 6 | 4 | 8.2745 | 8.2745 | | | |
| 5 | 4 | 8.5725 | 8.5725 | | | |
| 7 | 4 | 9.3425 | 9.3425 | 9.3425 | | |
| 4.5 | 4 | | 10.5950 | 10.5950 | 10.5950 | |
| 4 | 4 | | | 11.5700 | 11.5700 | |
| 3.5 | 4 | | | | 12.9700 | |
| 3 | 4 | | | | | 16.7275 |
| Sig. | | .404 | .230 | .274 | .206 | 1.000 |

Means for groups in homogeneous subsets are displayed.

5. Efek waktu inkubasi

Data hasil pengamatan efek waktu inkubasi

| Waktu inkubasi (hari) | Ulangan | Kadar etanol (%) | Kadar Nitrogen DDG (%) | Kadar karbon DDG (%) |
|---|---|---|---|---|
| 1 | 1 | 0 | 0.26 | 48.75 |
| | 2 | 0 | 0.32 | 46.61 |
| | 3 | 0 | 0.34 | 45.7 |
| | 4 | 0 | 0.37 | 45.21 |
| 2 | 1 | 0.43 | 0.67 | 39.15 |
| | 2 | 0.48 | 0.62 | 44.7 |
| | 3 | 0.24 | 0.68 | 44.43 |
| | 4 | 0.32 | 0.86 | 44.35 |
| 3 | 1 | 0.98 | 1.68 | 24.18 |
| | 2 | 0.869 | 1.93 | 23.74 |
| | 3 | 0.93 | 1.78 | 23.68 |
| | 4 | 0.98 | 1.83 | 23.59 |
| 4 | 1 | 3.43 | 3.11 | 13.4 |
| | 2 | 4.51 | 3.25 | 13.08 |
| | 3 | 4.09 | 3.24 | 12.56 |
| | 4 | 4.21 | 3.12 | 12.56 |
| 5 | 1 | 19.23 | 3.51 | 9.76 |
| | 2 | 20.15 | 3.64 | 9.75 |
| | 3 | 19.82 | 3.87 | 9.71 |
| | 4 | 19.65 | 3.28 | 9.65 |
| 6 | 1 | 20.23 | 4.36 | 9.56 |
| | 2 | 19.77 | 4.32 | 9.25 |
| | 3 | 20.54 | 4.35 | 9.02 |
| | 4 | 20.72 | 4.56 | 8.97 |
| 7 | 1 | 20.14 | 4.61 | 8.93 |
| | 2 | 20.93 | 4.72 | 8.84 |
| | 3 | 20.64 | 4.6 | 8.58 |
| | 4 | 20.65 | 4.73 | 8.38 |
| 8 | 1 | 20.25 | 4.37 | 8.34 |
| | 2 | 20.21 | 4.48 | 8.34 |
| | 3 | 21.07 | 4.5 | 8.32 |
| | 4 | 21.06 | 4.51 | 8.19 |
| 9 | 1 | 20.26 | 4.34 | 7.75 |
| | 2 | 20.34 | 4.07 | 7.55 |
| | 3 | 21.08 | 4.48 | 7.17 |
| | 4 | 21.12 | 4.5 | 6.68 |

Analisis statistika efek waktu inkubasi terhadap kadar etanol

**Descriptives**

etanol

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1 | 4 | .0000 | .00000 | .00000 | .0000 | .0000 | .00 | .00 |
| 2 | 4 | .3675 | .10813 | .05406 | .1954 | .5396 | .24 | .48 |
| 3 | 4 | .9398 | .05273 | .02636 | .8558 | 1.0237 | .87 | .98 |
| 4 | 4 | 4.0600 | .45563 | .22782 | 3.3350 | 4.7850 | 3.43 | 4.51 |
| 5 | 4 | 19.7125 | .38283 | .19141 | 19.1033 | 20.3217 | 19.23 | 20.15 |
| 6 | 4 | 20.3150 | .41589 | .20795 | 19.6532 | 20.9768 | 19.77 | 20.72 |
| 7 | 4 | 20.5900 | .32873 | .16437 | 20.0669 | 21.1131 | 20.14 | 20.93 |
| 8 | 4 | 20.6475 | .48238 | .24119 | 19.8799 | 21.4151 | 20.21 | 21.07 |
| 9 | 4 | 20.7000 | .46332 | .23166 | 19.9628 | 21.4372 | 20.26 | 21.12 |
| Total | 36 | 11.9258 | 9.67081 | 1.61180 | 8.6537 | 15.1979 | .00 | 21.12 |

**ANOVA**

etanol

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 3270.070 | 8 | 408.759 | 3.353E3 | .000 |
| Within Groups | 3.291 | 27 | .122 | | |
| Total | 3273.361 | 35 | | | |

**etanol**

Tukey HSD

| waktu | N | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| 1 | 4 | .0000 | | | | |
| 2 | 4 | .3675 | .3675 | | | |
| 3 | 4 | | .9398 | | | |
| 4 | 4 | | | 4.0600 | | |
| 5 | 4 | | | | 19.7125 | |
| 6 | 4 | | | | 20.3150 | 20.3150 |
| 7 | 4 | | | | | 20.5900 |
| 8 | 4 | | | | | 20.6475 |
| 9 | 4 | | | | | 20.7000 |
| Sig. | | .851 | .367 | 1.000 | .304 | .817 |

Means for groups in homogeneous subsets are displayed.

Analisis statistika efek waktu inkubasi terhadap kadar nitrogen DDG

**Descriptives**

nitogen

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1 | 4 | .3225 | .04646 | .02323 | .2486 | .3964 | .26 | .37 |
| 2 | 4 | .7075 | .10500 | .05250 | .5404 | .8746 | .62 | .86 |
| 3 | 4 | 1.8050 | .10408 | .05204 | 1.6394 | 1.9706 | 1.68 | 1.93 |
| 4 | 4 | 3.1800 | .07528 | .03764 | 3.0602 | 3.2998 | 3.11 | 3.25 |
| 5 | 4 | 3.5750 | .24664 | .12332 | 3.1825 | 3.9675 | 3.28 | 3.87 |
| 6 | 4 | 4.3975 | .10966 | .05483 | 4.2230 | 4.5720 | 4.32 | 4.56 |
| 7 | 4 | 4.6650 | .06952 | .03476 | 4.5544 | 4.7756 | 4.60 | 4.73 |
| 8 | 4 | 4.4650 | .06455 | .03227 | 4.3623 | 4.5677 | 4.37 | 4.51 |
| 9 | 4 | 4.3475 | .19822 | .09911 | 4.0321 | 4.6629 | 4.07 | 4.50 |
| Total | 36 | 3.0517 | 1.62100 | .27017 | 2.5032 | 3.6001 | .26 | 4.73 |

**ANOVA**

nitogen

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 91.515 | 8 | 11.439 | 682.572 | .000 |
| Within Groups | .452 | 27 | .017 | | |
| Total | 91.968 | 35 | | | |

**nitogen**

Tukey HSD

| waktu | N | Subset for alpha = 0.05 | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 1 | 4 | .3225 | | | | | | |
| 2 | 4 | | .7075 | | | | | |
| 3 | 4 | | | 1.8050 | | | | |
| 4 | 4 | | | | 3.1800 | | | |
| 5 | 4 | | | | | 3.5750 | | |
| 9 | 4 | | | | | | 4.3475 | |
| 6 | 4 | | | | | | 4.3975 | 4.3975 |
| 8 | 4 | | | | | | 4.4650 | 4.4650 |
| 7 | 4 | | | | | | | 4.6650 |
| Sig. | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | .928 | .127 |

Means for groups in homogeneous subsets are displayed.

Analisis statistika efek waktu inkubasi terhadap kadar karbon DDG

**Descriptives**

karbon

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1 | 4 | 46.5675 | 1.56636 | .78318 | 44.0751 | 49.0599 | 45.21 | 48.75 |
| 2 | 4 | 43.1575 | 2.67586 | 1.33793 | 38.8996 | 47.4154 | 39.15 | 44.70 |
| 3 | 4 | 23.7975 | .26235 | .13117 | 23.3801 | 24.2149 | 23.59 | 24.18 |
| 4 | 4 | 12.9000 | .41376 | .20688 | 12.2416 | 13.5584 | 12.56 | 13.40 |
| 5 | 4 | 9.7175 | .04992 | .02496 | 9.6381 | 9.7969 | 9.65 | 9.76 |
| 6 | 4 | 9.2000 | .26920 | .13460 | 8.7716 | 9.6284 | 8.97 | 9.56 |
| 7 | 4 | 8.6825 | .25038 | .12519 | 8.2841 | 9.0809 | 8.38 | 8.93 |
| 8 | 4 | 8.2975 | .07228 | .03614 | 8.1825 | 8.4125 | 8.19 | 8.34 |
| 9 | 4 | 7.2875 | .47105 | .23553 | 6.5379 | 8.0371 | 6.68 | 7.75 |
| Total | 36 | 18.8453 | 14.93455 | 2.48909 | 13.7922 | 23.8984 | 6.68 | 48.75 |

**ANOVA**

karbon

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 7775.777 | 8 | 971.972 | 856.069 | .000 |
| Within Groups | 30.656 | 27 | 1.135 | | |
| Total | 7806.432 | 35 | | | |

**karbon**

Tukey HSD

| waktu | N | Subset for alpha = 0.05 | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 |
| 9 | 4 | 7.2875 | | | | |
| 8 | 4 | 8.2975 | | | | |
| 7 | 4 | 8.6825 | | | | |
| 6 | 4 | 9.2000 | | | | |
| 5 | 4 | 9.7175 | | | | |
| 4 | 4 | | 12.9000 | | | |
| 3 | 4 | | | 23.7975 | | |
| 2 | 4 | | | | 43.1575 | |
| 1 | 4 | | | | | 46.5675 |
| Sig. | | .068 | 1.000 | 1.000 | 1.000 | 1.000 |

Means for groups in homogeneous subsets are displayed.