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Improving ethanol production by co-culturing of *Saccharomyces cerevisiae* with *Candida tropicalis* from rice husk hydrolysate media

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The use of agricultural by-product as feed stock and co-culture fermentation is a good strategy for improving the efficiency of fermentation and ethanol production. Most rice husks have low protein and nitrogen content and need to be supplemented with nitrogen for fermentation process. This research sought to determine the optimal supplementation of rice husk stream–based fermentation medium with nitrogen and molasses sources, initial pH and incubation time for maximizing ethanol production by co-culturing *Saccharomyces cerevisiae* with *Candida tropicalis*. Urea, sodium nitrate and ammonium nitrate were used as nitrogen sources and molasses was used as carbon sources. Co-cultures of *S. cerevisiae* and *C. tropicalis* can use different nitrogen sources and molasses for growth and ethanol production. Molasses supplemented with rice husk hydrolysate medium, initial pH and incubation period significantly influenced ethanol yield and content of nitrogen and carbon in distillers grains (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 containing 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 to 29°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 for improving the efficiency of fermentation and ethanol production. Lignocellulosic biomass including agricultural by-product has been considered as possible raw material for ethanol production due to its renewability, large quantities,

relatively low prices compared to grain or sugar, potential environmental benefits and competactiveness 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). In a previous study, the simple sugar content in rice husk hydrolysate consists of 35.97% glucose, 8.87% xylose and 1.21% arabinose (Sopandi and Wardah, 2015). *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). In addition, other problem associated with efficient conversion of cellulose and hemicellulose sugars to ethanol is that during dilution of 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 ethanol yield and productivity obtained during fermentation of lignocellulosic hydrolysates decreases due to the presence of inhibiting compounds, such as weak acids, furans and phenolic compounds formed or released during thermo-chemical pre-treatment step such as acid and steam explosion (Parawira and Tekere, 2011).

Although it varies, most rice husks have low protein and nitrogen content and need to be supplemented with nitrogen for fermentation process. In one study, crude protein and nitrogen of rice husks were 4.38 and 0.7%, respectively, with C/N ratio of 57.93 (Ofoefule et al., 2011). In another study, crude protein, crude fiber and gross energy of rice husks were 1.92%, 37.33% and 302.33 kcal/kg, respectively (Telew et al., 2013). Nitrogen sources such as ammonium (Jones et al., 1994; Srichuwong et al., 2009) and urea (Jones and Ingledew, 1994; Yue et al., 2010) are widely used to increase yeast growth, and rate of sugar utilization and to reduce fermentation time (Chniti et al., 2015). Urea not only promoted the specific growth rate and ethanol tolerance, but also increased the ethanol yield and reduced the formation of side products (Yue et al., 2010). However, several investigators have reported the 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).

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, *S. cerevisiae* cultivation influence cellular growth and metabolites biosynthesis (Thomas et al., 1996). Molasses is a waste product of the sugar industry which 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).

Co-culture is a potential bioprocess whereby, 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 increases ethanol productivity which might be due to enhanced substrate utilization (Tesfaw and Assefa, 2014). Co-culture of *S. cerevisiae* with other microbes reduces inhibitory compounds in lignocellulosic hydrolysates (Tom´as et al., 2013; Taherzadeh and Karimi, 2011; Wan et al., 2012) which increases ethanol yield and production rate (Singh et al., 2014; Wan, 2012), shortens fermentation time, and reduces process cost (Hickert et al., 2013; Tesfaw and Assefa, 2014).

C. tropicalis have been demonstrated to produce ethanol from a mixed-sugar stream (Oberoi et al., 2010) and acid hydrolysate olive pruning (Mateo et al., 2015). It is able to degrade acetate, furfural, and 5-hydromethylfurfural and metabolite xylose to ethanol under anaerobic simultaneous saccharification and fermentation (Cheng et al., 2014). In a previous study, ethanol production from rice husks hydrolysate medium by co-culturing of *S. cerevisiae* and *C. tropicalis* higher than mono cultures of *S. cerevisiae* or *C. tropicalis* and other mono and co-cultures fermentation was more efficient in metabolizing and converting fermentable sugars than other selected microorganisms (Sopandi and Wardah, 2015). The present study explored the supplementation of inorganic nitrogen sources and molasses used to improve ethanol production by co-culturing of *S. cerevisiae* with *C. tropicalis* from rice husk hydrolisate.

MATERIALS AND METHODS

Culture microorganism

S. cerevisiae Food and Nutrition Culture Collection (FNCC) 3012

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and *C. tropicalis* FNCC 3033 were obtained from Microbiology Laboratories, PPAU Gadjah Mada University, Yogyakarta, Indonesia. Sabouraud agar (Oxoid) was used to maintain the strains. Working stock cultures were prepared from stock in 7 days at 28°C SA plate cultures subcultures from the master stock. Colonies were aseptically sampled by scraping the top with an inoculating loop and transferred to 10 ml sterile water. Inoculum stock suspensions were prepared from working stock and diluted to 1.7×10^6 cell/ml, as enumerated using a haemocytometer.

Rice husk hydrolysis

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

Inorganic nitrogen supplementation

The effect of inorganic nitrogen supplemented with rice husk hydrolysate medium on ethanol yield, N and C content of DDG was conducted using completely randomized design with 4 treatments of N sources where each treatment was replicated 5 times. The basal medium containing 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 Erlenmeyer flasks. Each 1000 ml urea, sodium nitrate ($NaNO_3$) and ammonium nitrate (NH_4NO_3) was added to final individual concentrations of 9.0 g/l, respectively. Media were mixed thoroughly, adjusted to pH 5.5 with an addition of NaOH, which is autoclaved for 15 min at 121°C and cooled to room temperature.

One hundred millilitres was aseptically dispensed into individual Erlenmeyer flasks (250 ml), inoculated with 1.0 ml of *S. cerevisiae* FNCC 3012 and 1.0 ml of *C. tropicalis* FNCC 3033 inoculum stock suspension. All flasks were incubated at 28 to 29°C with 50% relative humidity in the dark, for 5 d in a rotary incubator at 60 rpm.

Molasses supplementation

The effect of molasses supplemented with rice husk hydrolysate medium on ethanol yield, N and C content of DDG was conducted using completely randomized design with 5 treatments of molasses proportion in a medium and each treatment was replicated 5 times. Molasses was obtained from locally sugar industry, Mojekerto, Indonesia. 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. Molasses was added to final concentrations of 0.0, 5.0, 10.0, 15.0 and 20.0 ml/l.

Media were mixed thoroughly, adjusted to pH 5.5 with an addition of NaOH or HCl 1 N, autoclaved for 15 min at 121°C and cooled to room temperature. One hundred millilitres was then aseptically dispensed into individual Erlenmeyer flasks (250 ml) with one ml of *S. cerevisiae* FNCC 3012 and *C.tropicalis* FNCC 3033 inoculum stock suspension and all flasks were incubated as described above.

Formulation of rice husk hydrolysate

The effect of formulation rice husk hydrolysate on ethanol yield, N and C content of DDG was conducted using completely randomized design with 4 treatments and each treatment was replicated five times. Four formulations of rice husk hydolysate media were examined to improve ethanol production by co-culturing *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 were 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), respectively.

Media were mixed thoroughly, adjusted to pH 5.5 with an addition of NaOH or HCl 1 N, autoclaved for 15 min at 121°C and cooled to room temperature. One hundred millilitres was then aseptically dispensed into individual Erlenmeyer flasks (250 ml) with one ml of *S. cerevisiae* FNCC 3012 and *C.tropicalis* FNCC 3033 inoculum stock suspension and all flasks were incubated as described above.

Initial medium pH

The effect of initial medium pH on ethanol yield, N and C content of DDG was conducted using completely randomized design with 8 treatments of initial pH medium (3.5 to 7.0) and each of the treatment was replicated 5 times.

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 was adjusted to 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, and 7.0 prior to autoclaving for 15 min at 121°C with NaOH or HCl 0.1 N added. After cooling to room temperature, flasks were inoculated with 1-ml *S. cerevisiae* FNCC 3012 and 1- ml *C.tropicalis* FNCC 3033 inoculum stock suspension and incubated as described above.

Incubation period

The effect of incubation period on ethanol yield, N and C content of DDG was conducted using completely randomized design with 9 treatments of incubation period (1 to 9 d) and each of the treatment was replicated 5 times. The effect of incubation period on ethanol yield, nitrogen and carbon content distillate 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 and adjusted to pH 5.5 by adding NaOH and autoclaved for 15 min at 121°C.

Erlenmeyer flasks (250 ml) containing 100-ml sterile medium were inoculated with 1-ml *S. cerevisiae* FNCC 3012 and 1- ml *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 yield

Whole flask cultures were distillated at 78°C for 60 min and ethanol in distillated were measured using a gas chromatograph Carbomax

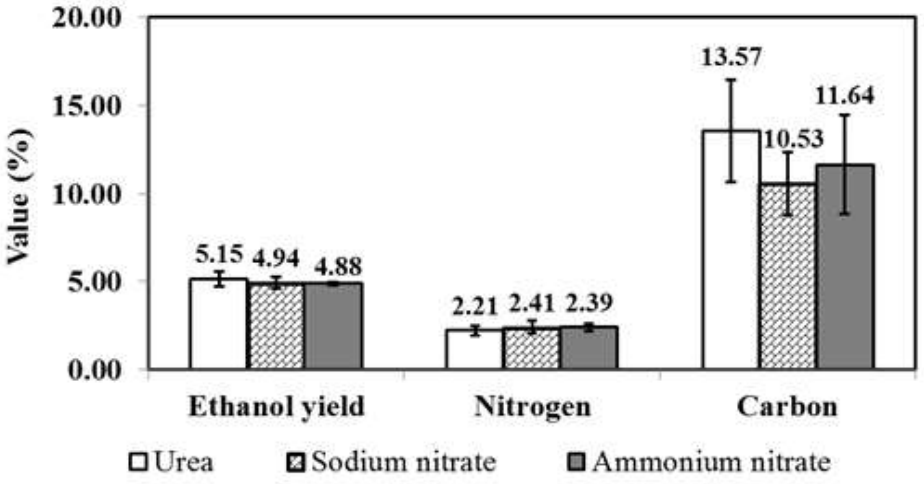

Figure 1. Ethanol yield, nitrogen and carbon content in DDG from rice husk hydrolysate medium supplemented inorganic nitrogen different that fermented by co-culture *S. cerevisiae* with *C. tropicalis*. Values and error bars represent means ± SD (n=5).

t70-10-0 column, FID t220 detector, helium as carrier gas with flow rate of 40.3 mL/min, tin column Porapack Q, detector temperature at 160°C and column temperature at 180°C with injection volume 1.0 µL. Fermented media were filtered through Whatmann No.1 paper prior to analysis.

$$\text{Ethanol yield (\%)} = \frac{\text{Concentration of ethanol from GC analysis (ml)}}{\text{Volume of medium (ml)}} \times 100$$

Determination of distillers dried grains

To analyze distillers' dried grains, whole flask cultures were distillated at 78°C for 60 min and residue was poured through predried (100°C) and preweighed Whatman No.1 filter paper. Retained material was washed with distilled water and ethanol until it became 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 millilitres (100 ml) 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 (NH_4-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. 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 were 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 and transferred to a new borosilicate glass test tube. 4 ml potassium sodium 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) were successively added, mixed and allowed to stand for 10 min. Four-millilitre of 5% NaOCl was also added, shaken and allowed to stand for 10 min with an absorbance measurement at 636 nm. $(NH_4)_2SO_4$ which 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 of inorganic nitrogen supplementation

No significant ($P>0.05$) differences in ethanol yield or nitrogen and carbon content of distillers' dried grains (DDG) was observed between types of nitrogen source (Figure 1). Also, no significant ($P > 0.05$) differences were

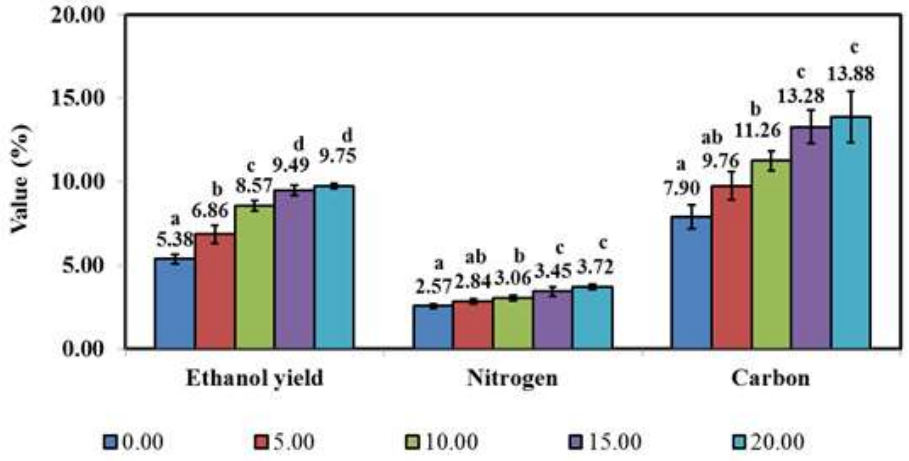

Figure 2. Effect of molasses addition on ethanol yield, nitrogen and carbon content of DDG rice husk hydrolysate medium that fermented by co-culture by co-culture *S. cerevisiae* with *C. tropicalis*. Values and error bars represent means ± SD (n=5) in same variable (ethanol yield, nitrogen and carbon) with different subscript shown ANOVA Tukey's test. a, b, c, d P<0.05 within respective groups.

observed between ethanol yields from rice husk hydrolysate basal media supplemented with urea. A similar lack of effect was observed for nitrogen content in DDG. Addition of inorganic nitrogen to the rice husk hydrolysate basal medium significantly affected (P > 0.05) carbon content of DDG.

Effect of molasses supplementation

Addition of 5 to 20 ml/l molasses to the rice husk hydrolysate basal medium significantly (P<0.05) increased ethanol yield, nitrogen and carbon content of DDG (Figure 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 is significantly (P<0.05) lower than that in the basal medium plus 5, 10, 15, and 20 ml/l molasses, but no significant (P>0.05) difference between 15 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 was not significantly different (P>0.05) from the basal medium plus of 5 ml/l molasses, but significantly (P<0.05) lower than that in basal medium plus 10, 15 and 20 ml/l molasses. However, there is no significant (P>0.05) difference between 15 ml/l and 20 ml/l molasses basal medium plus. This indicates molasses-concentration stimulates growth of yeast and ethanol production. While the mean carbon content of DDG in the basal medium was not significantly different (P>0.05) from that in the basal medium plus 5 ml/l molasses, it was significantly (P<0.05) lower than that in the basal medium plus 10, 15 and 20 ml/l molasses. However, no significant (P>0.05) difference was seen between 15 ml/l and 20 ml/l molasses.

Formulation of rice husk hydrolysate media

Formulation of rice husk hydrolysate media supplemented with inorganic nitrogen and molasses significantly (P<0.05) influenced ethanol yield, nitrogen and carbon content of DDG (Figure 3). The addition of nitrogen source and molasses to the rice husks hydrolysate fermentation media increased ethanol yield and nitrogen levels but lowered the carbon content of DDG.

Values and error bars represent means ± SD (n=5) in same variable (ethanol yield, nitrogen and carbon) with different subscripts shown in ANOVA Tukey's test. a, ab, b, bc, c P<0.05 within respective groups. 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; $7H_2O$, 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

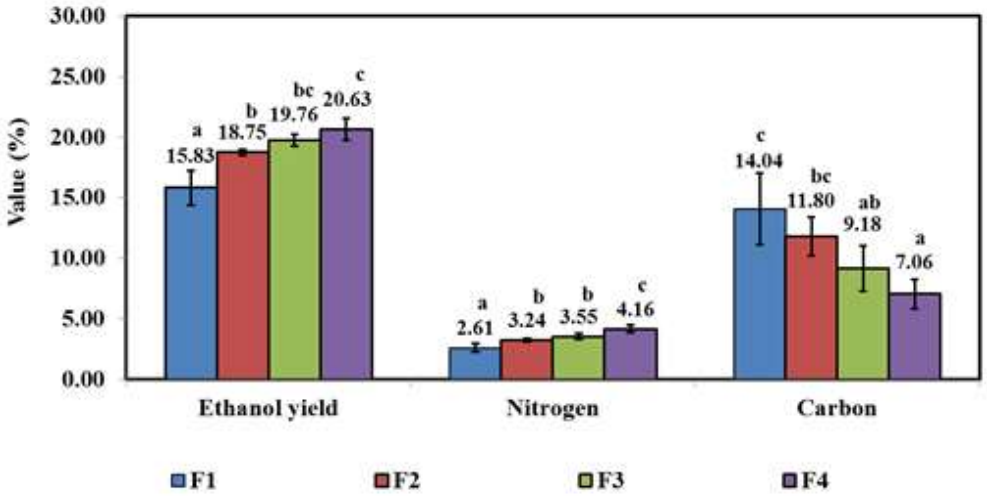

Figure 3. Effect of different formulation of rice husk hydrolysate culture medium on ethanol yield, nitrogen and carbon content of DDG were fermented by co-culture *S. cerevisiae* with *C. tropicalis*.

NH_4NO_3, 1.0 g/l KH_2PO_4, 0.7 g/l $MgSO_4 \cdot 7H_2O$, 20 ml/l molasses and 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.

Maximum ethanol yield, nitrogen and carbon content of DDG were obtained in the rice husk hydrolysate medium F4. Ethanol yield in F1 medium is significantly ($P<0.05$) lower than F2, F3 and F4. While mean nitrogen content of DDG in the F1 medium was significantly ($P<0.05$) lower than that in the F2, F3 and F4 medium, but no significant ($P > 0.05$) difference was observed between F2 and F3 medium. Carbon content of DDG in the F1 medium was also significantly ($P < 0.05$) higher than that in the F3 and F4 medium, but no significant ($P>0.05$) differences between F1 and F2 and between F3 and F4 also were observed in the medium.

Effect of initial medium pH

Initial medium pH significantly ($P<0.05$) affected ethanol yield, nitrogen and carbon content of DDG (Figure 4). This study showed that *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 to 6.5, decreased ethanol yield, nitrogen and carbon content of DDG. Ethanol yield at pH 5.5 and 6.0 was significantly ($P<0.05$) higher than that at pH 3.0, 3.5, 4.0, 4.5, 5.0, 6.5 or 7.0, with no significant ($P > 0.05$) difference observed between pH 5.5 and 6.0 and 6.0 and 6.5. Nitrogen contents of DDG pH 5.5, 6.0 and 6.5 were significantly ($P<0.05$) higher than those at pH 3.5, 4.0, 4.5, 5.0, or 7.0; no significant ($P > 0.05$) difference was observed between pH 5.0, 5.5, 6.0 and 6.5. There was significant diference in the carbon content mean of DDG at pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, or 7.0.

Effect of incubation period

Incubation period significantly ($P<0.05$) affected ethanol yield (Figure 5A), nitrogen and carbon content of DDG (Figure 5B). Ethanol yield increased between 3 and 5 d, then was stagnant from 6 to 9 d total incubation. Ethanol yields at 2 and 3 d were significantly ($P<0.05$) lower than that at 4 d; 4 d ethanol yield was significantly ($P < 0.05$) lower than that at 5, 6, 7, 8, and 9 d. Differences in ethanol yield between 5 and 6 d incubation were not significant ($P > 0.05$). Ethanol yield at 5 d was also significantly ($P < 0.05$) lower than 7, 8 or 9 d, but there were no significant ($P < 0.05$) differences between 6, 7, 8 and 9 d ethanol yields.

Nitrogen content of DDG increased between 1 and 7d, then relatively stagnant from 7 to 9 days of total incubation. Nitrogen contents of DDG 1, 2, 3, 4, 5, and 6 d were significantly ($P<0.05$) lower than those at 7, 8 and 9 d. Differences in nitrogen content of DDG between 1, 2, 3, 4, 5 and 6 d incubation were relatively small ($P < 0.05$), but no significant ($P > 0.05$) differences were observed

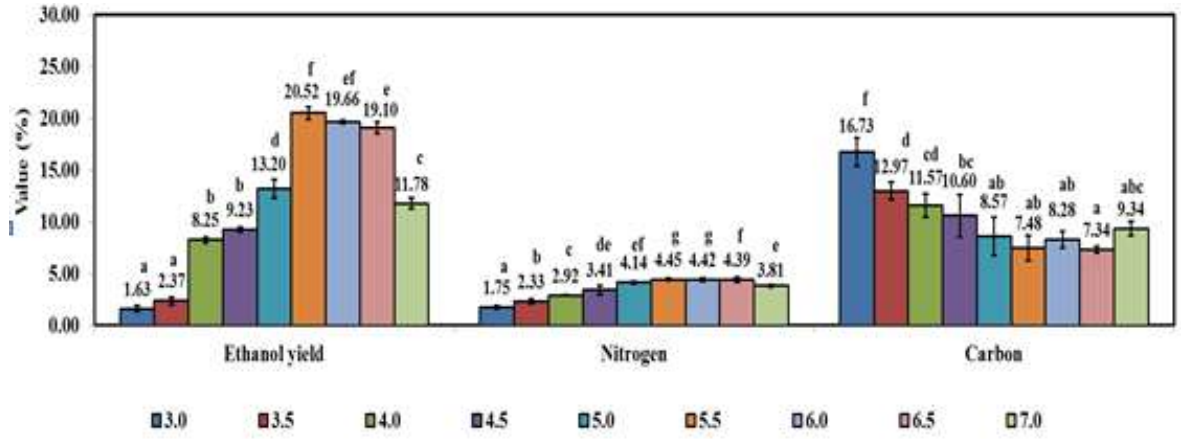

Figure 4. Effect of initial pH medium on ethanol yield, nitrogen and carbon content of DDG from rice husk hydrolysate culture medium with supplemented and fermented by co-culture *S. cerevisiae* and *C. tropicalis.* Values and error bars represent means ± SD (n=5) in same variable (ethanol yield, nitrogen and carbon) with different subscript shown ANOVA Tukey's test. a, b, c, d, e, g P<0.05 within respective groups.

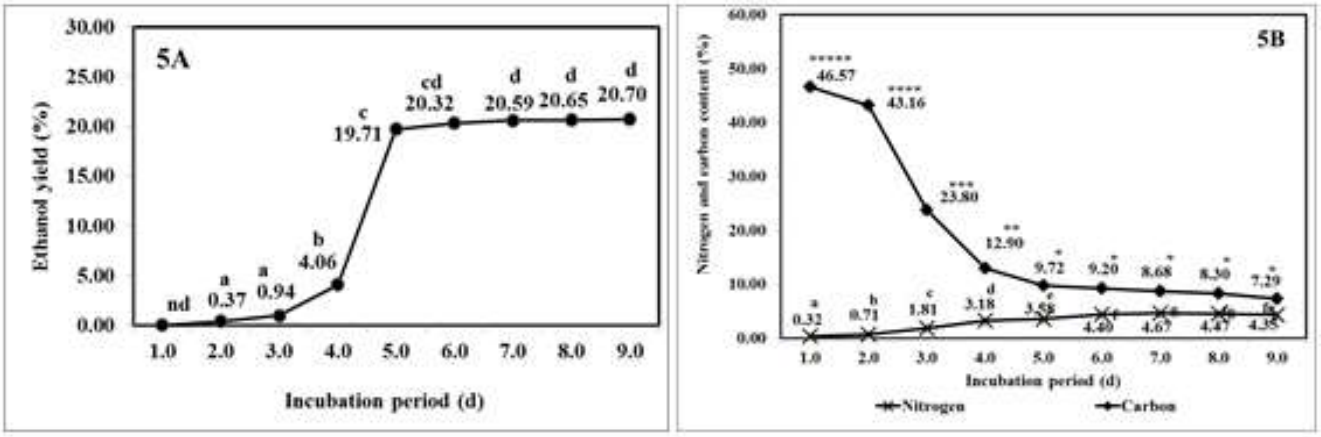

Figure 5. Effect of incubation period on ethanol yield (5A) and nitrogen and carbon content (5B) of distillate residue fermented rice husk hydrolysate medium by co-culture *S. cerevisiae* and *C. tropicalis.* Values and error bars represent means ± SD (n=5) in same variable (ethanol yield, nitrogen and carbon) with different subscript shown ANOVA Tukey's test. a, b, c, d, e, f, fg, g and *, **, ***, ****, **** P<0.05 within respective groups.

between 7, 8 and 9 d incubation. The mean nitrogen content of DDG decreased between 1 and 4 days, but was relatively stagnant from 5 to 9 days total incubation. Carbon contents of DDG 1, 2, 3, and 4 d were significantly (P<0.05) higher than those at 5, 6, 7, 8 and 9 d. Differences in nitrogen content of DDG between 1, 2, 3 and 4 days incubation were relatively small (P < 0.05), but no significant (P > 0.05) differences were observed between 5, 6, 7, 8 and 9 d incubation.

DISCUSSION

Studies in other fermentation systems have revealed that N deficiency in the fermentation medium leads to slow and stuck fermentation rate (Vilanova et al., 2007). N sources are very crucial and strongly influence the yeast growth and metabolism during fermentation (Beltran et al., 2005). The present study shows no significant differences in ethanol yield or N and C content of DDG at

exogenous N sources supplemented (NH_4NO_3, urea and $NaNO_3$) with rice husk fermentation media. This indicates that the co-culture of *S. cerevisiae* and *C. tropicalis* can be utilized on the various sources of N for growth and stimulation of ethanol production.

Some investigators have reported varying effects of exogenous N source supplemented with lignocellulosic fermentation media on ethanol production by yeast. The results of this work are similar to several studies which reported that supplementation of the various sources of N with fermentation media does not significantly affect the production of ethanol. 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 which was obtained during the second step of crystallization but did not improve ethanol productivity significantly. Wang et al. (2012) reported that, for 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 which reported that the supplementation of various N sources to fermentation media affected ethanol production. Mongkolchaiarunya et al. (2016) reported that ammonium nitrate is better than ammonium chloride, ammonium sulfate, urea and peptone as N sources for ethanol production from cattail. Li et al. (2016) reported that the combination of urea and ammonium sulfate as nitrogen sources synergistically enhanced ethanol production by *S. cerevisiae* in a very high gravity fermentation of corn starch.

Initial sugar concentrations before fermentation 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, such as *S. cerevisiae and Klyureomyces marxianus* (Parkash, 2015). The present study shows that supplementation of molasses in the growth media significantly ($P<0.05$) increased ethanol yield and the N and C content in the distillers grains. Production of ethanol from molasses-based media by co-culture fermentation has been reported. Eiadpum et al. (2012) reported that immobilized co-culture of *K. marxianus* and *S. cerevisiae* can improve ethanol production from both sugarcane juice and blackstrap molasses when the operating temperature ranged between 33°C and 45°C and generate 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 level of N sources significantly increased the efficiency of fermentation and yeast yield (Tyagi and Ghose, 1980). Increasing the N concentration in the fermentation medium can increase the rate of fermentation, decrease the duration and lack of nitrogen triggers sluggish fermentations **(**Alexandre and Charpentier, 1998; Fleet and Heard, 1992; Varela et al., 2004). The ratio of N sources to carbon sources in the medium can influence yeast growth and metabolism of *S. cerevisiae* (Larsson et al., 1993). N deficiency with a high sugar transporter turnover rate results in a loss of sugar uptake capacity in the cells (Salmon, 1989; Bisson, 1999). In the present study, 4 formulations of rice husk hydrolysate media with different supplemented inorganic nitrogen and molasses significantly ($P<0.05$) influenced 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 the present work, initial pH of the medium affected ethanol yield and the content of N and C at DDG. A wide range of optimum pH (4.0 to 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 to 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 reported the 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 wheat straw medium after 48 h of incubation, employing process of simultaneous saccharification and fermentation using *T. reesei* cellulase and *Kluyveromyces fragilis*. Sharma (2000) reported maximum ethanol yield and fermentation efficiency of 0.397 g/g and 77.84%, 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 at 48 h of incubation from starch medium in a single step process by co-culturing of amylolytic yeasts and *S.cerevisiae.*

In the present study, the maximum ethanol yield (20.32%) lower than the theoretical maximum ethanol yield of broth hexoses and pentoses is 0.511 kg/kg sugar, but higher than the ethanol yield from rice husk which has been reported by some investigators. Reddy and Pushpa (2012) reported the maximum ethanol yield (1.60%) obtained from rice husks, treated with 5% sodium hydroxide and fermented by *S. cereveceae* type 181 at pH 5.0 for 7 d. Sopandi and Wardah (2015) reported the maximum ethanol yield (2.13 %) gained from rice husk hydrolysate medium with supplement of 4 g/l urea, 3 g/l

$NaNO_3$, 3 g/l NH_4NO_3, 1 g/l KH_3PO_4 and 0.7 g/l $MgSO_4 \cdot 7H_2O$ fermented by co-culturing of *S. cerevisiae* and *C. tropicalis* for 3 d at 30°C, 60 to 70% relative humidity, under dark condition, and 150 rpm agitation) incubation. Gaffa and Krakwowiak (1997) reported the maximum ethanol yield (10.5%) by *S. cerevisiae* continuous fermentation process from molasses diluted tap water (1:2) for 14 d at 27°C.

Conclusion

Inorganic nitrogen and molasses supplementation can increase the production of ethanol from rice husk hydrolysate medium by co-culturing of *S. cerevisiae* and *C. tropicalis*. Initial pH medium and incubation period demonstrated can influence ethanol production by co-culturing of *S. cerevisiae* and *C. tropicalis* from the rice husk medium supplemented with molasses. The best formulation medium to obtain maximum production of ethanol with pH 5.5 and incubation period of 6 days comprised of 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 $MgSO_4 \cdot 7H_2O$, and 20 ml/l molasses in 1000 ml rice husks hydrolysate.

Conflict of Interests

The authors have not declared any conflict of interest.

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