Vol. 19, No. 3 (2020) 1477-1491
Revista Mexicana de Ingeniería Química

Sustainable bioconversion of saccharified agro residues into bioethanol by *Wickerhamomyces anomalus*

Bioconversión sostenible de residuos agrícolas sacarificados en bioetanol por *Wickerhamomyces anomalus*

Haq I.U.*, Tahir A., Nawaz A., Mustafa Z., Mukhtar H., Rehman A.U.

Institute of Industrial Biotechnology, GC University Lahore Pakistan 5400

Received: November 14, 2019; Accepted: March 9, 2020

Abstract

Snowballing levels of greenhouse gas emissions and concerns about climate change has led to an ongoing exploration of biofuels. Bioethanol produced from saccharified wheat straw employing yeast strains can be made readily available as a clean fuel for combusting the engines. Therefore, in current study, *Wickerhamomyces anomalus* yeast strain IHZ-26 was used to produce bioethanol from sugar solution obtained by enzymatic hydrolysis of wheat straw. Nineteen different fermentation media were assessed for this purpose in which sugar solution obtained from enzymatic hydrolysis of wheat straw was used as carbon source. Out of these fermentation media, maximum bioethanol yield (1.09 g/L; $p < 0.05$) was observed in 'C1 Yeast extract, peptone, glucose' medium. Surface culture fermentation for 5 days at 25 ºC resulted in maximum ethanol formation using 2, 1.5 and 2g of glucose, xylose and ammonium dihydrogen phosphate, respectively. Four hours old inoculum of *Wickerhamomyces anomalus* yeast strain IHZ-26 in a concentration of 3.5% was optimized for maximum bioethanol yield. These optimized parameters resulted in augmented bioethanol production (5.0 g/L) by 5.02 folds. This study further revealed that *W. anomalus* IHZ-26 have been able to covert pentoses and hexoses simultaneously into ethanol efficiently.

Keywords: Bioethanol, *Pichia anomala*, optimization, fermentation, green energy.

Resumen

Los niveles desorbitados de emisiones de gases de efecto invernadero y las preocupaciones sobre el cambio climático han llevado a una exploración continua de biocombustibles. El bioetanol producido a partir de paja de trigo sacarificada que emplea cepas de levadura puede estar fácilmente disponible como combustible limpio para la combustión de los motores. Por lo tanto, en el estudio actual, la cepa de levadura *Wickerhamomyces anomalus* IHZ-26 se usó para producir bioetanol a partir de una solución de azúcar obtenida por hidrólisis enzimática de la paja de trigo. Diecinueve medios de fermentación diferentes fueron evaluados para este propósito en el cual la solución de azúcar obtenida de la hidrólisis enzimática de la paja de trigo fue utilizada como fuente de carbono. Fuera de estos medios de fermentación, se observó un rendimiento máximo de bioetanol (1.09 g / L; $p < 0.05$) en medio de extracto de levadura 'C1, peptona, medio de glucosa. La fermentación del cultivo de superficie durante 5 días a 25 ° C dio como resultado la formación máxima de etanol usando 2, 1,5 y 2 g de glucosa, xilosa y dihidrógeno fosfato de amonio, respectivamente. El inóculo de cuatro horas de edad de la cepa de levadura *Wickerhamomyces anomalus* IHZ-26 en una concentración del 3,5% se optimizó para obtener el máximo rendimiento de bioetanol. Estos parámetros optimizados dieron como resultado una producción aumentada de bioetanol (5.0 g / L) en 5.02 veces. Este estudio reveló además que *W. anomalus* IHZ-26 ha sido capaz de convertir pentosas y hexosas simultáneamente en etanol de manera eficiente

Palabras clave: Bioetanol, *Pichia anomala*, optimización, fermentación, energía verde.

1 Introduction

The limited amount of fossil fuels and increasing environmental hazards due to their burning, especially greenhouse gas emissions (GHG) have made it necessary for the scientists to explore novel energy sources such as biofuels (Reyes *et al*., 2018). Biofuels provide an alternate source of energy to fossil fuels and other conventional sources of energy. The term biofuel is referred to a gaseous or a liquid fuel, made for the industrial sector, obtained from a variety of biomass. The usage of biofuels is rising worldwide and its possible applications are of great interest for the scientists in the current times.

* *Corresponding author. E-mail*: ikmhaq@yahoo.com
https://doi.org/10.24275/rmiq/Bio966
issn-e: 2395-8472

The chief task for the upcoming time is to develop a process for the production of biofuel which do not challenge the natural food chains. The biofuels msut be maintainable and proficient in terms of energy and expenses (Gadonneix *et al*., 2010; Chingono and Mbohwa 2016). Therefore, efficient bioethanol production involves lignocellulosic biomass as a potential feedstock (Anwar *et al*., 2014).

Yeast, *Saccharomyces cerevisiae* is generally utilized in industrial bioethanol production owing to reasons like persistence to wide range of pH, high ethanol endurance, easy access and cost effectiveness (Azhar *et al*., 2017). However, baker's yeast and many other strains of *S. cerevisiae* are proved to be less effective because of easily triggered toxicity during industrial processes. Various stress conditions such as high concentration of ethanol, osmotic pressure, high or low temperature and microbial toxicity are the limitations to the use traditional yeast cells in fermentation process (Nawaz *et al*., 2020). This is where *Pichia anomala*, currently known as *Wickerhamomyces anomalus*, comes into the spotlight. This strain is used for the bioethanol production by fermenting variety of sugars. The yeast *W. anomalus* is basically an ascomycetous heterothallic member of the *Wickerhamomycetaceae* family. *W. anomalus* exist in different environmental conditions and have been reported to be obtained from plant materials such as maize silage and cereal grain. A novel yeast strain, *W. anomalus* is branded as a level-1 biosafety microbe. This specie *W. anomalus* can even grow under stress conditions, like high and low pH, increased osmotic pressure, low water activity and high anaerobic condition (Satora *et al*., 2014). *W. anomalus* is studied for the economically viable second generation bioethanol production exhibiting pentose fermentation as its key feature (Azhar *et al*., 2017). In the present study, surface culture fermentation was used anaerobically for the optimized production of bioethanol. Liquid surface culture is the classic bioethanol production process characterized by high yield, low energy consumption and less man power employment (Darouneh *et al*., 2009). The fermentation of bioethanol depends on a number of factors such as pH, temperature, dissolved CO_2, dissolved O_2, inoculum size, composition and nature of medium etc. Change in these parameters may affect the rate of the fermentation process, yield of the product, appearance, texture, smell, taste of the product, nutritional quality, presence of toxins (if any) and other related physicochemical properties.

The type of fermentation medium also affects the product yield. Fermentation medium put into use must provide nitrogen, carbon, micronutrients, vitamins, trace elements etc in required amounts. In some cases the carbon and nitrogen ratio needs to be controlled as well. Additional factors such as the availability of the raw material, cost of the process and variability in batch to batch fermentation also affect the selection of medium (Olido, 2018; Paulová *et al*., 2015). Production of second generation fuels by using yeast is now demanding more research and study. Thereby, an inclusive process and economic analysis is needed to formulate a strategy which is industrially suitable for the production of biofuels such as bioethanol and biodiesel which will resolve our energy crisis in a sustainable way (Zabed *et al*., 2014; Tesfaw and Assefa 2014).

Therefore, objective of the current study was to select a fermentation medium having natural saccharified sugar of wheat starw as a carbon source for the economical production of bioethanol which can be used as a renewable energy source. In addition to this, bioethanol fermentation related cultural condition were also focused keenly to get a better bioethanol yield using appropriate medium components to make the product more viable economically.

2 Materials and methods

2.1 Microorganism

W. anomalus yeast strain IHZ-26 (accession number KT883963) and saccharified solution (main component of the fermentation media) was obtained from the project "Production of bioenergy from plant biomass" conducted at Institute of Industrial Biotechnology, Government College University, Lahore.

2.2 Fermentation media

Nineteen different fermentation media were studied for bioethanol production (Table 1). Fermentation was carried out using saccharification slurry as a carbon source. Inocula for these media were prepared in malt extract medium.

Table 1. Different fermentation media compositions for ethanol production.

Sr. No.	Name	Composition of different fermentation media for bioethanol production (g/40 mL)	References
1.	MZ	2 % residual sugar, 0.16 g $(NH_4)_2SO_4$, 0.24 g Yeast extract, 0.012 g $CaCl_2$, 0.06 g KH_2PO_4, 0.04 g $MgSO_4$. $7H_2O$	Vučurović et al., 2009
2.	C1 YPG	2 % residual sugar, 0.4 g Yeast extract and 0.8 g Bacto-peptone	Berłowska et al., 2016
3.	C2	2 % residual sugar, 0.2 g Yeast extract, 0.02 g NaCl, 1mM $CaCl_2$, 1mM $MgSO_4$, 1 mg/L thiamine HCl, 1mM betaine HCl, 0.04 g NH_4Cl,	Unrean and Srienc 2010
4.	C3	2 % residual sugar, 0.4 g Yeast extract, 0.8 g Bacto-peptone, 2 g soluble starch	Yamada et al., 2011
5.	C4	2 % residual sugar, 0.04 g $(NH_4)_2SO_4$, 0.04 g KH_2PO_4, 0.2 g $MgSO_4$, 0.16 g Yeast extract, pH 4.5	Plessas et al., 2007
6.	C5	2 % residual sugar, 0.12 g yeast extract, 0.12 g peptone, 0.12 g $(NH_4)_2SO_4$, 0.12 g NH_4NO_3	Raposo et al., 2017
7.	M1	2 % residual sugar, 0.4 g yeast, 0.8 g peptone.	Kadam and Newman, 1997
8.	M2	2 % residual sugar,0.4 g yeast extract, 0.2 g peptone, 0.04 g $(NH_4)_2SO_4$, 0.08 g KH_2PO_4, 0.02 g $MgSO_4.7H_2O$, 0.02 g $FeSO_4$	Cazetta et al., 2007
9.	M3	2 % residual sugar, Phosphate buffer 2.9 mL, 1.2g Glycerol, 0.04 g NH_4Cl, 0.01g KH_2PO_4, 0.01g Na_2SO_4, 0.01g NaCl, 0.01 $MgSO_4.7H_2O$	Cofré et al., 2012
10.	M4A	2 % residual sugar, 0.04 g Urea, 0.08 g $(NH_4)_2SO_4$	Pinal et al., 1997
11.	4B	2 % residual sugar, 0.2 g peptone, 0.06 g $NH_4H_2PO_4$	Ghosh and Prelas 2011
12.	4C	2 % residual sugar, 0.4 g peptone, 0.4 g $(NH_4)_2SO_4$, 0.4 g Casamino Acid	Cruz et al., 2003
13.	4C-Ca	2 % residual sugar, 0.4 g peptone, 0.4 g $(NH_4)_2SO_4$	Cruz et al., 2003
14.	4D	2 % residual sugar, 0.2 g $(NH_4)_2SO_4$	Navarro et al., 2000
15.	4E	2 % residual sugar, 0.5 g peptone	Kiran et al., 2003
16.	4F	2 % residual sugar, 0.256 g Urea	Yu and Zhang 2004
17.	4G	2 % residual sugar, 0.09 g $NH_4H_2PO_4$	Najafpour et al. 2004
18.	4H	2 % residual sugar, 0.08 g peptone, 0.16 g $(NH_4)_2SO_4$	Lin and Tanaka, 2006
19.	4I	2 % residual sugar, 0.5 g Xylose, 0.14 g Peptone, 0.12 g $(NH_4)_2SO_4$	Sanchez et al., 1999

2.3 Methodology

2.3.1 Revival of yeast strain and inoculum preparation

The given yeast strain of *W. anomalus* (IHZ-26) was revived from glycerol stocks on YPDA medium paltes at 30 ºC after an incubation of 48 h. Furthermore, inoculum was prepared by inoculating loopful of *W. anomalus* (IHZ-26) in potato dextrose broth after overnight incubation at 30 ºC and 120rpm.

2.3.2 Fermentation

Nineteen different fermentation media (table 1) were prepared using sugar slurry containing 2% reducing sugar as a carbon source. Surface culture fermentation was carried out by inoculating 2% *W. anomalus* starter culture for bioethanol production at 30 ºC for 5 days. Additionally, MZ fermentation medium was prepared (table 1) with the synthetic sugar instead of using saccharified sugar to probe difference in bioethanol production.

2.3.3 Extraction

After the completion of fermentation process, broth was centrifuged at 6000 rpm for 5 minutes. Supernatant was transferred to a falcon tube for further analysis and the cell mass pellet was discarded.

2.3.4 Analytical methods

2.3.4.1 Ethanol and reducing sugar estimation

Quantification of bioethanol produced was carried out by the method undertaken by Gupta *et al*. (2012) and Babu *et al*. (2014). Residual sugar was estimated using DNS method (Miller, 1959).

2.3.5 *Optimization of fermentation parameters*

Nineteen different fermentation media as shown in Table 1 were studied for the production of bioethanol. Optimization of various parameters like incubation time (1-6 days), temperature (20-40 ºC), pH (6-8), different carbon sources such as glucose, fructose, maltose, mannitol, soluble starch, sucrose, xylose, mannose, lactose, saccharose, CSL and various nitrogen sources like peptone, yeast extract, NH_2SO_4, di ammonium hydrogen phosphate, KNO_3, Urea, $NaNO_3$, $NH_4H_2PO_4$, NH_3NO_2, NH_4Cl was carried out for maximum ethanol yield. In this regard, varying concentrations of carbon and nitrogen sources were also assessed. Additionally, various inoculum sizes of *W. anomalus* were checked to get the most suitable inoculum size for the fermentation. Age of inoculum being an important factor contributing to the fermentation yield was also assessed for its effect was also investigated.

2.3.6 *Statistical analysis*

Statistical examination and validation of the result obtained was performed using SPSS version 16.00 (IBM Analytics, New York USA). Significant variance in the values of probability was calculated by employing one way ANOVA.

3 Results and discussion

3.1 *Influence of sugar source*

Effect of saccharified and synthetic sugar was observed separately on the production of bioethanol in MZ I and MZ II media, respectively, at 30 ºC for 5 days at 120 rpm.

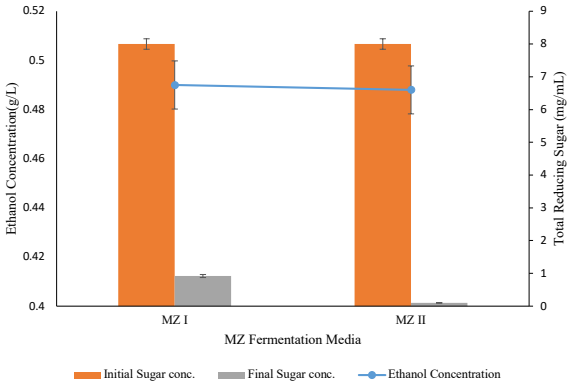

Fig. 1. Effect of nature of sugar on maximum bioethanol production using *W. anomalus* (IHZ-26).

A very little difference was observed while using synthetic (MZ I) and natural sugar (MZ II) i.e. 0.49 g/L ($p < 0.05$) and 0.488 g/L ($p < 0.05$) bioethanol production, respectively, as given in figure 1. Therefore, saccharified sugar slurry was selected for further analysis to make the process economical. This was a very promosing finding as as yeast was conerting both synthetic and natural sugars at same rate might be due to its ability to get over the contaminants or inhibitors which are present in saccharified slurries produced from lignocellulosic biomass. Keshav *et al.* (2016) also reported similar strategy for ethanol production but employing *S. cerevisiae*.

3.2 *Impact of various media*

Nineteen different fermentation media (Table 1) were studied for the production of bioethanol using *W. anomalus* at 30 ºC and pH 7 for 5 days with agitation speed of 120 rpm. The maximum bioethanol production (1.09 g/L) ($p < 0.05$) was observed in C1 (YPG) medium as shown in figure 2.

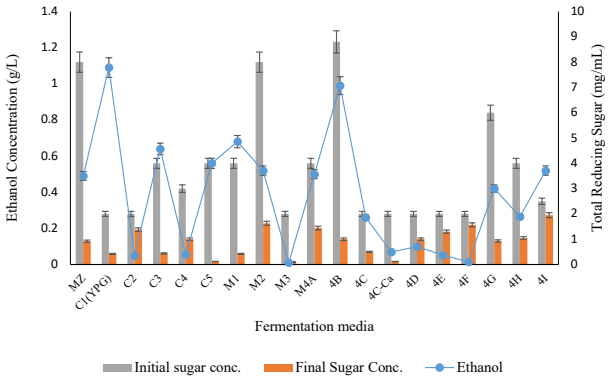

Fig. 2. Optimization of fermentation media for maximum bioethanol production using *W. anomalus* (IHZ-26).

The C1(YPG) medium gave maximum bioethanol production 1.09 g/L (figure 2). This is because the components such as yeast extract, glucose and peptone present in C1(YPG) medium may have provided carbon, essential minerals, vitamins and trace elements to promote yeast growth. Amino acids and nitrogenous compounds necessary for microbial cell synthesis were provided by yeast extract (YE) present in the medium (Phillips *et al*., 2014 and Dickinson, 2006). It was followed by 4B medium which gave 0.99 g/L ($p < 0.05$) bioethanol production. Other media gave less bioethanol production as compared to C1(YPG) and 4B medium. However, minimum bioethanol production was obtained using M3 medium i.e. 0.01 g/L ($p < 0.05$). Same fermentation medium was used by Gao *et al*. (2013) to get a better bioethanol yield (3.5 g/L) using *Clostridium ragsdalei* compared to current study. This contradiction might be owed to use of different mciroogranisms for fermentation. Further, this contradiction can be attribute dto the use of synthetic sugars in fermentation medium by Gao *et al*. (2013) instead of saccharified slurry which was used as carbon source in current study.

3.3 Optimization of incubation period

Effect of different incubation periods i.e. 1, 2, 3, 4, 5 and 6 days was analyzed for the maximum production of bioethanol. The production of bioethanol was observed to increase from day 1 i.e. 0.7 g/L ($p < 0.05$) to day 4 i.e. 1.0 g/L ($p < 0.05$), which reached to its maximum on day 5 with yield of 1.09 g/L ($p < 0.05$). However, further increase in incubation time resulted in decreased bioethanol production as shown in figure 3. Therefore, incubation period of 5 days was selected for further analysis. Thenmozhi and Victoria (2013) also reported maximum bioethanol (7.83 g/L) with 5 days of incubation while using *S. cerevisiae* which is greater yield compared to current study owing to use of *S. cerevisiae* and synthetic sugars instead of *W. anomalus* and natural sacchariefied sugar. The process of fermentation might remain incomplete if carried out for short durations, owing to the fact that there was inadequate population of microbes (Bokulich and Bamforth 2013). On the other hand, prolonged fermentation might be lethal for the growth of microbes because of the accumulation product in high concentration. It was observed that as the concentration of ethanol increased in the fermentation medium, the inhibition of yeast cells also increased resulting in decreased microbial mass and rate of fermentation.

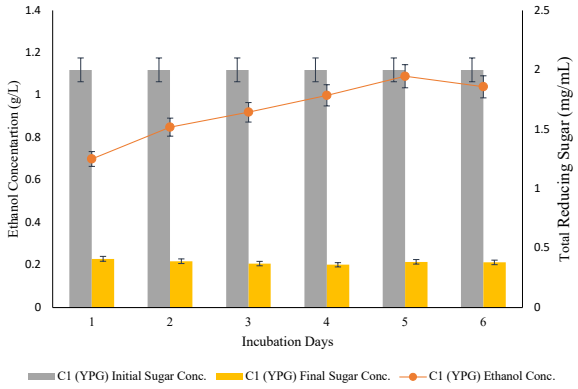

Fig. 3. Effect of Incubation period for maximum bioethanol production using *W. anomalus* (IHZ-26).

The decrease in the bioethanol production after day 5 might be due to the depletion of the nutrients, decrease in media pH and feedback inhibition (Tesfaw & Assefa, 2014). However, the results of Rayabova *et al*. (2003) and Cazetta *et al*. (2007) disagree with our findings as they reported maximum ethanol production in 48 hours (2 days) using *Pichia stipitis* and using *Zymomonas mobilis*,respectively. These yeast strains might have fast metabolic system due to which they achieved the task earlier or the use of synthetic sugars without the presence of any inhibitors and conatminants, which are usually present in sccharified sugars (as in current study), could have contributed to robust bioethanol production in their experiments.

3.4 Effect of fermentation method

Effect of surface culture fermentation (static incubation) and submerge fermentation (shaking incubation) was analyzed for their effect on bioethanol production. It was observed that maximum bioethanol production (1.17 g/L ($p < 0.05$) was obtained in surface culture fermentation as shown in figure 4. It was followed by 1.02 g/L g/L ($p < 0.05$) of bioethanol produced in submerged culture fermentation. Therefore, surface culture fermentation was selected for further experiments. Holker *et al*. (2004) and Darouneh *et al*. (2009) also observed maximum bioethanol yield using surface culture fermentation with *Pichia anomala*. It might be due to low water activity and zero oxygen availability as improved production of ethanol takes place in anaerobic conditions. Submerged fermentation on the other hand have multiple reuirements such as high water content, energy for agitation, space and large waste disposal area which makes the process of ethanol production more costly (Baldwin *et al*., 2019).

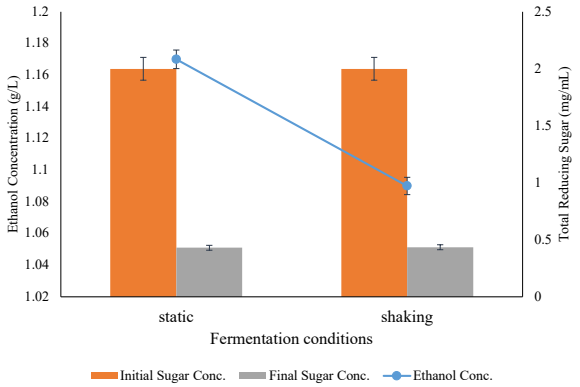

Fig. 4. Effect of fermentation methods (shaking and static) on bioethanol production using *W. anomalus* (IHZ-26).

3.5 *Optimization of temperature*

The effect of different incubation temperatures i.e., 20, 25, 30, 35 and 40 ºC on the production of bioethanol was investigated in C1(YPG) medium at pH 7.0. It was observed that the rate of bioethanol production increased from 0.49 g/L g/L ($p < 0.05$) (20 ºC) to 1.39 g/L ($p < 0.05$) (25 ºC) which is maximum production as shown in figure 5. However, further increase in temperature failed to boost bioethanol production. Least production was attained at 40 ºC i.e. 0.22 g/L ($p < 0.05$), bioethanol. Elevated temperature is stressful for the growth of microbes and results into their cessation. The microbes may switch to the formation of heat shock proteins (HSPs) which are responsible for disturbance in ribosomal activity (Philasaphong *et al*., 2006). In addition, high temperature is thought to cause negative effect on enzymes, ribosome and membrane fluidity (Kurian *et al*., 2010). Most suitable temperature i.e. 25 ºC was opted for further experimentations.

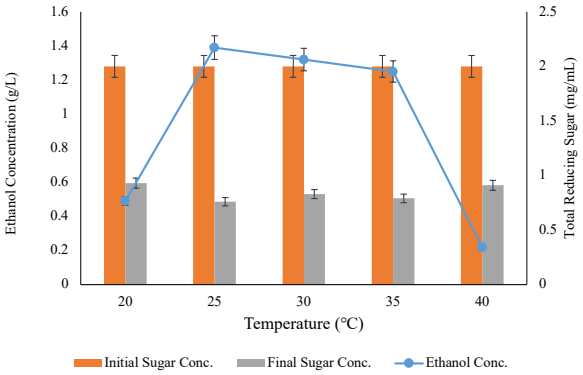

Fig. 5. Effect of varying temperature for maximum bioethanol production using *W. anomalus* (IHZ-26).

Lin *et al*. (2012), Davis *et al*. (2006) and Cazetta *et al*. (2007) also reported 25-30 ºC temperature for maximum bioethanol yield using *S. cerevisiae* which is in accordance to the current studt and might be attributed to the fact that both *S. cerevisiae* and *W. anomalus* belongs to Saccharomycetaceae family. Torija *et al*. (2003), Thenmozhi and Victoria (2013) have got diverging results andeported maximum ethanol yield at 35 ºC using *S. cerevisiae*. Contradiction in results can be explained on the basis that yeast used might have been isolated from habitats that support the metabolic process to work best at higher temperature.

3.6 *Optimization of pH*

Effect of different medium pH i.e., 6.0, 6.5, 7.0, 7.5 and 8.0 was assessed for bioethanol production using C1(YPG) fermentation medium. The rate of bioethanol production increased from pH 6.0 (0.19 g/L ($p < 0.05$)) to pH 7.0 (1.4 g/L g/L ($p < 0.05$)) where maximum bioethanol production was observed (Figure 6). Least amount of bioethanol (0.19 g/L ($p < 0.05$)) was obtained at pH 6.0. It was observed that further increase in pH declined the bioethanol production. Therefore, pH 7.0 with 1.4 g/L bioethanol production was selected for further investigation. Maximum ethanol formation at neutral pH might be due to the possibility that the yeast strain used in current study may have isolated from the habitat having neutral pH. However, decreae ethanol production at low and high pH values are due to the fact that change in hydrogen ion concentration in the medium may affect proteins such as enzymes which are responsible for carrying out bioethanol fermentation (Liu *et al*., 2012).

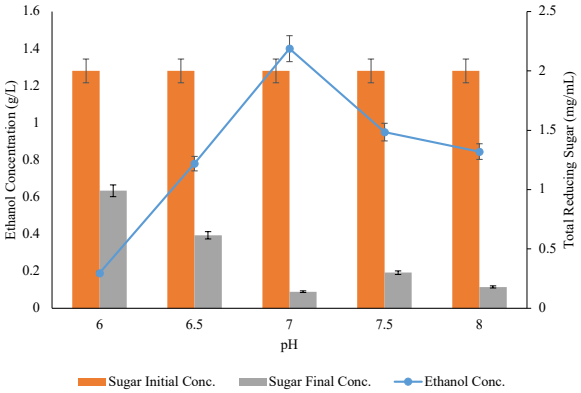

Fig. 6. Optimization of fermentation medium pH for maximum bioethanol production using *W. anomalus* (IHZ-26).

Therefore, bioethanol production seems to decline at pH other than optimum pH (Acharya & Chaudhary 2012 and Lin *et al*., 2012). Similar results were obtained by Temudo *et al*. (2007) and Temudo *et al*. (2008) as they reported maximum production of ethanol at neural pH as well.

3.7 Optimization of carbon source

Different carbon sources such as glucose, fructose, maltose, mannitol, soluble starch, sucrose, xylose, mannose, lactose and a combination of glucose and xylose were tested for bioethanol production. Among mentioned carbon sources, maximum bioethanol production was observed using a medium having a combination of glucose and xylose (3.45 g/L ($p < 0.05$)) as shown in figure 7. Least amount of bioethanol (0.12 g/L ($p < 0.05$)) was obtained in a medium containing lactose as a carbon source. Effect of different concentrations of glucose such as 0.5, 1, 1.5, 2, 2.5 and 3% was analyzed for bioethanol production. The rate of bioethanol production increased with the increase in glucose concentration and reached to maximum when 2% glucose was used which gave 3.6 g/L ($p < 0.05$), bioethanol yield. With the further increase in the concentration of glucose, the rate of bioethanol production decreased from 3.6 g/L ($p < 0.05$) to 2.99 g/L ($p < 0.05$) as shown in figure 8. Least amount of bioethanol 2.99 g/L ($p < 0.05$) was obtained when 3% glucose was used. Similarly, effect of different xylose concentrations such as 0.5, 1, 1.5, 2, 2.5 and 3% was also analyzed to study its effects on the bioethanol production. It was observed that the bioethanol yield increased from 2.8 to 3.8 g/L when the xylose concentration was increased from 0.5 to 1.5%, respectively.

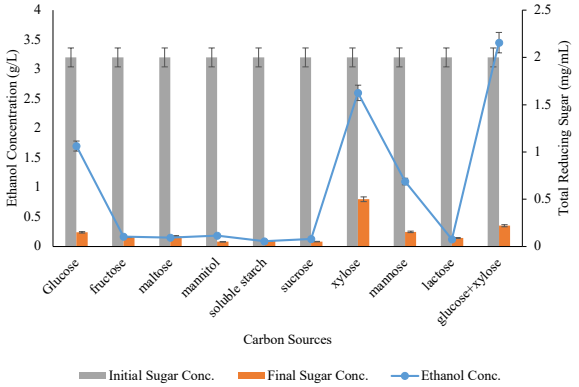

Fig. 7. Effect of various carbon sources on maximum bioethanol production using *W. anomalus* (IHZ-26).

However, further increase in xylose concentration above 1.5% dropped off bioethanol production as shown in figure 9. Least amount of bioethanol (0.99 g/L ($p < 0.05$)) was obtained when 3% of xylose was used. Xylose concentration (1.5g ($p < 0.05$)) with maximum bioethanol production was selected for further experimentation.

Carbon source is another major factor which significantly influences the yeast growth (Zaldivar *et al*., 2001). The yeast *W. anomalus* used in the present study is capable of assimilating all the complex and simple sugars simultaneously (Almeida *et al*., 2007 and Van *et al*., 2006). Therefore, several sugars like glucose, mannose, fructose, xylose, maltose and sucrose were optimized. Among different carbon sources 2% xylose and 2% glucose gave 2.3 and 1.7 g/L bioethanol yield, respectively. It was observed that when these two carbon sources were combined, the rate of bioethanol production was enhanced significantly (3.45 g/L).

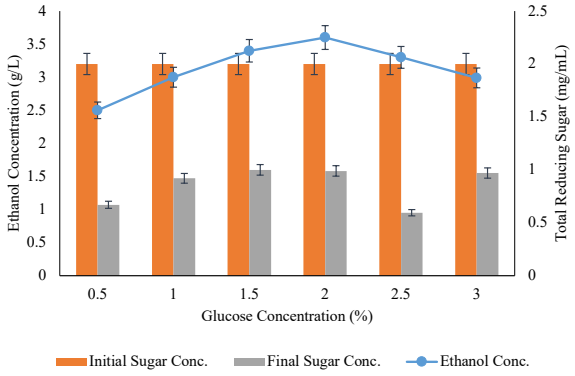

Fig. 8. Optimization of carbon source (Glucose) concentration for maximum bioethanol production using *W. anomalus* (IHZ-26).

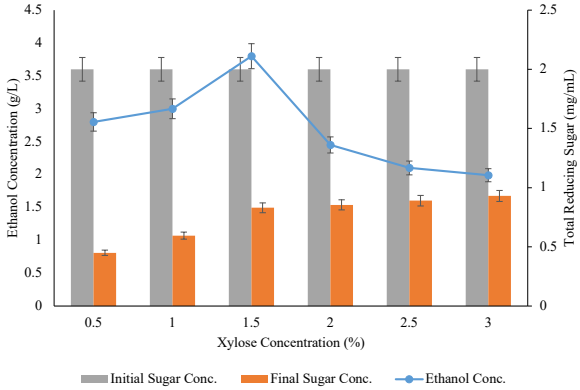

Fig. 9. Effect of varying xylose concentrations on maximum bioethanol production using *W. anomalus* (IHZ-26).

The utilization of hexoses and pentoses simultaneously may be attributed to the ability of *W. anomalus*, to break down the sugars by the activity of enzymes such as invertase and α amylase (Lee *et al*., 2011). Singh *et al*. (2014), Karagöz and Ozkan (2014) and Jyotsana *et al*. (2015) also reported maximum bioethanol yield using *S. cerevisiae* and *Scheffersomyces stipites* in a fermentation medium containing both xylose and glucose. But yield of ethanol as per provided substrate is greater in present study as compared to reports in which other yeasts were used showing superiority of *W. anomalus* in simultabeous utilization of pentoses and hexoses.

Glucose and xylose were optimized separately as 2 and 1.5%, respectively (figure 8 and 9). Our findings related to the optimization of concentrations were similar to the results of Gikonyo *et al*. (2015) who reported maximum bioethanol yield while using *Kluyveromyces* sp. IIPE453 yeast strain. The similarity in results might be due to the use of similar kind of yeast strains. Bioethanol production declined with further increase in the amount of carbon sources than the optimum amount may be associated to the finding that growth of microbes in the of excess of carbon source under anaerobic conditions may generate acidic by-products in the so-called 'overflow' metabolism (Zhu and Shimizu 2005; Chang *et al*., 2018). The concentration of sugar higher than the optimum amount may have repressed the enzymes of yeast involved in glycolysis resulting into reduced ethanol yield (Yamaoka *et al*., 2014). The increase in amount of glucose, ethanol production was also increase but to a certain extent. After this limit, microbes accept no more glucose resulting in steady state or may lead to decrease in fermentation rate (Shin *et al*., 2009).

3.8 Optimization of nitrogen source

Effect of different nitrogen sources such as peptone, yeast extract, $(NH_4)_2SO_4$, di AHP, KNO_3, urea, $NaNO_3$, $NH_4H_2PO_4$, NH_3NO_2 and NH_4Cl was studied for the production of bioethanol in C1(YPG) fermentation medium. Maximum bioethanol production (4.0 g/L) was obtained when $NH_4H_2PO_4$ nitrogen source was used. Rest of the sources such as peptone, yeast extract, $(NH_4)_2SO_4$, KNO_3, urea, $NaNO_3$ and NH_3NO_2 gave 0.09 g/L ($p < 0.05$), 0.61 g/L ($p < 0.05$), 0.04 g/L ($p < 0.05$), 0.06 g/L ($p < 0.05$), 0.03 g/L ($p < 0.05$), 0.05 g/L ($p < 0.05$) and 0.07 g/L ($p < 0.05$) bioethanol, respectively. Least amount of bioethanol production was given by NH_4Cl with 0.01 g/L ($p < 0.05$) bioethanol as shown in figure 10. The effect of varying nitrogen concentrations such as 0.5, 1, 1.5, 2 and 2.5% were assessed for maximum bioethanol production. The rate of bioethanol production increased from 3.8 g/L ($p < 0.05$) to 4.4 g/L ($p < 0.05$) when the concentration of nitrogen was increased from 0.5 to 2%, respectively. It was observed that further increase in the concentration of nitrogen resulted in the decrease in bioethanol production from 4.4 g/L ($p < 0.05$) to 4.3 g/L ($p < 0.05$) as shown in figure 11.

Nitrogen source greatly influence bioethanol production asit contribute to the synthesis of important enzymes which paly important role in their metabolic pathway for the production of ethanol (Slininger *et al*., 2006; Pérez *et al*., 2011; Harde *et al*., 2014). Ammonium dihydrogen phosphate, in the medium, may have led to synthesis of essential proteins involved in the process of transportation of sugars to the membrane interior.

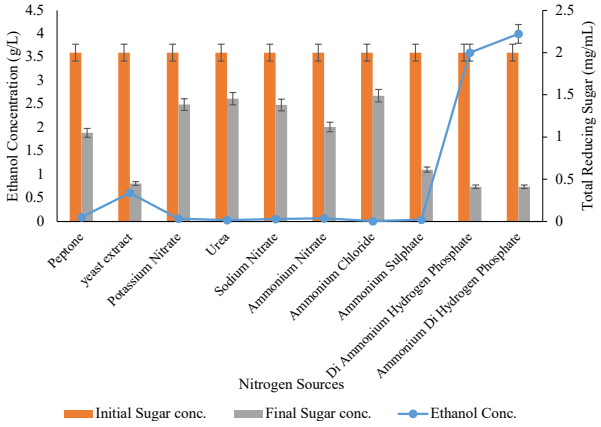

Fig. 10. Optimization of nitrogen source for maximum bioethanol production using *W. anomalus* (IHZ-26).

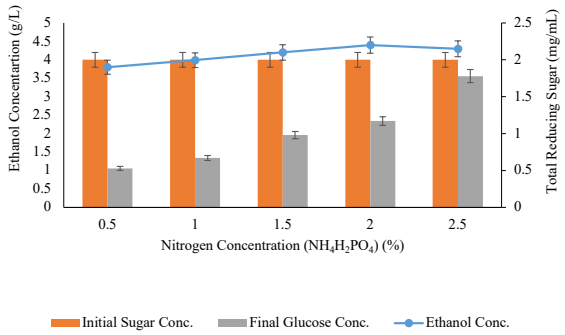

Fig. 11. Effect of different concentrations of $NH_4H_2PO_4$ on bioethanol production using *W. anomalus* (IHZ-26).

With the deficiency of nitrogen such proteins may not be synthesized resulting in restricted cell growth and bioethanol production. The ability of yeast cells to utilize ammonium salt is perhaps much more widespread among ethanol producing species. Our findings were similar to Fadel *et al*. (2013) who reported their work on the optimization of D-xylose conversion to ethanol by the yeast *Pichia stipitis* NRRL Y-7124. It was observed that lack of nitrogenous compounds can cause slow or stuck fermentation (Júnior *et al*., 2009). Further increase in the nitrogen concentration resulted in the decreased rate of bioethanol production. This is due to the fact that accumulation of ethanol in the fermentation medium inhibits growth of yeast cells (Santos *et al*., 2012 and Li *et al*., 2017), hence, the rate of ethanol production decreases (Thenmozhi and Victoria 2013; Bafrncová *et al*., 1999). Chan-u-tit *et al*. (2013) reported contrary results using *Saccharomyces cerevisiae* which gave 1.89 g/L bioethanol yield by consuming 3% nitrogen source. This might be associate dto the use of different yeast species in both studies.

3.9 Optimization of inoculum size

The influence of inoculum size such as 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5 and 6% was assessed on the production of bioethanol in C1(YPG) medium. The rate of bioethanol production increased from 3.6 g/L ($p < 0.05$) to 4.8 g/L ($p < 0.05$) when the inoculum size increased from 1 to 3.5% respectively, as shown in figure 12. It was observed that further increase in inoculum size (3.5%) declined the rate of bioethanol production from 4.8 g/L ($p < 0.05$) to 3.7 g/L ($p < 0.05$). Least bioethanol yield (3.6 g/L ($p < 0.05$)) was obtained by using 1% inoculum.

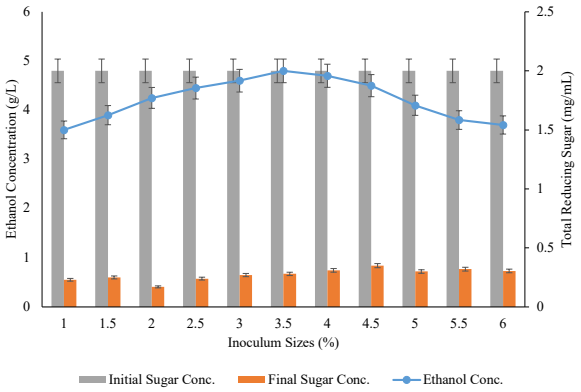

Fig. 12. Optimization of inoculum size for bioethanol production using *W. anomalus* (IHZ-26).

Therefore, 3.5% inoculum size yielding maximum bioethanol production (4.8 g/L ($p < 0.05$)) was used for further analysis. More yeast cells will grow in the medium when the inoculum size is increased as it may lead to a competition for nutrients resulting in slow growth and metabolism which decreases the ethanol yield (Laopaiboon *et al*., 2007). Moreover, high inoculum level might result in rapid exhaust of nutrients in the medium, hence, reducing the ethanol yield (Franca *et al*., 2009 and Tesfaw & Assefa, 2014). On contrary, if inoculum size is less than the amount required, limited amount of substrates will be converted into product as there will be less yeast cells present to carry out that task (Niladevi and Prema 2008). Results slightly differ from the findings of Laluce *et al*. (2009) and Tahir *et al*. (2010) who obtained maximum bioethanol yield while using 3% inoculum size of *S. cerevisiae* as it may require more numer of cells to achieve the same task which can be accomplished by little of number of *W. anomalus* cells.

3.10 Optimization of inoculum age

The effect of time required for inoculum development on the production of bioethanol was analyzed. The inoculum age varied from 0 to 24 hours with an interval of 4 hours. It was observed that maximum bioethanol (5.0 g/L ($p < 0.05$)) was produced when inoculum 4 h old inoculum was used. Further increase in the age of inoculum from 4 to 24 hours, declined bioethanol production from 5.0 g/L ($p < 0.05$) to 3.6 g/L ($p < 0.05$) as shown in figure 13. Least amount of bioethanol was obtained when 0 hour old inoculum was used. Increase in the age of inoculum decreased the fermentation efficiency of yeast cells as they might have entered into their stationary phase.

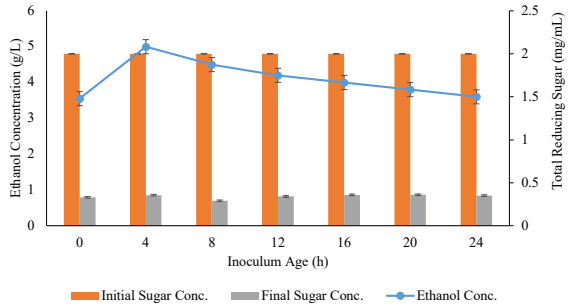

Fig. 13. Effect of varying inoculum age for maximum bioethanol production using *W. anomalus* (IHZ-26).

Formation of by products in this phase that hinder yeast growth and its potent enzymes also reduce bioethanol yield (Zhang *et al*., 2015). Thenmozhi and Victoria (2013) reported similar findings but Rekha and Vijayalakshmi (2018) reported contradicted results to our finding with best results obtained after 72 h of inoculum age. The reason for contradiction might be the use of *S. cerevisiae* which have slow metabolic growth rate instead of *W. anomalus*.

Conclusions

The research finding concluded that *W. anomalus* is a potnet yeast strain with efficient ability to convert pentoses and hexoses simultaneously. Furthermore, optimization of various process parameters enhanced the rate of bioethanol production significantly by five folds . Applying optimization finding of this research, feasible and economical fermentation process can be developed for the efficient production of bioethanol using saccharification mixture of lignocellulosic biomass. The process will provide long term benefits of economical bioethanol production in relation to reduction in environmental pollutants produced by burning of by fossil fuels.

References

Acharya, S. and Chaudhary, A. (2012). Alkaline cellulase produced by a newly isolated thermophilic *Aneurinibacillus thermoaerophilus* WBS2 from hot spring, India. *African Journal of Microbiology Research 6*, 5453-545. https://doi.org/10.5897/AJMR11.1491

Almeida, J. R., Modig, T., Petersson, A., Hähn-Hägerdal, B., Lidén, G. and Gorwa-Grauslund, M.F. (2007). Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *Journal of Chemical Technology and Biotechnology 82*, 340-349. https://doi.org/10.1002/jctb.1676

Anwar, Z., Gulfraz, M. and Irshad, M. (2014). Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: a brief review. *Journal of Radiation Research and Applied Sciences 7*, 163-173. https://doi.org/10.1016/j.jrras.2014.02.003

Azhar, S.H.M., Abdulla, R., Jambo, S.A., Marbawi, H., Gansau, J.A., Faik, A.A.M. and K.F. Rodrigues. (2017). Yeasts in sustainable bioethanol production: A Review. *Biochemistry and Biophysics Reports 10*, 52-61. https://doi.org/10.1016/j.bbrep.2017.03.003

Babu, S., Harinikumar, K., Singh, R.K. and Pandey, A. (2014). Optimization of bioethanol production from fruit wastes using isolated microbial strains. *International Journal of Advanced Biotechnology and Research 5*, 598-604. http://dx.doi.org/10.1590/S1517-83822014000200012

Bafrncova, P., Slavikova, I., Patkova, J. and Domeny, Z. (1999). Improvement of very high gravity ethanol fermentation by media supplementation using *Saccharomyces cerevisiae*. *Biotechnology Letters 21*, 337-341. https://doi.org/10.1023/A:1005436816047

Baldwin, E.L., Karki B., Zahler, J.D., Rinehart, M. and Gibbons, W.R. (2019). Submerged vs. solid-state conversion of soybean meal into a high protein feed using *Aureobasidium pullulans. Journal of the American Oil Chemists' Society 96*, 989-998. https://doi.org/10.1002/aocs.12251

Berłowska, J., Pielech-Przybylska, K., Balcerek, M., Dziekońska-Kubczak, U., Patelski, P., Dziugan, P. and Kregiel, D. (2016). Simultaneous saccharification and fermentation of sugar beet pulp for efficient bioethanol production. *BioMed Research International 2016*. https://doi.org/10.1155/2016/3154929

Bokulich, N.A. and Bamforth, C.W. (2013). The microbiology of malting and brewing.

Microbiology and Molecular Biology Reviews 77, 157-172. https://doi/10.1128/MMBR.00060-12

Cazetta, M.L., Celligoi, M.A.P.C., Buzato, J.B. and Scarmino, I.S. (2007). Fermentation of molasses by Zymomonas mobilis: Effects of temperature and sugar concentration on ethanol production. *Bioresource Technology 98*, 2824-2828. https://doi.org/10.1016/j.biortech.2006.08.026

Chang, Y.H., Chang, K.S., Chen, C.Y., Hsu, C.L., Chang, T.C. and Jang, H. D. (2018). Enhancement of the efficiency of bioethanol production by *Saccharomyces cerevisiae* via gradually batch-wise and fed-batch increasing the glucose concentration. *Fermentation 4*, 45. https://doi:10.3390/fermentation4020045

Chan-u-tit, P., Laopaiboon, L., Jaisil, P. and Laopaiboon, P. (2013). High level ethanol production by nitrogen and osmoprotectant supplementation under very high gravity fermentation conditions. *Energies 6*, 884-899. https://doi.org/10.3390/en6020884

Chingono, T. and Mbohwa, C. (2016). Study on regulations, policies and permits for implementation of bioenergy systems. http://ieomsociety.org/ieomdetroit/pdfs/300.pdf

Cofré, O., Ramírez, M., Gómez, J.M. and Cantero, D. (2012). Optimization of culture media for ethanol production from glycerol by *Escherichia coli. Biomass and Bioenergy 37*, 275-281. http://dx.doi.org/10.1016/j.biombioe.2011.12.002

Cruz, S.H., Batistote, M. and Ernandes, J. R. (2003). Effect of sugar catabolite repression in correlation with the structural complexity of the nitrogen source on yeast growth and fermentation. *Journal of the Institute of Brewing 109*, 349-355. https://doi.org/10.1002/j.2050-0416.2003.tb00609.x

Darouneh, E., Alavi, A., Vosoughi, M., Arjmand, M., Seifkordi, A. and Rajabi, R. (2009). Citric acid production: Surface culture versus submerged culture. *African Journal of Microbiology Research 3*, 541-545. http://doi/10.1.1.919.5493&rep=rep1&type=pdf

Davis, L., Rogers, P., Pearce, J. and Peiris, P. (2006). Evaluation of Zymomonas-based ethanol production from a hydrolysed waste starch stream. *Biomass and Bioenergy 30*, 809-814.

Dickinson, B. 2006. Bionutrients tech manual. Sparks, Md.: Becton-Dickinson. Retrieved from www.bd.com/ds/technicalCenter/misc/br_3_2547.pdf

Fadel, M., Keera, A.A., Mouafi, F.E. and Kahil, T. (2013). High level ethanol from sugar cane molasses by a new thermotolerant *Saccharomyces cerevisiae* strain in industrial scale. *Biotechnology Research International 2013*, 1-6. https://doi.org/10.1155/2013/253286

Franca, A. S., Gouvea, B., Torres, C., Lean-dro, S. O., & Oliveira, E. S. (2009). Feasibility of ethanol production from coffee husks. In *13th International Biotechnology Symposium and Exhibition* (pp. 269-269).

Gadonneix, P., De Castro, F.B., De Medeiros, N.F., Drouin, R., Jain, C.P., Kim, Y.D. and Naqi, A.A. (2010). *Biofuels: Policies, Standards and Technologies*. World Energy Council. http://environmentportal.in/files/biofuelsformatedmaster.pdf

Gao, J., Atiyeh, H.K., Phillips, J.R., Wilkins, M.R. and Huhnke, R.L. (2013). Development of low cost medium for ethanol production from syngas by *Clostridium ragsdalei. Bioresource Technology 147*, 508-515. https://doi.org/10.1016/j.biortech.2017.09.060

Ghosh, T.K. and Prelas, M.A. (2011). Ethanol. In *Energy Resources and Systems*. Springer Netherlands, pp. 419-493. https://link.springer.com/chapter/10.1007/978-94-007-1402-1_7

Gikonyo, B. (2015). Design and optimization of ethanol production from bagasse pith hydrolysate by a thermotolerant yeast *Kluyveromyces* sp. IIPE453 using response surface methodology. In *Sugarcane as Biofuel Feedstock* (pp. 109-128). Apple Academic Press.

Gupta, P., Samant, K. and Sahu, A. (2012). Isolation of cellulose-degrading bacteria and determination of their cellulolytic potential. *International Journal of Microbiology 2012*, 1-5. https://doi.org/10.1155/2012/578925

Harde, S.M., Bankar, S.B., Ojamo, H., Granström, T., Singhal, R.S. and Survase, S.A. (2014). Continuous lignocellulosic ethanol production using *Coleus forskohlii* root hydrolysate. *Fuel 126*, 77-84. https://doi.org/10.1016/j.fuel.2014.02.046

Hölker, U., Höfer, M. and Lenz, J. (2004). Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. *Applied Microbiology and Biotechnology 64*, 175-186. https://doi.org/10.1007/s00253-003-1504-3

Júnior, M.M., Batistote, M., Cilli, E.M. and Ernandes, J.R. (2009). Sucrose fermentation by Brazilian ethanol production yeasts in media containing structurally complex nitrogen sources. *Journal of the Institute of Brewing 115*, 191-197. https://doi.org/10.1002/j.2050-0416.2009.tb00368.x

Jyotsana, K.P., Rao, A.R. and Devaki, K. (2015). Effect of nutritional factors on cellulase production by *Streptomyces albaduncus* from the gut of earthworm, Eisenia foetida. *Pest Management In Horticultural Ecosystems 21*, 75-80.

Kadam, K.L. and Newman, M.M. (1997). Development of a low-cost fermentation medium for ethanol production from biomass. *Applied Microbiology and Biotechnology 47*, 625-629. https://doi.org/10.1007/s002530050985

Karagöz, P. and Özkan, M. (2014). Ethanol production from wheat straw by *Saccharomyces cerevisiae* and *Scheffersomyces stipitis* co-culture in batch and continuous system. *Bioresource Technology 158*, 286-293. http://doi.org/10.1016/j.biortech.2014.02.022

Keshav, P.K., Naseeruddin, S. and Rao, L. V. (2016). Improved enzymatic saccharification of steam exploded cotton stalk using alkaline extraction and fermentation of cellulosic sugars into ethanol. *Bioresource Technology 214*, 363-370. https://doi.org/10.1016/j.biortech.2016.04.108

Kiran, S, Ali, S. and Haq, I.U. 2003. Time course study for yeast invertase production by submerged fermentation. *Journal of Biological Sciences 3*, 984-988. https://doi.org/0.3923/jbs.2003.984.988

Kurian, J. K., Ashok, M.K., Banerjee, A. and Kishore, V.V.N. (2010). Bioconversion of hemicellulose hydrolysate of sweet sorghum bagasse to ethanol by using *Pichia stipitis* NCIM 3497 and *Debaryomyces hansenii* sp. *Bioresources 5*, 2404-2416. https://doi.org/10.15376/biores.5.4.2404-2416

Laluce, C., Tognolli, J.O., De Oliveira, K.F., Souza, C.S. and Morais, M.R. (2009). Optimization of temperature, sugar concentration, and inoculum size to maximize ethanol production without significant decrease in yeast cell viability. *Applied Microbiology and Biotechnology 83*, 627-637. https://doi.org/10.1007/s00253-009-1885-z

Laopaiboon, L., Thanonkeo, P., Jaisil, P. and Laopaiboon, P. (2007). Ethanol production from sweet sorghum juice in batch and fed-batch fermentations by *Saccharomyces cerevisiae*. *World Journal of Microbiology and Biotechnology 23*, 1497-1501. https://doi.org/10.1007/s11274-007-9383-x

Lee, Y.J., Choi, Y.R., Lee, S.Y., Park, J.T., Shim, J.H., Park, K.H. and Kim, J.W. (2011). Screening wild yeast strains for alcohol fermentation from various fruits. *Mycobiology 39*, 33-39. https://doi:10.11648/j.ajbio.20160405.11

Li, Z., Wang, D. and Shi, Y.C. (2017). Effects of nitrogen source on ethanol production in very high gravity fermentation of corn starch. *Journal of the Taiwan Institute of Chemical Engineers 70*, 229-235. http://dx.doi.org/10.1016%2Fj.jtice.2016.10.055

Lin, Y. and Tanaka, S. (2006). Ethanol fermentation from biomass resources: current state and prospects. *Applied Microbiology and Biotechnology 69*, 627-642. https://doi.org/10.1007/s00253-005-0229-x

Lin, Y., Zhang, W., Li, C., Sakakibara, K., Tanaka, S. and Kong, H. (2012). Factors affecting ethanol fermentation using *Saccharomyces cerevisiae* BY4742. *Biomass and Bioenergy 47*, 395-401. https://doi.org/10.1016/j.biombioe.2012.09.019

Liu, K., Atiyeh, H.K., Tanner, R.S., Wilkins, M.R. and Huhnke, R.L. (2012). Fermentative production of ethanol from syngas using novel moderately alkaliphilic strains of *Alkalibaculum bacchi*. *Bioresource Technology 104*, 336-341. https://doi.org/10.1016/j.biortech.2011.10.054

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical chemistry 31*, 426-428. https://doi.org/10.1021/ac60147a030

Najafpour, G., Younesi, H. and Ismail, K.S.K. (2004). Ethanol fermentation in an immobilized cell reactor using *Saccharomyces cerevisiae*. *Bioresource Technology 92*, 251-260. https://doi.org/10.1016/j.biortech.2003.09.009

Navarro, A.R., Sepúlveda, M.D.C. and Rubio, M.C. (2000). Bio-concentration of vinasse from the alcoholic fermentation of sugar cane molasses. *Waste management 20*, 581-585. http://dx.doi.org/10.1016/S0956-053X(00)00026-X

Nawaz, A., Ashfaq, A., Zaidi, S. M. A. M., Munir, M., Haq, I. U., Mukhtar, H., & Tahir, S. F. (2020). Comparison of fermentation and medical potentials of *Saccharomyces* with *Wickerhamomyces genera. Revista Mexicana de Ingeniería Química 19*, 33-47. https://doi.org/10.24275/rmiq/Bio379

Niladevi, K.N. and Prema, P. (2008). Effect of inducers and process parameters on laccase production by *Streptomyces psammoticus* and its application in dye decolourization. *Bioresource Technology 99*, 4583-4589. https://doi.org/10.1016/j.biortech.2007.06.056

Olido, E.L.Y. (2018). Xylanase enzyme production by *Aspergillus fumigatus* in solid state fermentation and submerged fermentation. *Revista Mexicana de Ingeniería Química 17*, 47-61.

Paulova, L., Patakova, P., Branska, B., Rychtera, M. and Melzoch, K. (2015). Lignocellulosic ethanol: technology design and its impact on process efficiency. *Biotechnology Advances 33*, 1091-1107. https://doi.org/10.1016/j.biotechadv.2014.12.002

Pérez-Carrillo, E., Cortés-Callejas, M.L., Sabillón-Galeas, L.E., Montalvo-Villarreal, J.L., Canizo, J.R., Moreno-Zepeda, M.G. and Serna-Saldivar, S.O. (2011). Detrimental effect of increasing sugar concentrations on ethanol production from maize or decorticated sorghum mashes fermented with *Saccharomyces cerevisiae* or *Zymomonas mobilis*. *Biotechnology Letters 33*, 301-307. https://doi.org/10.1007/s10529-010-0448-9

Phillips, J.R., Atiyeh, H.K. and Huhnke, R.L. (2014). Method for design of production medium for fermentation of synthesis gas to ethanol by acetogenic bacteria. *Biological Engineering Transactions 7*, 113-128.

Phisalaphong, M., Srirattana, N. and Tanthapanichakoon, W. (2006). Mathematical modeling to investigate temperature effect on kinetic parameters of ethanol fermentation. *Biochemical Engineering Journal 28*, 36-43. https://doi.org/10.1016/j.bej.2005.08.039

Pinal, L., Ceden, M., Gutie, H. and Alvarez-Jacobs, J. (1997). Fermentation parameters influencing higher alcohol production in the tequila process. *Biotechnology Letters 19*, 45-47. https://doi.org/10.1023/A:1018362919846

Plessas, S., Bekatorou, A., Koutinas, A. A., Soupioni, M., Banat, I.M. and Marchant, R. (2007). Use of *Saccharomyces cerevisiae* cells immobilized on orange peel as biocatalyst for alcoholic fermentation. *Bioresource Technology 98*, 860-865. https://doi.org/10.1016/j.biortech.2006.03.014

Raposo, S., Constantino, A., Rodrigues, F., Rodrigues, B. and Lima-Costa, M. E. (2017). Nitrogen sources screening for ethanol production using carob industrial wastes. *Applied Biochemistry and Biotechnology 181*, 827-843. https://doi.org/10.1007/s12010-016-2252-z

Rayabova, O.B., Chmil, O. M. and Sibirny, A.A. (2003). Xylose and cellobiose fermentation to ethanol by the thermotolerant methylotrophic yeast *Hansenula polymorpha*. *FEMS Yeast Research 4*, 157-164. https://doi.org/10.1016/S1567-1356(03)00146-6

Rekha, Y.G.G. and Vijayalakshmi, S. (2018). Production and optimization techniques of bioethanol from withered flowers of *Allamanda schottii* L. by Activated Dry Yeast. *Journal of Pure and Applied Microbiology 12*, 943-952. http://dx.doi.org/10.22207/JPAM.12.2.57

Reyes, I., Hernandez-Jaimes, C., Meraz, M. and Rodríguez-Huezo, M.E. (2018). Physicochemical changes of corn starch during lactic acid fermentation with *Lactobacillus bulgaricus*. *Revista Mexicana de Ingeniería Química 17*, 279-288. https://doi.org/10.24275/uam/izt/dcbi/revmexingquim/2018v17n1/Reyes

Sanchez, S., Bravo, V., Castro, E., Moya, A.J. and Camacho, F. (1999). Comparative study of the fermentation of D-glucose/D-xylose mixtures with *Pachysolen tannophilus* and *Candida shehatae*. *Bioprocess Engineering 21*, 525-532. https://doi.org/10.1007/PL00009091

Santos, J., Sousa, M.J. and Leao, C. (2012). Ammonium is toxic for aging yeast cells, inducing death and shortening of the chronological lifespan. *PloS one 7*. https://doi.org/10.1371/journal.pone.0037090

Satora, P., Tarko, T., Sroka, P. and Blaszczyk, U. (2014). The influence of Wickerhamomyces anomalus killer yeast on the fermentation and chemical composition of apple wines. *FEMS Yeast Research 14*, 729-740. https://doi.org/10.1111/1567-1364.12159

Shin, S., Chang, D.E. and Pan, J.G. (2009). Acetate consumption activity directly determines the level of acetate accumulation during *Escherichia coli* W3110 growth. *Journal of Microbiology and Biotechnology 19*, 1127-1134. https://doi.org/10.4014/jmb.0902.0097

Singh, A., Bajar, S. and Bishnoi, N. R. (2014). Enzymatic hydrolysis of microwave alkali pretreated rice husk for ethanol production by *Saccharomyces cerevisiae*, *Scheffersomyces stipitis* and their co-culture. *Fuel 116*, 699-702. https://doi.org/10.1016/j.fuel.2013.08.072

Slininger, P.J., Dien, B.S., Gorsich, S.W. and Liu, Z.L. (2006). Nitrogen source and mineral optimization enhance D-xylose conversion to ethanol by the yeast *Pichia stipitis* NRRL Y-7124. *Applied Microbiology and Biotechnology 72*, 1285-1296. https://doi.org/10.1007/s00253-006-0435-1

Tahir, A., Aftab, M. and Farasat, T. (2010). Effect of cultural conditions on ethanol production by locally isolated *Saccharomyces cerevisiae* BIO-07. *Journal of Applied Pharmaceutical Science 3*, 72-78.

Temudo, M. F., Kleerebezem, R. and Van-Loosdrecht, M. (2007). Influence of the pH on (open) mixed culture fermentation of glucose: a chemostat study. *Biotechnology and Bioengineering 98*, 69-79. https://doi.org/10.1002/bit.21412

Temudo, M. F., Muyzer, G., Kleerebezem, R. and Van-Loosdrecht, M. C. (2008). Diversity of microbial communities in open mixed culture fermentations: impact of the pH and carbon source. *Applied Microbiology and Biotechnology 80*, 1121-1130. https://doi.org/10.1007/s00253-008-1669-x

Tesfaw, A. and Assefa, F. (2014). Current trends in bioethanol production by *Saccharomyces cerevisiae*: substrate, inhibitor reduction, growth variables, coculture, and immobilization. *International Scholarly Research Notices 2014*, https://doi.org/10.1155/2014/532852

Thenmozhi, R. and Victoria, J. (2013). Optimization and improvement of ethanol production by the incorporation of organic wastes. *Pelagia Research Library 4*, 119-23.

Torija, M.J., Rozes, N., Poblet, M., Guillamón, J.M. and Mas, A. (2003). Effects of fermentation temperature on the strain population of *Saccharomyces cerevisiae*. *International Journal of Food Microbiology 80*, 47-53. https://doi.org/10.1016/S0168-1605(02)00144-7

Unrean, P. and Srienc, F. (2010). Continuous production of ethanol from hexoses and pentoses using immobilized mixed cultures of *Escherichia coli* strains. *Journal of Biotechnology 150*, 215-223. https://doi.org/10.1016/j.jbiotec.2010.08.002

Van, M.A.J., Abbott, D.A., Bellissimi, E., VanDen Brink, J., Kuyper, M., Luttik, M.A. and Pronk, J.T. (2006). Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: Current status. *Antonie Van Leeuwenhoek 90*, 391-418. https://doi.org/10.1007/s10482-006-9085-7

Vucurovic, V. M., Razmovski, R.N. and Popov, S.D. (2009). Ethanol production using *Saccharomyces cerevisiae* cells immobilised on corn stem ground tissue. *Zbornik Matice Srpske Za Prirodne Nauke 116*, 315-322. https://doi.org/10.2298/ZMSPN0916315V

Yamada, R., Yamakawa, S. I., Tanaka, T., Ogino, C., Fukuda, H. and Kondo, A. (2011). Direct and efficient ethanol production from high-yielding rice using a *Saccharomyces cerevisiae* strain that express amylases. *Enzyme and Microbial Technology 48*, 393-396. https://doi.org/10.1016/j.enzmictec.2011.01.002

Yamaoka, C., Kurita, O. and Kubo, T. (2014). Improved ethanol tolerance of *Saccharomyces cerevisiae* in mixed cultures with *Kluyveromyces lactis* on high-sugar fermentation. *Microbiological Research 169*, 907-914. https://doi.org/10.1016/j.micres.2014.04.007

Yu, Z. and Zhang, H. (2003). Ethanol fermentation of acid-hydrolyzed cellulosic pyrolysate with *Saccharomyces cerevisiae*. *Bioresource Technology 90*, 95-100. https://doi.org/10.1016/S0960-8524(03)00093-2

Zabed, H., Faruq, G., Sahu, J.N., Azirun, M.S., Hashim, R. and Nasrulhaq B.A. (2014). Bioethanol production from fermentable sugar juice. *The Scientific World Journal 2014*. https://doi.org/10.1155/2014/957102

Zaldivar, J., Nielsen, J. and Olsson, L. (2001). Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. *Applied Microbiology and biotechnology 56*, 17-34. https://doi.org/10.1007/s002530100624

Zhang, Q., Wu, D., Lin, Y., Wang, X., Kong, H. and Tanaka, S. (2015). Substrate and product inhibition on yeast performance in ethanol fermentation. *Energy and Fuels 29*, 1019-1027. https://doi.org/10.1021/ef502349v

Zhu, J. and Shimizu, K. (2005). Effect of a single-gene knockout on the metabolic regulation in *Escherichia coli* for D-lactate production under microaerobic condition. *Metabolic Engineering 7*, 104-115. https://doi.org/10.1016/j.ymben.2004.10.004