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Bioethanol production from starchy part of tuberous plant (potato) using *Saccharomyces cerevisiae* MTCC-170

Joginder Singh Duhan[1]*, Ashok Kumar[1] and Sunil Kumar Tanwar[2]

[1]Department of Biotechnology, Ch. Devi Lal University, Sirsa-125055, India.
[2]Department of Biotechnology and Microbiology, Meerut Institute of Engineering and Technology, Meerut-250005, India.

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Ethanol is one of the bio-energy sources with high efficiency and low environmental impact. Various raw materials have been used as carbon source for ethanol production. In the present study, one varieties of potato that is, Kufri Bahar (KB) flour was chosen as a carbon source. In order to obtain maximum conversion of starch into fermentable sugar, optimum parameters for the liquefaction were determined as 104 to 105°C, 0.15% v/w of α-amylase enzyme solution (300 U/ml) and 30 g dry-weight potato mash/100 ml distilled water, respectively with a 68.86% loss in dry weight during the process. For saccharification process, the optimum dose of amyloglucosidase was 0.35% w/v (300 U/ml) with 16.95% glucose production at pH 5.0 and temperature 60°C after 1 h. The maximum ethanol concentration 7.89% (v/v) was obtained with 10% inoculum size at pH 6.0 after 48 h. Furthermore, out of the three nitrogen (yeast extract, peptone and ammonium sulphate) sources tested for ethanol production, peptone at 1.5 g/l was found to be best (7.58%). In conclusion, this study demonstrates the potential utilization of potato powder for ethanol production.

Key words: Potato starch, bioethanol, liquefaction, saccharification, *Saccharomyces cerevisiae* MTCC-170.

INTRODUCTION

In the 21st century, the demand of energy for transportation, heating and industrial processing is increasing day by day. Environmental issues are a point of concern (Hahn-Hagerdal et al., 2006). Renewable energy sources receive attention not only to protect the environment but also to supply energy needs by reducing dependence on foreign oil. In recent years, bio-energy sources have become more important as a viable and economical alternative source. Ethanol is one of the bio-energy sources with high efficiency and low environmental impact. Worldwide production of ethanol is approximately 51,000 million litters. Fuel encompassed 73% of produced ethanol, while beverage and industrial ethanol constitute 17 and 10%, respectively (Sanchez and Cardona, 2008). As a fuel enhancer, ethanol has some advantages. Woodson and Jablonowskiy (2008) reported that "as an additive (ethanol), serves as a fuel volume extender, an oxygenate and an octane enhancer." Most of the countries have either ethanol blended gasoline or direct ethanol as fuel, such as: Brazil, USA, Canada, Colombia, Spain and France (Sanchez and Cardona, 2008). In 2005, 45.42 billion litters of ethanol were produced worldwide (Balat et al., 2008) with Brazil and the U.S as the two major producers of ethanol. Ethanol is an alcohol that is a product of microbial fermentation. Microorganisms meet their energy demand by converting carbon sources to by-products such as: carbon dioxide, lactic acid, ethanol, etc. *Saccharomyces cerevisiae*, *Zymomonas*

*Corresponding author. E-mail: duhanjs68@gmail.com, duhanjs@rediffmail.com. Fax: +91 1666 248123, +91 1666 247127.

mobilis, *Kluyveromyces* spp. and *Schizosaccharomyces pombe* are microorganisms able to convert sugars to ethanol.

Various feedstock and chemically defined media can be used for ethanol fermentation. The most commonly used types of feedstock for ethanol production are corn, sugar cane and wheat (Balat et al., 2008). Sugarcane, sugar beets and molasses are feasible for ethanol fermentation and have been used; however, these carbon sources are high value products as food sources (Nalley and Hudson, 2003; USDA, 2006). In order to meet the low cost requirement, lignocellulosic biomass is another option for ethanol fermentation. However, lignocellulosic biomass is complex and requires expensive pre-treatments. Currently, potatoes are an alternative feedstock for ethanol production. Minal and Deshpande (2010) stated that potatoes are the second most used food in the world. Potatoes are starchy crops which do not require complex pre-treatments. Although, it is also a high value crop, but 5 to 20% of crops that are waste potato by-products from potato cultivation could be utilized for bio-ethanol production (Limatainen et al., 2004; Adarsha et al., 2010). Moreover, during processing of potato, particularly in the potato chip industry, approximately 18% of the potatoes are generated as waste. Therefore, the waste from potato industry can also be utilized as growth media (economical carbon source) for the fermentation processes for the production of ethanol as it has high starch content. The wastes of potato industry are currently being utilized as animal feed (Yamada et al., 2009).

Starchy materials require a reaction of starch with water (hydrolysis) to break down the starch into fermentable sugars (saccharification). Hydrolysis is carried out at high temperature (90 to 110°C); however, at low temperatures, it is also possible and can contribute to energy savings (Sanchez et al., 2008). To convert starch into the fermentable sugars, either acid hydrolysis or enzymatic hydrolysis needs to be performed. Each has their own set of advantages and disadvantages for use. Enzyme hydrolysis is generally chosen even though high cost of enzymes and initial investment because of high conversion yield of glucose (Tasic et al., 2009). Amylases (α-amylase, β-amylase and glucoamylase) are employed for hydrolysis of starchy materials. Although, amylases are derived from plants, animals and microorganisms, microbial amylases are in use commonly (Kunamneni et al., 2005). α-Amylase hydrolyses the 1,4-α-D-glucosidic linkages in the linear amylase chain, randomly. However, amyloglucosidase cleaves the 1,6-α-linkages at the branching points of amylopectin as well as 1,4-α-linkages (Pandey et al., 2000). However, production of ethanol from waste potato still needs to be optimized because limited research has been conducted about the utilization of potato waste for ethanol production. Fadel (2000) and Liimatainen et al. (2004) showed that different wastes of potato industry can be a good carbon source for yeast during alcohol fermentation by studying waste from potato to chips industry (98.67% total carbohydrate) and different potato cultivations (starch content in a range of 11.2% to over 19.3%), respectively.

So, the aim of the present study was to use potato starch, a very cheap substrate for the production of ethanol and to optimize fermentation process.

MATERIALS AND METHODS

Raw materials

Potato tubers were procured from CCS HAU, Hisar and analyzed for different components by standard methods (AOAC, 1990). Thoroughly, washed peeled potato (1 kg) were dried overnight at 70°C and grounded to fine powder.

Enzyme for liquefaction and saccharification

Commercial α-amylase (specific activity 300 DUN U/ml) and amyloglucosidase (specific activity 400 GA U/ml) were obtained from SIGMA – ALDRICH PVT. LTD., India.

Preparation of potato flour slurry

Slurries of various concentrations (10, 15, 20, 25 and 30% w/v) of potato flour starch was prepared in water and treated with liquefying enzyme (0.15% v/w) at 104 to 105°C for 60 min in an autoclave. The slurry prepared by mixing 25 g flour in 100 ml water (1:4) being homogenous, loose, easy to handle was used for further experiments. Liquefaction of potato flour (100 ml slurry) was carried out at 104 to 105°C in an autoclave using varying concentration of alpha amylase (0.05 to 0.20% v/w) for different time intervals (10 to 240 min). The progress of liquefaction was monitored by employing starch-iodine (1 g of iodine and 2 g KI in 100 ml water) reaction. Saccharification of liquefied starch was carried out at 60°C for different time intervals using varying concentration (0.05 to 0.45% v/w) of amyloglucosidase. The reaction was monitored by the yield of total reducing sugars estimated by dinitrosalicylic acid method (Miller, 1959).

Yeast strain

A fast fermenting strain of *Saccharomyces cerevisiae* MTCC170 was obtained from Microbial Type Culture Collection, Chandigarh and maintained on yeast extract peptone dextrose (YEPD) agar medium containing yeast extract (1%), peptone (2%), dextrose (2%) and agar (2%). Dextrose inoculum medium (IM) used for inoculum preparation contained dextrose (6%), peptone (0.5%) and yeast extract (0.5%). Yeast cells pre-grown in inoculum medium for 18 h under shaking condition (120 rpm) was directly used as an inoculum at 10% (v/v).

Liquefaction

Potato slurry was liquefied with α- amylase at different enzyme dosages (0.05 to 0.20%v/w), incubation times (10 to 150 min) and temperatures (85 to 105°C). The initial pH of the slurry was 5.6. The extent of liquefaction was determined by disappearance of blue colour. The effects of enzyme dosage, incubation time and heating temperature on liquefaction ratio were evaluated.

Saccharification

Liquefied-mash of potato-powder obtained by employing the optimized conditions of liquefaction process were saccharified at 60°C for 1 h by using another enzyme that is, amyloglucosidase. This enzyme completes the process of breakdown of the starch into simple sugar that is, glucose. Saccharification process was determined by using dinitrosalicylic acid method (Miller, 1959).

Optimization of fermentation conditions

Effect of inoculum concentration

The hydrolysate was inoculated with different concentrations of inoculum that is 5, 10, 15 and 20% (v/v) and kept for fermentation at 35°C for 48 h.

Effect of temperature

The hydrolysate inoculated with the best combination of nutrients and fermentation was carried out at various temperatures namely 25, 30, 35 and 40°C. Ethanol content in fermented samples was estimated after 48 h of incubation.

Effect of pH

The pH of hydrolysate was adjusted to different levels and it was fermented after supplementation with the best combination of nutrients after inoculating with 10% inoculum (v/v). The fermentation was carried out at 35°C for 48 h.

Effect of nutrient concentration

To 100 ml hydrolysate, different nutrients like ammonium sulphate (0.3%), yeast extract (0.5%) and peptone (0.5%) was added in their single and double concentration. The flasks were inoculated with 10% yeast cells (v/v). The fermentation was carried out at 35°C for 48 h.

Analytical methods

Estimation of reducing sugars

The DNS method of Miller (1959) was used to estimate reducing sugars of the samples.

Ethanol determination

Ethanol concentration was determined by the method of Caputi et al. (1968).

Statistical analysis

All experiments were carried out in a completely randomized design and in triplicates. The results were subjected to analysis of variance (one-way ANOVA) and the treatment means were compared using the least significant difference (LSD) values at a significance level of $P < 0.05$. Simple ANOVA were evaluated using SPSS 16.0 software (SPSS, O.P. Sheoran Programmer, Computer Section, CCS HAU, Hisar).

RESULTS AND DISCUSSION

Potato flour contained 8.39% moisture, 73.25% starch and 4.86% proteins (Table 1).

Optimization of condition for liquefaction process

The optimum combination of temperature, dose of enzyme (α- amylase) and amount of potato flour slurry was determined as 104 to 105°C, 0.15% v/w of α-amylase enzyme solution (300 U/ml) and 30 g dry-weight potato mash/100 ml distilled water, respectively with a 68.86% loss in dry weight during the liquefaction process (Table 2).

Optimization of saccharification

For the saccharification process, dose of enzyme, temperature and saccharification time were also determined. The optimum dose of amyloglucosidase was 0.35% v/w (300 U/ml) with 16.82 g/100 ml glucose production after 1 h at 60°C for potato (KB) as shown in Table 3.

Optimization of fermentation conditions

Optimization of inoculum size for ethanol production

To determine the economic inoculum size of SSF of potato flour hydrolysate, different innoculum size that is 5, 10, 15 and 20% were used by keeping initial substrate concentration (100 g/l), initial pH (6.0), inoculum age (17 h old culture) and agitator speed (120 rpm) for 24, 36 and 48 h fermentation period as shown in Figure 1, there was significant difference among the inoculum size tested (5, 10, 15 and 20%) regarding kinetic parameters in ethanol production. The maximum ethanol concentration (7.89%) was produced by *S. cerevisiae* MTCC-170. Sugar utilization (94.83%) and ethanol yield that is, 91.39% was obtained with an initial inoculum of 10%, which is economic and environment friendly. It was observed that when the innoculum size was increased from 5 to 10%, ethanol production was also increased but above 10%, rate of alcohol production decreased after 48 h of incubation. Breisha (2010) reported that increasing the yeast inoculum volume from 3 to 6% showed positive effects on fermentation from 25% sucrose and reduced the fermentation time from 72 (3) to 48 h (6%). The fermentation time shorten along with the raise in inoculum size which was due to the fast cell growth within the reactor. Most of the substrate was immediately converted to ethanol. A maximum ethanol production of 88 from 200 g/l sucrose medium at 10% inoculum size in 16 to 18 h was obtained by Singh and Jain (1994).

According to the study of Fadel (2000), the maximum

Table 1. Composition of starchy raw materials.

Raw material	Source	Chemical composition % (w/w)					
		Starch					
		Acid hydrolysis	Enzymatic hydrolysis	Nitrogen contents	Protein contents	Phosphorus contents	Ash contents
Potato (KB)	CCS HAU, Hisar	72.13	73.25	0.81	4.86	0.61	4.40

Table 2. Summary of liquefaction.

Raw material	Slurry % (w/v)	Enzymes % (v/w)	pH	Temperature (°C)	Time (h)	Ca^{++} (mM)	K^{+} (mM)
Potato (KB)	25	0.10	6.2-7.0	104-105	1	0.36	0.30
	30	0.10	6.2-7.0	104-105	1	0.72	0.30

Table 3. Summary of saccharification.

Raw material	Slurry % (w/v)	Enzymes % (v/w)	pH	Temperature (°C)	Time (h)	Sugar production (Kufri Bahar) % (w/v)
Potato	25	0.10	5.0	60	1	15.34
	30	0.10	5.0	60	1	16.82

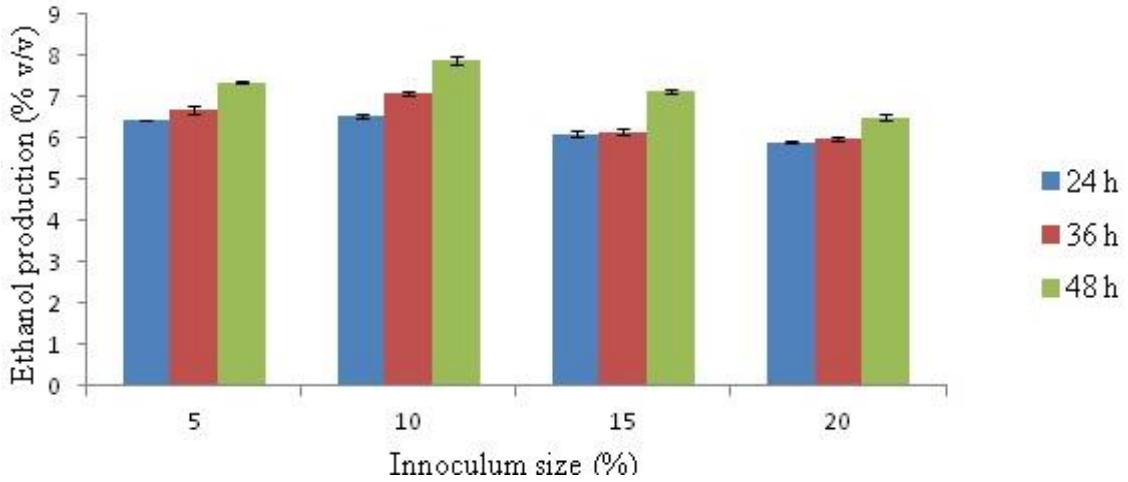

Figure 1. Effect of inoculum size on ethanol production from supplemented potato (Kufri Bahar) powder hydrolysate.

alcohol production (12.9%) was obtained when inoculated with 10% culture of *S. cerevisiae*.

Afifi et al. (2011) produced maximum ethanol from industrial solid potato wastes when inoculated with 10% (v/w) inoculum size of *S. cerevisiae.* Neelakandan and Usharan (2009) studied different inoculum size (2, 4, 6, 8 and 10% v/v) for a period of 24 h and observed that the maximum ethanol concentration that is, 8.8% was obtained at 10% inoculum size. In comparison of these results, Izmirlioglu and Demirci (2012) showed that 3% inoculum size was optimum for maximum ethanol concentration and production rate. Turhan et al. (2010) reported the ethanol production from carob extract by using *S. cerevisiae* and found that maximum ethanol concentration; ethanol productivity and ethanol yield were 42.90 g/L, 3.7 g/L/h and 45.0%, respectively, obtained

Table 4. Effect of temperature on ethanol production from supplemented[b] potato (Kufri Bahar) flour hydrolysate[a].

Yeast strain[c]		Ethanol % (v/v)[d]		
		35°C		
		Kufri Bahar		
	Temperature[e] (°C)	24 h	36 h	48 h
S. cerevisiae	30	5.90	6.28	7.50
	35	6.11	6.59	7.99
	40	4.07	2.02	4.30
	45	2.14	1.67	2.26

[a]Initial sugars 16 to 17% (w/v).
[b]Potato (KB) flour hydrolysate was supplemented with ammonium sulphate (0.2% w/v), peptone (0.25% w/v) and yeast extract (0.25% w/v).
[c]Inocula were grown in YEPD shake flask (210 rpm) at 35°C and used at 10% (v/v).
[d]Ethanol values are mean of three replications.
[e]Fermentation process was carried out at different temperatures.

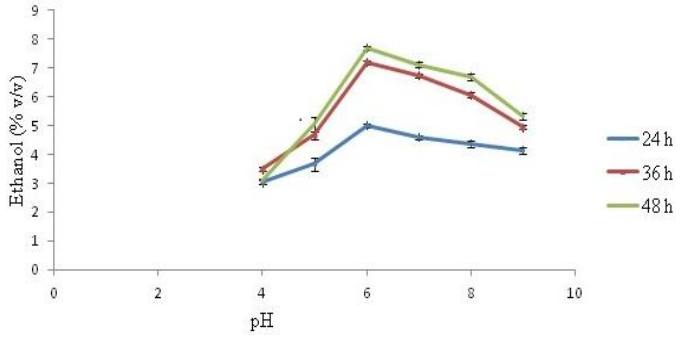

Figure 2. Effect of pH on ethanol production from potato (Kufri Bahar) powder hydrolysate.

with an initial inoculum of 3%.

Effect of temperature on ethanol production

Temperature is one of the major constraints that determine the ethanol production because temperature exerts a profound effect on growth, metabolism and survival of the fermenting organism. To know the optimum temperature for ethanol production, the fermentation media were kept at 25, 30, 35 and 40°C. The maximum ethanol concentration (7.99%) was obtained from *S. cerevisiae* MTCC-170 when culture was grown at 35°C. Above 35°C ethanol production was decreased to 4.30% (Table 4). Hashem and Darwish (2010) observed that production of ethanol by *S. cerevisiae* y-1646 was favoured at 35°C temperature and reached its maximum value (5.29 g/l) after 36 h. At 37°C, ethanol production was reduced to 4.38 g/l. Rani et al. (2010) observed that maximum ethanol content of 56.8 g/l was recorded after 48 h of fermentation at 30°C. However, at temperature 35, 37 and 40°C, the corresponding values were 53.6, 50.0 and 46.0 g/l, respectively showing a decline with increase in temperature of fermentation. Asli (2010) observed best ethanol production rate at 32°C temperature. Bio-ethanol production increases with increased in temperature and reaches its maximum value at 35°C.

Further, the increasing temperature reduced the percentage of ethanol production and it is mainly due to denaturation of the yeast cells (Periyasamy et al., 2009). Khan et al. (2012) studied the effects of temperature on bioethanol production and observed that maximum bioethanol was produced at 35°C as compared to bioethanol produced at 23 and 28°C, respectively.

Effect of pH on ethanol production

The initial pH is one of the important factors that affect the performance of SSF. The effect of pH on ethanol fermentation is studied by conducting batch experiments at different pH ranging from pH 4.0 to 7.0 for yeast strains namely *S. cerevisiae* MTCC- 170 by keeping initial substrate concentration (100 g/l), initial temperature (35°C), inoculum age (17 h old culture) and agitator speed (120 rpm) for 24, 36 and 48 h of fermentation period. As shown in Figure 2, the ethanol concentration was increased from pH 4.0 to 6.0 and then decreased marginally above this value. The maximum ethanol concentration 7.70% was obtained from *S. cerevisiae* MTCC- 170 culture grown at pH 6.0. Fadel (2000) reported that high ethanol production was obtained by using initial pH 5.0 to 6.0. It was also shown that no ethanol production exists lower than pH 4.0 (Graves et al., 2006). Turhan et al. (2008) reported that maximum ethanol yield, maximum growth rate and biomass concentration were obtained at pH 5.5 on carob as a medium for ethanol production. Osman et al. (2011) tested wide initial pH range and found that at pH 3.0 no growth was observed and no ethanol was produced, while pH 6.0 was the optimum for both biomass and ethanol production. Similar results were obtained by Kadambini (2006). Mohanty et al. (2009) reported that pH 6.0 was optimum for bioethanol production from mahula (*Madhuca latifolia* L.) flowers by

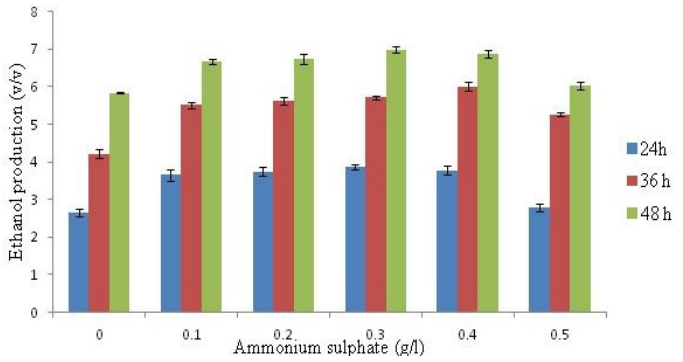

Figure 3. Effect of addition of ammonium sulphate on ethanol production from potato (Kufri Bahar) flour hydrolysate.

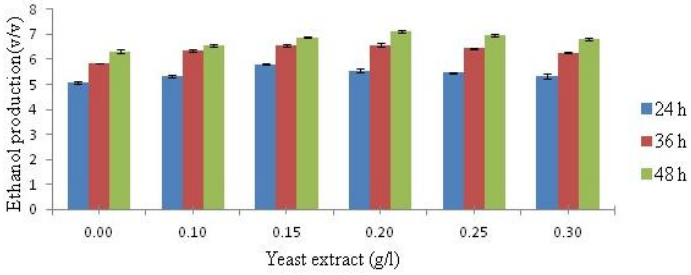

Figure 4. Effect of addition of yeast extract on ethanol production from potato (Kufri Bahar) flour hydrolysate.

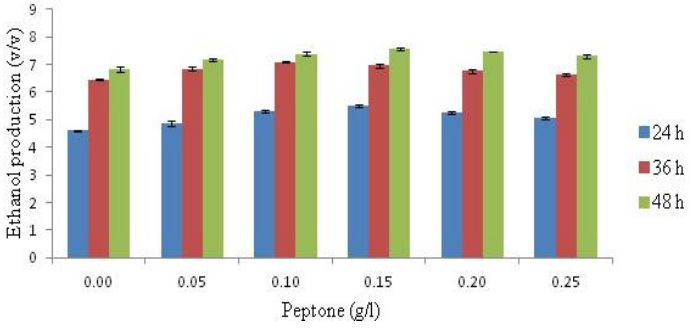

Figure 5. Effect of addition of peptone on alcohol production from potato (Kufri Bahar) flour hydrolysate.

production from mahula (*Madhuca latifolia* L.) flowers by solid-state fermentation.

Similar results were obtained by Togarepi et al. (2012) when *Ziziphus mauritiana* fruit pulp was used as a substrate (Akponah and Akpomie, 2011).

Effect of nutrients on ethanol production

Addition of nutrients such as ammonium sulphate, yeast extract and peptone play a vital role in boosting the ethanol production and its rate. Effect of ammonium sulphate as a nitrogen source was studied by varying its concentration between 1.0 to 5.0 g/l keeping rest of the parameters at their optimal conditions. Figure 3 shows that as the concentration of ammonium sulphate increased from 1.0 to 3.0 g/l, ethanol production also increased from to 5.84 to 6.98% for *S. cerevisiae*; above that concentration ethanol production was decreased when potato (Kufri Bahar) was used as substrates. Beltran et al. (2007) studied the effect of ammonium sulphate with different concentrations ranging from 0.01 to 0.09 g/l and observed that maximum production was obtained at 0.06 g/l concentration of ammonium sulphate. Amutha and Gunashekaran (2000) obtained higher ethanol yield of 44.2 and 54.9 g/l, respectively by supplementation of liquefied cassava starch with ammonium sulphate (1.0 g/l). Srichuwong et al. (2009) studied the SSF simultaneous saccharification and fermentation (SSF) of very high gravity (VHG) potato mash for the production of ethanol and results revealed that 2 to 2.5% ethanol concentration was increased with ammonium sulphate supplementation which corresponded to a decrease in residual glucose (0.25 to 3.3% w/v).

Anupama et al. (2010) obtained optimum ethanol yield of 5.6% with 3 g/l concentration of $(NH_4)_2SO_4$ as a nitrogen source.

Effect of yeast extract on ethanol production

Effect of yeast extract was studied by varying its concentration from 1.0 to 3.0 g/l keeping rest of the parameters at their optimal conditions. Figure 4 shows that as the concentration of yeast extract increased from 1.0 to 2.0 g/l, ethanol production was also increased from 6.55 to 7.11% for *S. cerevisiae* but above this concentration, ethanol production was decreased when potato (Kufri Bahar) was used as substrates. Nuanpeng et al. (2011) studied that sugar consumption, ethanol production and yeast cell viability during batch VHG fermentation of *S. cerevisiae* NP 01 from sweet sorghum juice supplemented with various yeast extract concentrations and observed that the highest ethanol concentration in the EP medium containing 9.0 g/l of yeast extract. Laopaiboon et al. (2009) observed that ethanol production efficiency was improved when 3.0 g/l of yeast extract (120.68 ± 0.54 g/l) was added to sweet sorghum juice under VHG conditions.

Effect of peptone on ethanol production

To examine the effect of peptone on ethanol production various concentrations that is, 0.5 to 2.5 g/l were used keeping rest of the parameters at their optimal conditions. Data in Figure 5 shows that as the concentration of peptone increased from 0.5 to 1.5 g/l, ethanol production increases from 6.83 to 7.58% for *S. cerevisiae*, above this concentration ethanol production was decreased when potato (Kufri Bahar) was used as substrate. Wang et al. (2007) observed that peptone was a critical factor

for ethanol production and 1.5% (w/v) peptone in the medium increased the final ethanol titre from 14.2 to 17% (v/v) in 48 h. Dake et al. (2010) observed that maximum ethanol was produced at 0.5% (w/v) of peptone concentration.

Conclusion

According to the results, it could be concluded that potato can be an attractive feedstock for the bioethanol production, especially in India where 5 to 20% waste potato by-products are obtained from potato cultivar and also due to poor storage facility (Adarsha et al., 2010). Since it provided the necessary nutrient element and the appropriate hydrogen balance for the fermentation, there was no need for supplementing these additionally or making any pH adjustment. Potato were dried overnight at 70°C and grounded to fine powder was used for ethanol fermentation by *S. cerevisiae* MTCC-170. Homogenised (1:4) slurry was obtained on treatment with α-amylase (300 U/ml) at 104 to 105°C for 60 min which was saccharified with glucoamylase at 60°C for 1 h. Optimum parameters for ethanol fermentation by this strain are pH 6.0, temperature at 35°C, initial sugar concentration of 16.82 g/100 ml, $(NH_4)_2SO_4$, yeast extract and peptone used as nitrogen source. The yeast concentration of 2.0 g/l of potato flour yielded the optimum ethanol concentration that is 7.11% v/v. Addition of peptone, ammonium sulphate, to the production medium also markedly influences the level of ethanol concentration. Evidently, treating the hydrolysate with nutrients after formation of hydrolysate enhanced the degree of ethanol production significantly ($p<0.05$). Holding the hydrolysate at 35°C for 48 h could increase in the ethanol production, consequently allowing greater growth of the yeast strain to act on the hydrolysate.

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