

ETHANOL PRODUCTION AND SUGAR CONSUMPTION OF CO-CULTURE *Saccharomyces cerevisiae* FNCC 3012 WITH *Candida tropicalis* FNCC 3033 IN MEDIA CONTAINING INHIBITOR FERMENTATION

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ABSTRACT

Inhibitor fermentation is one of the problems that arise in the ethanol production from lignocellulose waste. This work examined the ethanol yield and sugar consumption of mono and co-culture *S. cerevisiae* with *C. tropicalis* in media containing inhibitor fermentation. Furfural and phenol were used for inhibitor fermentation in basal medium with concentrations of 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 NaNO₃, 3 g.L⁻¹ NH₄NO₃, 1 g.L⁻¹ KH₂PO₄ and 0.7 g.L⁻¹ MgSO₄·7H₂O with pH adjusted to 5.5 with 1 mol.L⁻¹ HCl. After furfural or phenol addition separately, and inoculation by mono and co-culture *S. cerevisiae* FNCC 3012 with *C. tropicalis* FNCC 3033, all media were 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 yields 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* were more tolerant to furfural and phenol. Ethanol yield 8.52%, 5.37% and 3.83% obtained from basal medium, basal medium plus 2.5 or 5.0 % furfural, respectively and efficiency of fermentation 27.00%, 17.00% and 12.20%. Ethanol yields 8.13%, 5.62% and 3.19% obtained from basal medium, basal medium plus 2.5 or 5.0 % phenol, respectively and efficiency of 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 lignocellulosic media content inhibitors. The use of this co-culture effectively utilizes hexose and pentose sugars in the substrate, increasing the yield and efficiency of fermentation for ethanol production.

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

INTRODUCTION

The limitations of fossil fuels and environmental conditions have drawn attention to development of alternative energy sources that have a lower impact on the environment (Abreu-Cavalheiro and Monteiro, 2013). Ethanol is an alternative energy source with the potential to replace fossil energy sources and has received a lot of 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 its renewability, large quantities, relatively low prices compared to grain or sugar and potential environmental benefits, lignocellulosic biomass has been considered a possible raw material for ethanol production (Cardona and Sánchez, 2007; Kumar et al., 2008; Lee and Huang, 2000; Mielenz, 2001; Service, 2007; Zaldivar et al., 2001). Lignocellulosic biomass is preferred as a raw material for ethanol production over sugar or starch derived from crop products because it does not compete in terms of food needs and concerns 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 are the main products of lignocellulosic hydrolysates (Lee and Huang, 2000; Service, 2007; Eiteman et al., 2008). However, the lack of a microorganism capable of efficiently fermenting all sugars released by hydrolysis from lignocellulosic materials has been one of the main factors preventing the utilization of lignocellulose (Zaldivar et al., 2001). *Saccharomyces cerevisiae* is the dominant yeast used for ethanol production but cannot metabolize xylose and convert it into ethanol (Jeffries and Jin, 2004; Lin and Tanakan, 2006).

In addition, another problem associated with efficient conversion of cellulose and hemicellulose sugars to ethanol is that during dilute acid hydrolysis a broad range of compounds that 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 are formed or released during thermochemical pretreatment steps such as acid and steam

explosion can decrease the ethanol yield and productivity of lignocellulosic fermentation (Parawira and Tekere, 2011). Reduction of the ethanol yield and productivity by inhibiting components can influence the performance of microorganisms during the fermentation stage (Almeida et al., 2007).

The choice of the fermenting microorganism, complete substrate utilization, inhibitor tolerance and ethanol productivity are important aspects in the production of ethanol 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 all glucose and xylose in the lignocellulose hydrolysate efficiently and it should have high tolerance to the inhibitors present in the lignocellulose hydrolysate (Cheng et al., 2014).

Strategies for using a single microorganism to convert glucose and xylose simultaneously have limitations (Eiteman et al., 2008). Co-culture among microorganisms could potentially increase ethanol production and the efficiency of fermentation from lignocellulosic hydrolysate. Co-culture of *S. cerevisiae* and other microorganisms 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 of *S.cerevisiae* with *C. tropicalis* has the ability to generate and convert fermentable sugars from a waste stream rice husk to ethanol (Sopandi and Wardah, 2015). This work examined the ethanol yield and sugar consumption of mono and co-culture *S. cerevisiae* with *C. tropicalis* in medium containing inhibitor fermentation.

MATERIAL AND METHODS

Culture of microorganism

Saccharomyces cerevisiae FNCC 3012 and *Candida tropicalis* FNCC 3033 were obtained from Microbiology Laboratories, PPAU Gadjah Mada University, Yogyakarta, Indonesia. Saburoad agar (Oxoid) was used to maintain the strains *S. cerevisiae* and *C. tropicalis*. Working stock cultures were prepared from stock for 7 days at 28 °C in SA plate subcultures from the master stock. Colonies were aseptically sampled by scraping the top with an inoculating loop and transferring to 10 ml sterile water. Inoculum stock suspension was prepared from working stock and diluted to 1.7 x 10⁶ cell.mL⁻¹, as enumerated, using a haemocytometer.

Fermentation

The batch fermentation experiments were carried out in a 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 NaNO₃, 3.0 g.L⁻¹ NH₄NO₃, 1.0 g.L⁻¹ KH₂PO₄ and 0.7 g.L⁻¹ MgSO₄·7H₂O with pH adjusted to 5.5 with 1.0 mol.L⁻¹ HCl. Liquid basal medium (9.0 l) was mixed thoroughly and 100 ml individually dispensed into 250 mL Erlenmeyer flasks, autoclaved and cooled to room temperature. Media in Erlenmeyer were divided into two groups, Individually flask, one group was added furfural until final concentrations of 0.0, 2.5 and 5.0 % and another group was added phenol until final concentration of 0.0, 2.5, and 5.0 %. A 1.0 mL inoculum stock suspension of *S. cerevisiae* and 1.0 mL *C. tropicalis* for mono-culture were aseptically dispensed into individual Erlenmeyer flasks and 0.5 mL *S. cerevisiae* with 0.5 mL *C. tropicalis* for co-culture were 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

Serial dilution 10¹⁰ using sterile water was conducted to yeast count observation in 10 mL media before and after 5 days fermentation. Each serial dilution (0.1 mL) was inoculated and spread onto Saburoad agar (Oxoid), and incubated at 28–29 °C, 50% r.h. in the dark for 3 d. A colony counter was used for counting colonies on the media.

Determination of ethanol

Ethanol was measured using a gas chromatograph Carboxmax t70-10-0 column, an FID t220 detector, with helium as carrier gas with a flow rate of 40.3 mL.min⁻¹, and a tin column Porapak Q, with a detector temperature of 160 °C and a column temperature of 180 °C with an injection volume of 1.0 µL. Fermented media were filtered through Whatman Grade 1 paper.

Determination of sugar

Glucose, D(+) xylose and arabinose were determined using HPLC (Shimadzu, Kyoto, Japan) at 85 °C, a Metacharb 87C column, with H₂O as eluent, with a flow rate of 0.6 mL/min and an RID detector. After fermentation, the media were mixed and aseptically filtered through Whatman Grade 1 paper. The filtrate was centrifuged at 12,000 rpm for 15 min, refiltered through millex 0.45 µm and 25 µL of sample was injected for HPLC. Glucose, D(+) xylose and arabinose (Merck) were used as standard with concentrations of 62.5, 125, 250 and 500 ppm, respectively.

Efficiency of fermentation

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

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

Statistical analysis

Tukey's honestly significant difference multiple comparison test and a paired sample t-test were used to segregate significantly different treatment using SPSS 16 software. Analysis of variance (ANOVA) was performed to determine differences between experiments with 5% level of significance (P < 0.05).

RESULTS AND DISCUSSION

Yeast count

Addition of furfural (Fig. 1A) to the growth medium significantly (p < 0.05) decreased the yeast colony count in mono- or co-culture *S. cerevisiae* with *C.*

tropicalis. This work indicated that furfural inhibits the growth of yeast in monoculture and co-culture *S. cerevisiae* with *C. tropicalis*. Some investigators have reported the effect of furfural on the growth of microorganisms. **Palmqvist and Hahn-Hägerdal (2000)** reported that furfural inhibited the specific growth and fermentation rate of yeasts. **Agbogbo et al. (2007)** reported that a concentration of furfural of 1.5 g.L⁻¹ could interfere with the respiration and growth of microorganisms. **Hristozova et al. (2000)** reported that a concentration of furfural of 0.04% inhibited glutamate dehydrogenase and ion ammonia assimilation in the alanine metabolism of *C. blankii* 35 and *C. pseudotropicalis* 11. **Kelly et al. (2008)** reported that a concentration of furfural of 1 g.L⁻¹ or higher inhibited growth of *C. guilliermondii*. **Jones (1989)** and **Almeida et al. (2009)** suggested that furfural and 5-hydroxymethyl furfural (HMF) can be consumed by *S. cerevisiae* but will lose ATP. **Mattam et al. (2016)** reported that an increase in the level of furfural, HMF and acetic acid in growth media led to a gradual decrease in *C. tropicalis* biomass.

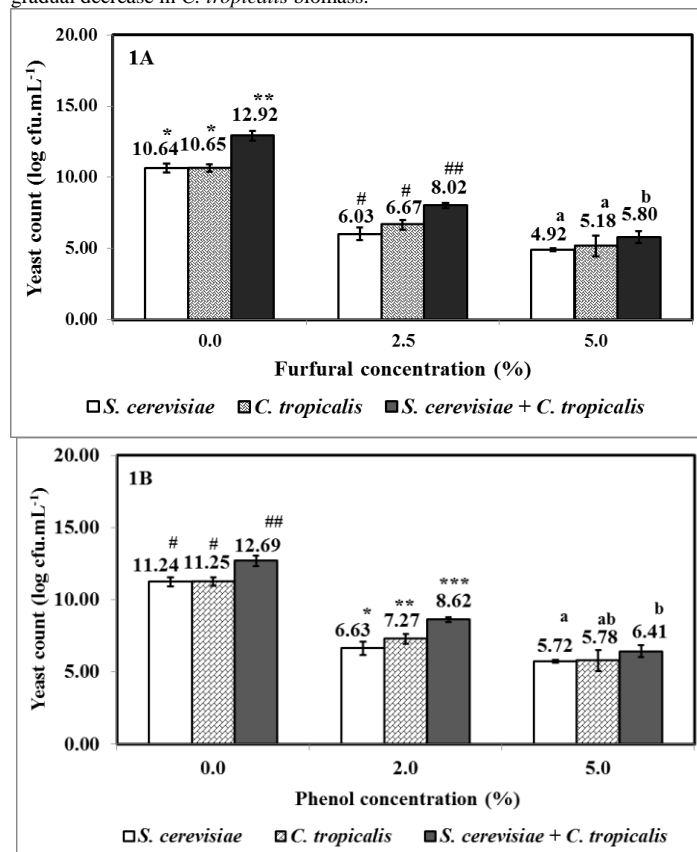


Figure 1 Yeast count in medium plus different concentrations of furfural (1A) and phenol (1B) after 5 days' fermentation by mono and co-culture *S. cerevisiae* with *C. tropicalis*. The values with different superscripts (^a and ^b, * and ** or # and ##) are different significant (p < 0.05) of means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

However, this work shows that the yeast count colony in co-culture of *S. cerevisiae* with *C. tropicalis* is significantly (P < 0.05) higher than mono-culture *S. cerevisiae* or *C. tropicalis* in the medium growth with or without furfural. We hypothesized that there is a synergistic mechanism to stimulate yeast growth through simultaneous utilization of fermentable sugars such as glucose and xylose and degradation of furfural in growth medium by co-culture of *S. cerevisiae* with *C. tropicalis*. Some investigators have reported consumption of fermentable sugars (glucose and xylose) simultaneously by co-culture fermentation. **Hikert et al. (2013)** reported that co-culture of *C. sheatae* HM 52.2 with *S. cerevisiae* ICV D254 can simultaneously consume glucose and xylose in synthetic medium and rice hull hydrolysate. **Fu and Peiris (2008)** reported that co-culture of *Zymomonas mobilis* with *Pachysolen tannophilus* was fully consumed in a mixture of glucose and xylose media. Some investigators have reported the degradation and conversion of furfural by yeast. **Taherzadeh et al. (1999)** reported that *S. cerevisiae* converted furfural to furfuryl alcohol in exponentially growing cells. **Cheng et al. (2014)** reported that *C. tropicalis* W103 degraded furfural and 5-hydroxymethylfurfural under aerobic conditions after 60 h of aerobic incubation. Some investigators reported that co-culture of *S. cerevisiae* with other microbes can reduce inhibitory compounds in lignocellulosic hydrolysates. **Tomás et al. (2013)** reported that co-culture of *Thermoanaerobacter pentosaceus* with *S. cerevisiae* was able to grow 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 that co-cultures of *S. cerevisiae* Y5 with *Pichia stipitis* CBS6054 can grow and metabolize furfural and HMF in a medium of a mixture of xylose and glucose.

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

Residue and sugar consumption

Addition of furfural to the growth medium significantly ($p < 0.05$) influenced glucose residue (Fig. 3) and xylose (Fig. 4), but did not significantly ($p > 0.05$) influence 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 did not significantly ($p > 0.05$) influence 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). Effects of inhibitory furfural and phenol on glucose consumption were reported by several researchers (Almeida et al., 2009; Larsson et al., 1999; Lin et al., 2015; Palmqvist et al., 2000; Sluiter et al., 2010). Wikandari et al. (2010) reported that glucose consumed 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 showed that glucose consumption by co-culture of *S. cerevisiae* with *C. tropicalis* was significantly ($P < 0.05$) higher than mono-culture *S. cerevisiae* or *C. tropicalis* in the medium with or without furfural. It was suspected that the higher glucose consumption by co-culture than mono-culture in this study was due to degradation of furfural by each yeast in the mixture fermentation. Under anaerobic conditions, *S. cerevisiae* can convert furfural to furfuryl alcohol (Díaz de Villegas et al., 1992; Sárvári Horváth et al., 2003) and the reduction of furfural has been linked to the co-factor NADH (Wahlbom et al., 2002).

In addition, the higher sugar consumption by co-culture than mono-culture in this study was also suspected to be 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 less than *S. cerevisiae*. This observation similar to that of 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 that co-culture of *C. shehatae* with *S. cerevisiae* completely consumed D-glucose from a mixture medium containing 70% of D-glucose and 30% of D-xylose after 14 h fermentation, while D-xylose, in practice, was not consumed. These researchers suggest that xylose consumption by *C. shehatae* can be inhibited in the presence of glucose.

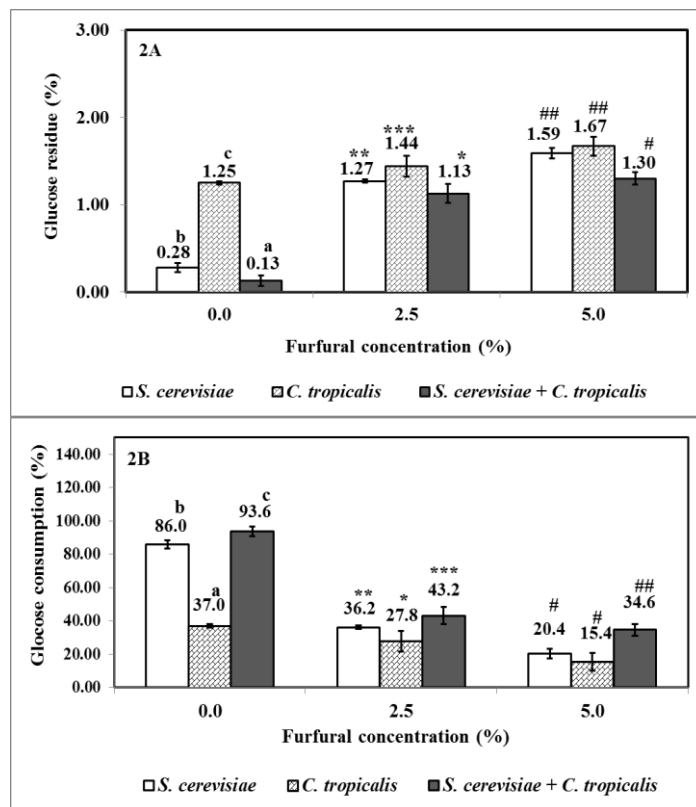


Figure 2 Glucose residue (2A) and consumption of glucose (2B) after 5 days fermentation by mono- and co-culture of *S. cerevisiae* with *C. tropicalis* in the media plus different furfural concentrations. The values with different superscripts (^a and ^b, * and ** or ^{##} and ^{###}) are different significant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

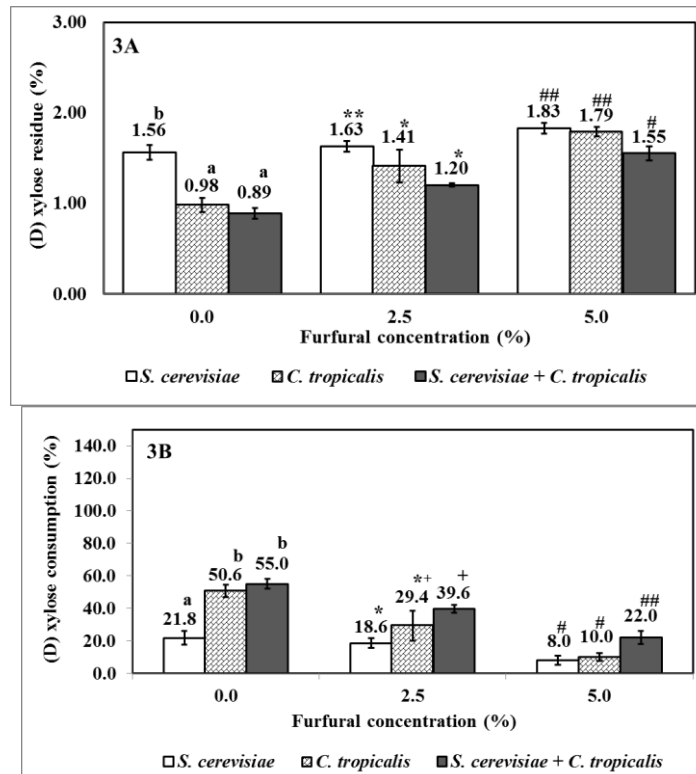


Figure 3 (D) xylose residue (3A) and consumption of xylose (3B) after 5 days' fermentation by mono- and co-culture of *S. cerevisiae* with *C. tropicalis* in media plus different furfural concentrations. The values with different superscripts (^a and ^b, * and ** or [#], ^{##} and ^{###}) are different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

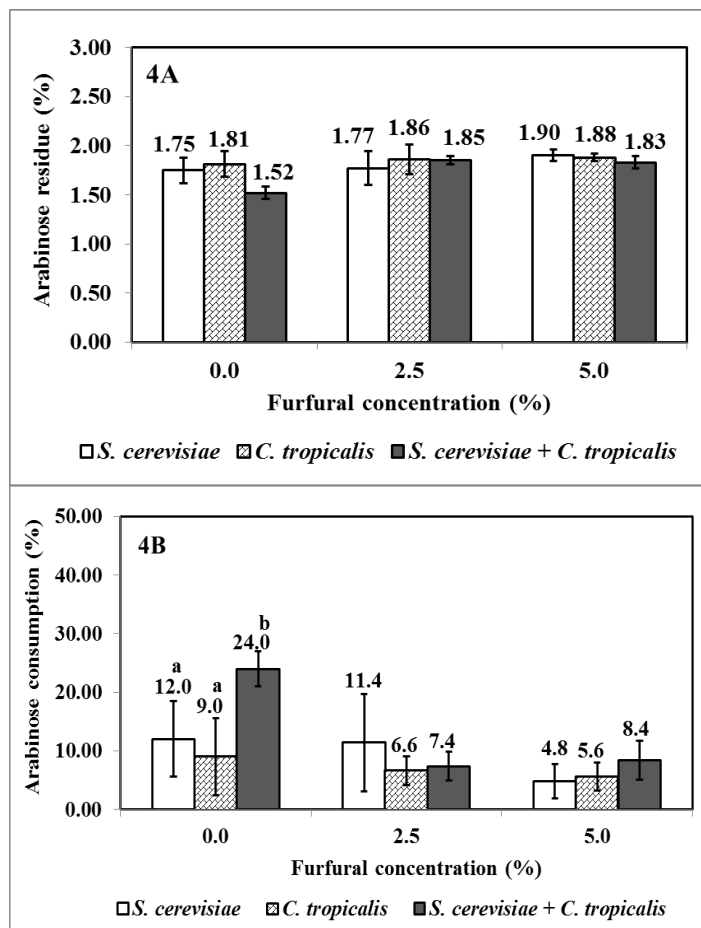


Figure 4 Arabinose residue (4A) and consumption of arabinose (4B) after 5 days' fermentation by mono- and co-culture of *S. cerevisiae* with *C. tropicalis* in media plus different furfural concentrations. The values with different superscripts (^a and ^b) are different significant ($p < 0.05$) of means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

In this work, D(+) xylose consumption by mono-culture of *C. tropicalis* and co-culture of *S. cerevisiae* with *C. tropicalis* was higher than *C. tropicalis*. Cheng et al. (2014) reported that *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 was allegedly due to inhibitor degradation by each yeast in the mixture fermentation as described before, as well as the contribution of *S. cerevisiae* in consuming 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 the 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 shows the glucose, D(+) xylose and arabinose residues in the media after being fermented by *S. cerevisiae*. Shin et al. (2015) suggested that *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 of *S. cerevisiae* with *C. tropicalis* significantly ($p < 0.05$) increased arabinose consumption in the fermentation media. Schimer-Michel et al. (2008) argued that arabinose was metabolized in a later phase, when both glucose and xylose were exhausted. Generally, in this work *S. cerevisiae* and *C. tropicalis* shown very low consumption of arabinose in the media with or without furfural. We found that arabinose consumption depends on the availability of glucose and xylose in the media.

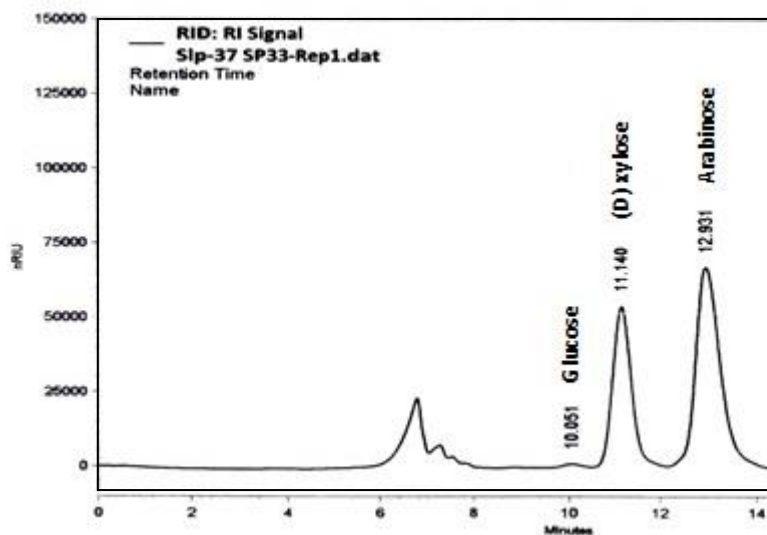


Figure 5. An HPLC chromatogram of residue glucose, D(+) xylose and arabinose in medium after 5 days' fermentation by *S. cerevisiae*.

Addition of phenol to the growth medium significantly ($p < 0.05$) influenced glucose (Fig. 6A) and xylose (Fig. 7A) residue, but did not significantly ($p > 0.05$) influence 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 did not significantly ($p > 0.05$) affect arabinose consumption (Fig. 8B). The toxicity of phenolics is very variable as it depends on the functional groups (Adeboye et al., 2014; Ando et al., 1986; Jonsson et al., 2013); more methoxy groups are related to high hydrophobicity and toxicity (Klinke et al., 2004). Yeast *S. cerevisiae* can assimilate many phenolics which can be part of the detoxification process occurring during fermentation (Delgenes et al., 1996; Mills et al., 1971). Phenolic compounds mainly interfere with the function of proteins and trigger changes in the protein-to-lipid ratio (Keweloh et al., 1990). Hence, these compounds affect cellular functions like sorting and signalling, and also cause membrane swelling (Caspeta et al., 2015).

Richard et al. (2003) stated that 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 a reduced nicotinamide adenine dinucleotide phosphate (NADPH)-consuming reaction. Xylitol will be oxidized by an NAD β -consuming reaction to form D-xylulose. In fungi, L-arabinose goes through four redox reactions. Two oxidations are coupled to NAD β consumption and two reductions to NADPH consumption. Furthermore, Richard et al. (2003) reported that *S. cerevisiae* enables growth on L-arabinose and under anaerobic conditions ethanol is produced from L-arabinose, but at a very low rate. Similarly to furfural, in this work *S. cerevisiae* and *C. tropicalis* shown very low consumption of arabinose in the media with or without phenol. We found that arabinose consumption depends on the availability of glucose and xylose in the media.

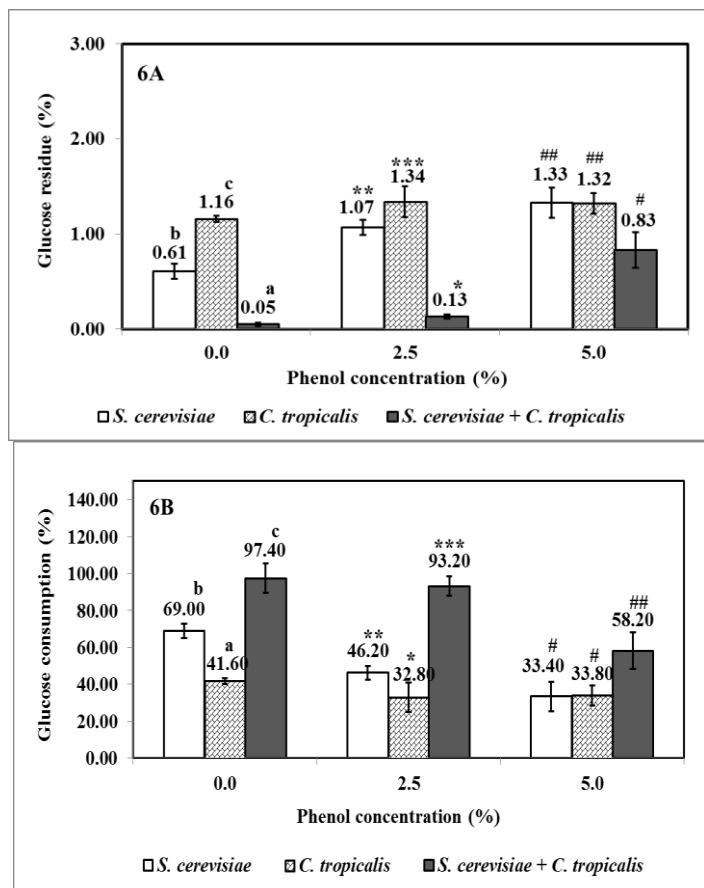


Figure 6 Glucose residue (6A) and consumption of glucose (6B) after 5 days' fermentation by mono- and co-culture of *S. cerevisiae* with *C. tropicalis* in media plus different phenol concentrations. The values with different superscripts (a and b, * and ** or #, ## and ###) are different significant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

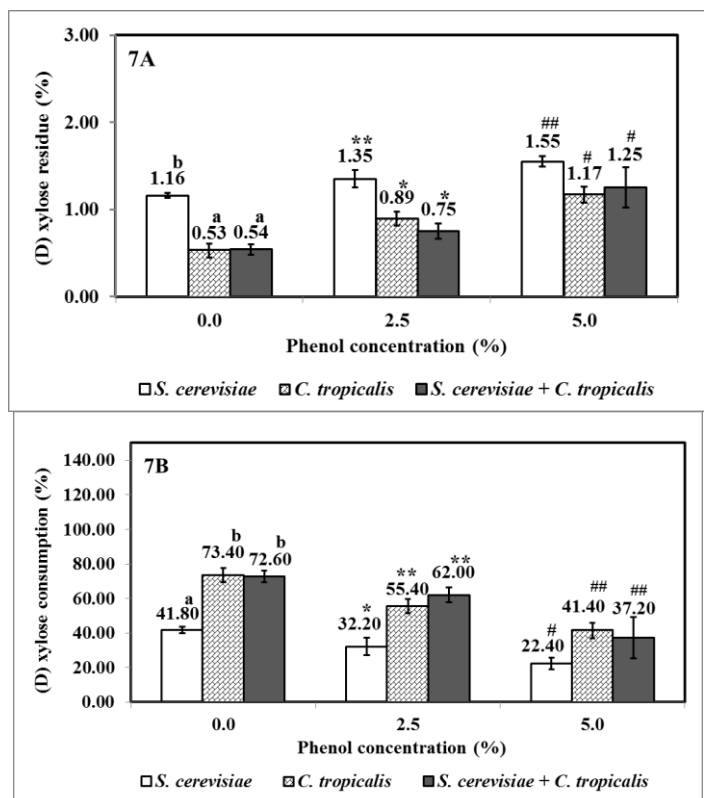


Figure 7 Xylose residue (7A) and consumption of xylose (7B) after 5 days' fermentation by mono- and co-culture of *S. cerevisiae* with *C. tropicalis* in media plus different phenol concentrations. The values with different superscripts (a and b, * and ** or #, ## and ###) are different significant (p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

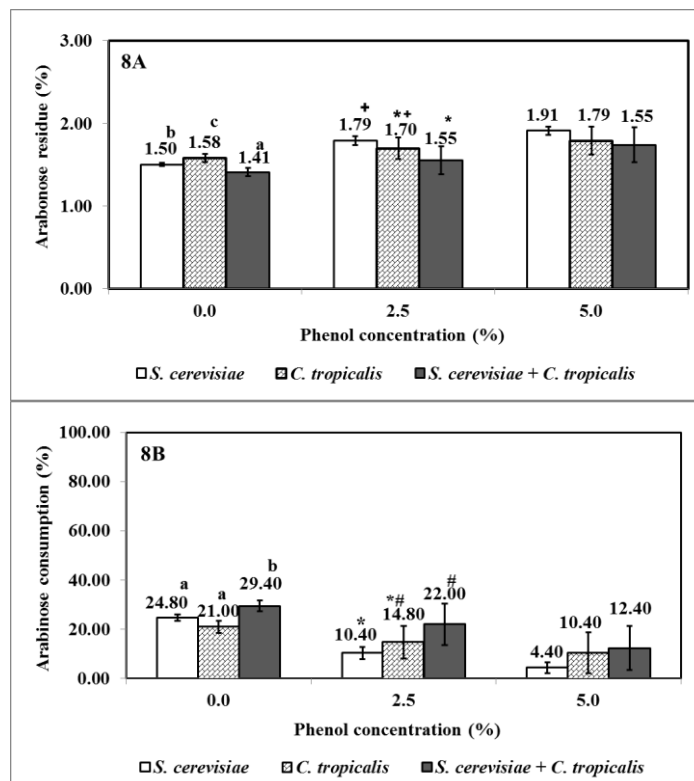


Figure 8 Arabinose residue (8A) and consumption of arabinose (8B) after 5 days' fermentation by mono- and co-culture of *S. cerevisiae* with *C. tropicalis* in media plus different phenol concentrations. The values with different superscripts (a and b, * and ** or #, ## and ###) are different significant (p < 0.05) of means from five independent observations in the same phenol concentration.

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) decreases ethanol yields. This work indicated that furfural inhibits sugar conversion to ethanol in mono-culture and co-culture of *S. cerevisiae* with *C. tropicalis*. Some investigators have reported an inhibitory effect of furfural on yeast growth and ethanol production. **Zaldivar et al. (1999)** reported that furfural and HMF compromised membrane integrity leading to extensive membrane disruption/leakage, which will eventually cause a reduction in the cell replication rate and ATP production and consequently lower ethanol production. **Agbogbo and Wenger (2007)** reported that a furfural concentration of 1.5 g.L⁻¹ can inhibit respiration and growth of microorganisms, leading to reduced ethanol production (90.4%) and productivity (85.1%). **Ylivero et al. (2013)** reported that furfural in lower concentrations (0.8 and 1.5 g L⁻¹) decreases ethanol yields by less than 10% and in higher concentrations decreases ethanol yield by 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 and dissipation of proton/ion gradients, thereby compromising the ability of cellular membranes to act as selective barriers (**Heipieper et al., 1994**). **Kuntiya et al. (2013)** reported that the isolate *C. tropicalis* No. 10 was fully able to degrade a phenol concentration of 100 mg.L⁻¹ at 20–42 °C, but this degradation was inhibited by a decreasing concentration of oxygen in media.

However, this work showed ethanol yields in the co-culture of *S. cerevisiae* with *C. tropicalis* significantly (P < 0.05) higher than mono-culture *S. cerevisiae* or *C. tropicalis* with or without the inhibitors furfural or phenol in the medium growth. We hypothesized that there is synergistic mechanism to stimulate ethanol production through simultaneous utilization of fermentable sugars such as glucose and xylose and degradation of furfural or phenol in the growth medium by co-culture *S. cerevisiae* with *C. tropicalis*. **Chen (2011)** suggested that co-culture fermentation is a strategy for efficient conversion of glucose and xylose to ethanol and increases ethanol yield and production rate. **Thurnheer et al. (1988)** and **Shim et al. (2002)** suggested that a co-culture as a mimic of the natural environment has been used for biodegradation of aromatic compounds. **Bader et al. (2010)** suggested that in co-cultures, degradation and metabolization of substrates occur through the combined metabolic activity of known microbial strains under aseptic conditions. Some investigators have reported on ethanol production by co-culture of microorganisms in a medium containing furfural. **Wan et al. (2012)** reported that co-culture of *S. cerevisiae* Y5 and *P. stipitis* CBS6054 effectively converted glucose and xylose to ethanol, as well as effectively degrading inhibitors in the hydrolysate. Furthermore, **Wan et al.**

(2012) reported that co-culture of *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 that *C. tropicalis* can use a phenol concentration of 500 mg.L⁻¹ as a source of carbon and energy.

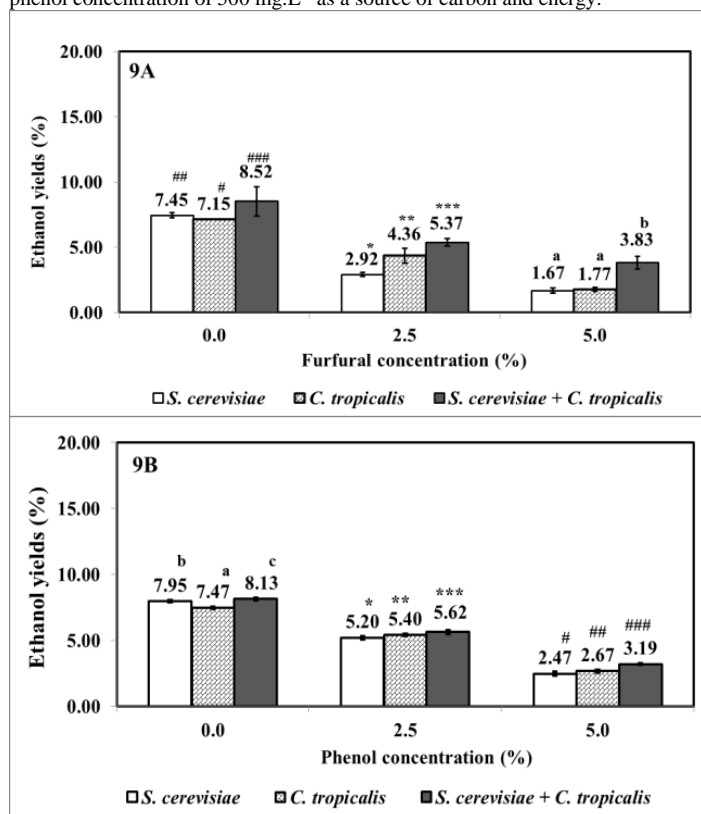


Figure 9 Ethanol yields in media plus different furfural (9A) and phenol (9B) concentrations after 5 days' fermentation by mono- and co-culture *S. cerevisiae* with *C. tropicalis*. The values with different superscripts (a and b, *, **, and *** or #, ## and ###) are different significant (p < 0.05) of means from five independent observations in the same furfural

Efficiency of fermentation

Addition of furfural (Fig. 10A) and phenol (Fig. 10B) to the growth medium significantly (p < 0.05) decreased the efficiency of fermentation of ethanol production by mono- and co-culture of *S. cerevisiae* with *C. tropicalis*. However, co-culture of *S. cerevisiae* with *C. tropicalis* shown significantly (p < 0.05) higher efficiency fermentation of ethanol production than mono-culture of *S. cerevisiae* or *C. tropicalis* from medium with or without furfural and phenol. Although *S. cerevisiae* efficiently converts hexoses into ethanol, this native yeast is not able to metabolize pentose sugars present in lignocellulosic hydrolysate. This work indicates that co-culture of *S. cerevisiae* and *C. tropicalis* more efficient to use of sugar in media and convert into ethanol. Co-culture of *S. cerevisiae* with *C. tropicalis* exhibits a higher consumption of glucose and D(+) xylose than *C. tropicalis* and *S. cerevisiae* alone. Wang et al. (2012) suggested that co-culture between two microorganisms in a single process is an alternative way to reduce the effects of inhibitors present in the media. Hickert et al. (2012) reported that co-culture of *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 of *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 consumption with yeast more preferred pentose consumption to produce ethanol efficiently is one alternative for optimizing the production of ethanol in hydrolysate containing xylose (Gutiérrez-Rivera et al., 2012; Karagoz and Ozkan, 2014; Licht, 2006). Gutiérrez-Rivera et al. (2012) reported ethanol production by co-culture of *S. cerevisiae* ITV-01 and *Pichia stipitis* NRRL Y-7124 five times higher than ethanol production by mono-culture of *S. cerevisiae* ITV-01 and mono-culture of *P. stipitis* NRRL Y-7124. Increased ethanol productivity can cause enriched substrates that can be utilized as *S. cerevisiae* use glucose and *P. stipitis* use pentose to produce ethanol (Tesfaw and Assefa, 2014). Co-culture fermentation of *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 of *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 of *S. cerevisiae* ATCC 26602 and *P. stipitis* DSM 3651

(7.36 g.L⁻¹) shown higher than mono-culture *S. cerevisiae* (6.68 g.L⁻¹) from wheat straw media with pretreatment H₂O₂ and enzyme hydrolysis (Karagoz and Ozkan, 2014). Tolerant microorganisms including co-culture fermentation to inhibitors and ethanol are one of the problems of the production of ethanol from lignocellulosic waste. Gutiérrez-Rivera et al. (2012) reported that *P. stipitis* NRRL Y-7124 has a low tolerance to ethanol produced by *S. cerevisiae* ITV-01 and prevents further ethanol production by *P. stipitis* NRRL Y-7124. This work showed that co-culture of *S. cerevisiae* and *C. tropicalis* has a high tolerance to inhibitors and higher ethanol yield than mono-culture of *S. cerevisiae* or mono-culture of *C. tropicalis* in basal medium and basal medium plus furfural or phenol. Co-culture of *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 were allegedly due to the contribution of *C. tropicalis* to convert xylose into ethanol. Karagoz and Ozkan (2014) suggested that ethanol production was increased by co-culture of *S. cerevisiae* and *P. stipitis* due to the contribution of *P. stipitis* to convert xylose into ethanol. Hickert et al. (2013) reported that co-culture of *C. shehatae* HM 52 with *S. cerevisiae* ICV D254 in synthetic medium and rice hull hydrolysate effectively converted glucose and xylose simultaneously, maximizing the utilization rate of the substrate, and increasing the yield and rate of ethanol production.

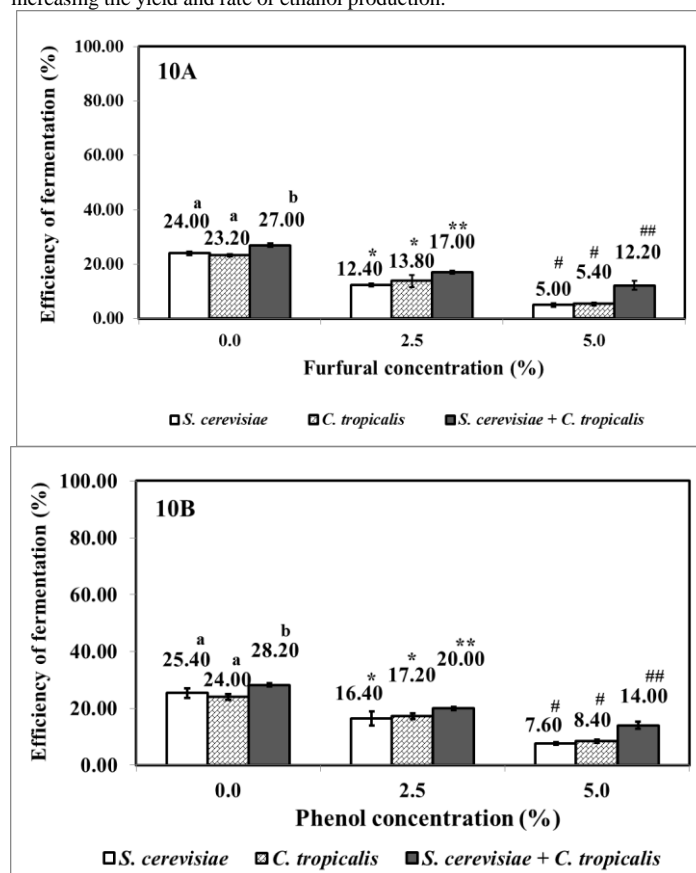


Figure 10 Efficiency of fermentation of ethanol production by mono- and co-culture of *S. cerevisiae* with *C. tropicalis* from basal media plus furfural (10A) and phenol (10B) with different concentrations.

CONCLUSIONS

Sugar consumption and ethanol production by co-culture of *S. cerevisiae* with *C. tropicalis* in media fermentation with or without inhibitors are higher than mono *S. cerevisiae* or *C. tropicalis*. Co-culture of *S. cerevisiae* with *C. tropicalis* demonstrated a higher tolerance to inhibitor 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 mixed substrate. Co-culture of *S. cerevisiae* FNCC 3012 with *C. tropicalis* FNCC 3033 demonstrated potential as a fermentation process for ethanol production from lignocellulosic medium or media content inhibitors. The use of this co-culture effectively utilizes hexose and pentose sugars in the substrate, increasing the yield and efficiency of fermentation ethanol production.

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