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Isolation and Optimization of Ethanol Producing Bacteria from Natural Environments of Mazandaran Province in Iran

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Abstract

Ethanol producing bacteria are useful in industrial production of biofuel. There are interesting for screening of active bacteria from natural resources and introduce to biofuel industries. The present study aims to isolation of ethanol producing bacteria with characterization, optimization, and evaluation of their ethanol productivity. Samples from various fruits, plant saps and soils were screened for isolation of ethanol producing bacteria then evaluate to find the highest ethanol producer. Of all the 37 ethanol producing isolates, 6 highest producers were selected for characterization. Bacterial growth and ethanol production conditions were optimized based on pH, temperature, agitation, time and initial glucose concentration. Most isolates were occurred single or in pairs. All of isolates were motile and catalase positive but failed to hydrolyze gelatin and produce H_2S. Among them, Zym6 was exhibited highest ethanol yield 6.28 gL^{-1} with optimum pH 6 and growth temperature 35 ˚C. In addition, Zym5 and Zym6 were exhibited highest ethanol yield 19.52 gL^{-1} and 18.75 gL^{-1} with xylose and tryptophan, respectively. Thus the optimum condition for ethanol production was a medium composed of pH 6, growth temperature 35 ˚C for 24-48 hours and xylose and tryptophan as carbon and nitrogen sources.

Keywords: Bacteria, Biofuel, Ethanol, Isolation, Natural resources.

Introduction

The ethanol is one of the most important clean fuels and renewable energy resources, which would play an important role in effectively solving the problem of the forthcoming oil storage. (Rogers *et al.,* 2007; Rogers *et al.,* 1982; Swings and Deley, 1977). Isolation of ethanol producing bacteria from natural resources and assay of their ethanol production to produce higher ethanol as a supplement and replacement for fossil fuels has an ignorable importance in the world future energy trends (Chum *et al.,* 2001). Microbial processes have been proved useful for production of alternate energy products from renewable resources (Wheals *et al.,* 1999). Alcoholic fermentation is one of the most important examples. Ethanol is the most promising liquid fuel since it can be readily produced from various agriculture-based renewable materials (Wigmosta *et al.,* 2011). Currently, yeast *Saccharomyces cerevisiae* is used as the major ethanol producing microorganism worldwide (Najafpour *et al.,* 2004). Despite its expensive use, it has a number of disadvantages, such as high aeration cost, high biomass production and low temperature and ethanol tolerances (Desiniotis *et al*., 2012; Panesar et *al.,* 2006; Remize et al., 1999). *Zymomonas mobilis* has emerged as a potential bacterium for ethanol production. The studies have clearly demonstrated that it has a high specific rate of sugar uptake (Yamashita *et al.,* 2008), high ethanol yield (Yanase *et al*., 2012), low biomass production and non-requirement of controlled addition of oxygen to maintain the viability of the cells (Rogers *et al.,* 1997). It is widely distributed in natural habitats and classified into the family *Sphingomonadaceae*. This study aims to isolate

ethanol producing bacteria from natural environments, optimize the ethanol production and evaluate their productivity. Since province of Mazandaran is located in a dump area, it is likely that high diversity of ethanol bacteria will be found in the north areas of Iran.

Materials and methods

Screening of bacteria

To isolate ethanol producing bacteria, various samples were collected from natural resources including fruits (apple, fig, grape, apricot, nectarine, orange, tangerine, pomegranate, peach, pear and cane) and soils during June- November 2012. Samples were crushed aseptically then inoculated into Zymomonas sucrose medium (ZSM) contained (gL^{-1}): sucrose, 20 g; yeast extract, 10 g; ammonium sulfate, 2 g; KH_2PO_4, 2 g; $MgSO_4$ $7H_2O$, 0.5 g; pH 6.8. ZSM bottles with Durham were incubated at 35 ˚C for 1-7 days (Swings *et al.*, 1977). Those bottle were produced CO_2 gas then plated out on RM medium (contain gL^{-1}: 20 g glucose, 10 g yeast extract, 2 g Ammonium sulfate, 2 g KH_2PO_4, 0.5 g $MgSO_4$, $7H_2O$, 15 g agar, pH 6.8) supplemented with 0.083 mg mL^{-1} of nystatin to inhibit the growth of yeasts. Plates were incubated at 35 ˚C for 2 days under aerobic conditions. Colonies growing on RM medium were isolate for further studies (Shihui et al., 2013).

Identification of the isolates

For identification of the bacterial isolates, morphological and physiological characteristics were examined using the methods described in Bergey's manual of systematic bacteriology (Brenner *et al*., 2004).

Ethanol production and assay

The strains were evaluated for producing of ethanol. The strains were cultured in ZSM medium at 35 ˚C. After 48 hours incubation, the ethanol was assayed using both methods including GC Mass and dichromate colorimetric method. The ethanol concentration that produced in ZSM culture was estimated using microprocessor based gas chromatograph equipped with flame ionization detector and DB-5 column. The injector, detector and oven temperature of gas chromatograph were maintained at 200, 210 and 100 ˚C, respectively (Panesar *et al.*, 2006). In dichromate colorimetric method, the reaction mixture containing 1 ml each of the sample, potassium dichromate 50 gL-1 and saturated diphenylcarbazide was heated at 90 ˚C for 5-15 minutes until it turned brown. Then, 1 ml of sodium potassium tartrate (40%) was added for stabilization of the produced color. The absorbance was measured at 575 nm. The concentration of ethanol was calculated from a standard curve covering the concentration range 0.01-0.1% of ethanol (Grootjen et al., 1990).

Optimization of nutrition sources and culture conditions

To find a suitable medium and condition for ethanol production by isolates, different carbon and nitrogen sources was examined (Mohseni and Ebrahimi, 2013). Different carbon sources.including glucose, xylose, fructose, maltose, sucrose, ribose, galactose, mannose and arabinose were used at 20 g L-1 in RM basal medium. In addition, the effect of glucose on ethanol production was studied using different concentrations as 5, 10, 15 and 20 g L-1.

Nitrogen sources for optimization process were yeast extract, peptone, cysteine, ammonium sulfate, alanine, arginine and tryptophan. The nitrogen sources were added at 10 gL^{-1} in RM basal medium.

To examine the effects of temperature, initial pH, time of fermentation and agitation on ethanol production, isolates were cultivated at a range of temperatures 25, 30, 35, 40 ˚C; various pH 2, 4, 6 and 8; different fermentation time 24, 48, 72, 96 hours and various agitation rate 50, 100, 150, 200 rpm.

Results

A total of 37 isolates were selected as ethanol producing bacteria. They were Gram-negative and Gram-positive, rod-shaped and entire-edged with non-pigmented colonies (Fig. 1). The isolates were tested for ethanol production in the RM medium. These isolates were inoculated on RM broth and all isolates showed signs of gas production. Of all the 37 isolates, 6 isolates that -

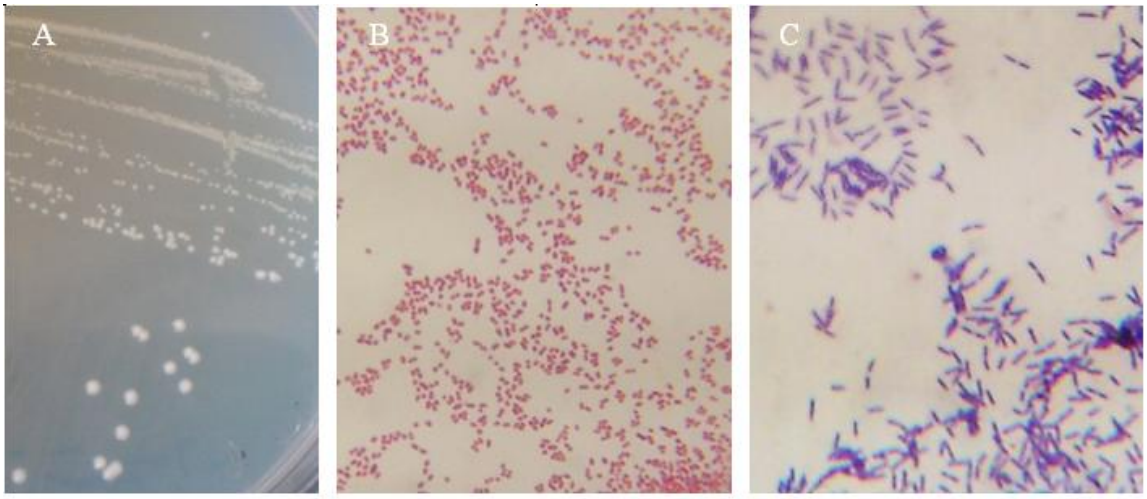

Fig 1. Colonies of pure isolate ZYM2 on RM agar (A) and rod shaped, Gram negative ZYM1 (B) and Gram positive ZYM3 (C).

produce high gas production in Durham tube were selected for further studies. These bacterial strains that isolated from pomegranate, apple, grape, peach, pear, sap of plants and soils were shown a highest productivity of ethanol (data not shown).

Morphological and physiological characteristics of isolates were summarized in table 1. Most isolates were occurred single or in pairs and all of them were motile. The same characteristics were observed in the reference organism, *Z. mobilis* PTCC 1718. When plates were incubated aerobically, the resulting colonies were smaller than those incubated anaerobically. The average diameter of colonies that grown aerobically after 48 hours incubation was 1.0-1.2 mm while those isolates incubated anaerobically ranged from 1.8-2.0 mm.

Physiological and biochemical tests showed uniform reaction of all isolates (Table 1).

Table 1. Biochemical test of the isolated ethanol producing bacteria.

Isolates	Morphological and physiological characteristics								
	Morphology	**Gram**	**Catalase**	**Oxidase**	**Indole**	**Mobility**	**MR**	**VP**	**Gelatinase**
ZYM1	Rod shape	-	+	-	-	+	+	-	-
ZYM2	Rod shape	-	+	-	-	+	+	-	-
ZYM3	Rod shape	+	+	-	-	+	+	-	-
ZYM4	Rod shape	-	+	-	-	+	-	-	-
ZYM5	Rod shape	+	+	-	-	+	+	-	-
ZYM6	Rod shape	-	+	-	-	+	+	-	-

+, positive and -, negative reaction

All of them were catalase positive, failed to hydrolyze gelatin, and did not produce H_2S. To investigate the effect of pH on ability of isolate Zym1-Zym6 to produce ethanol, pH of RM medium was adjusted from 2 to 8 then incubated at 35 ˚C in static conditions for 48 hours. The results indicated that maximum ethanol was produced at pH 6–8 (Table 2).

Among the isolates, Zym6 was exhibited highest ethanol yield 6.28 gL^{-1} at pH 6. In addition, the results obtained from Table 2 revealed that Zym3 was able to produce as low ethanol 0.21 gL^{-1} and Zym1, Zym6 were unable to produce at pH 8 (Fig. 2). To find out optimum sugar level for fermentation, batch fermentation was carried out with varying levels of initial glucose concentration. Ethanol production by 6 isolates in different glucose concentration was summarized in table 2.

Table 2 Effect of pH, time, initial glucose concentration, temperature and agitation on production of ethanol by isolated bacteria.

Strain	Time		pH		Temperature		Initial glucose		Agitation	
	Hours	Ethanol (gL^{-1})	pH	Ethanol (gL^{-1})	˚C	Ethanol (gL^{-1})	Glucose (gL^{-1})	Ethanol (gL^{-1})	rpm	Ethanol (gL^{-1})
ZYM1	24	0	2	0	25	0	5	0	50	0
	48	5.70	4	0	30	0	10	0	100	0
	72	4.21	6	4.21	35	4.21	15	0	150	6.05
	96	2.53	8	0	40	0	20	4.00	200	3.95
ZYM2	24	0.30	2	0	25	0	5	0	50	3.37
	48	0.35	4	0	30	1.62	10	0	100	3.95
	72	6.28	6	6	35	6.00	15	2.37	150	4.95
	96	2.45	8	4.24	40	0	20	0	200	4.74
ZYM3	24	0	2	0	25	0	5	0	50	1.00
	48	0	4	0	30	0	10	0	100	0
	72	0.39	6	0	35	0.39	15	0	150	1.17
	96	0	8	0.21	40	0	20	0.21	200	0.48
ZYM4	24	0	2	0	25	0	5	0	50	0
	48	0	4	0	30	0	10	0	100	0
	72	3.91	6	0	35	0.79	15	0	150	0.09
	96	0	8	0.52	40	0	20	0.52	200	0.07
ZYM5	24	0	2	0	25	0	5	0	50	3.63
	48	2.21	4	0	30	0.94	10	0	100	0
	72	0	6	0	35	0	15	0	150	0
	96	0	8	0.26	40	0	20	0.26	200	0
ZYM6	24	0	2	0	25	1.62	5	0	50	0
	48	5.74	4	6.28	30	6.28	10	0	100	0
	72	4.74	6	0.94	35	0.94	15	1.21	150	5.49
	96	1.00	8	0	40	0	20	4.74	200	3.95
Z. mobilis	24	0	2	0	25	0	5	0	50	0
	48	6.00	4	4.00	30	0.20	10	0.21	100	3.00
	72	2.53	6	0.30	35	3.37	15	1.00	150	0.30
	96	0.31	8	0	40	0	20	3.37	200	0

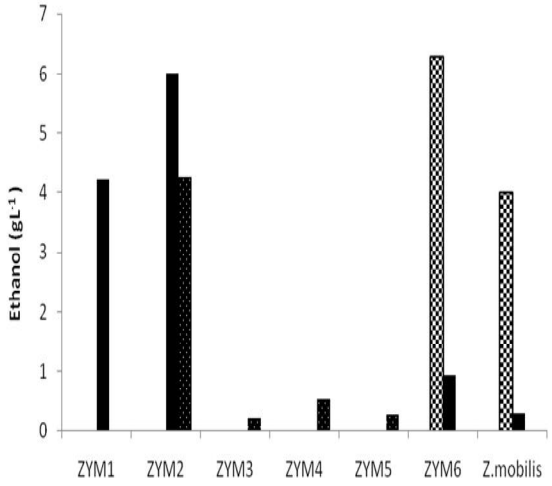

Fig 2. Effect of pH on ethanol production by isolated bacteria. (▩) pH 2, (▩) pH 4, (■) pH 6 and (▩) pH 8.

The maximum efficiency of fermentation was observed at 20% (w/v) glucose by Zym1 and Zym6 with 4.00 gL^{-1} and 4.74 gL^{-1} ethanol, respectively (Fig. 3). The isolates Zym2 was produced 2.37 gL^{-1} ethanol at 15% (w/v) glucose, respectively. The results in Table 2 indicated that all strains were unable to produce ethanol at low initial glucose concentration 5% (w/v).

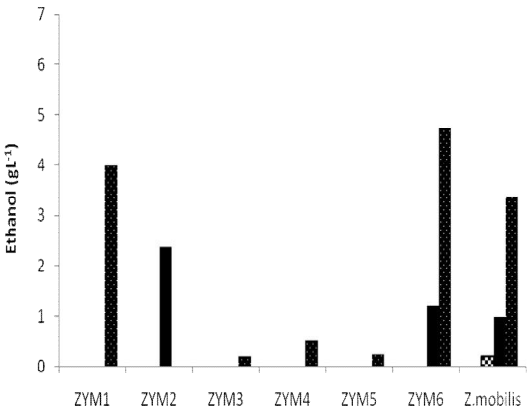

Fig 3. Effect of initial glucose concentration on producing ethanol by isolated bacteria. **(▩)** 5 g, (▩) 10 g, (■) 15 g and **(▩)** 20 g.

To determine the effect of temperature on ethanol production, the isolates were cultured at different temperature. The results obtained from Table 2 demonstrated that the optimum growth temperature was 35 ˚C and high ethanol (6.28 gL^{-1}) was produced by Zym6 at 35 ˚C. In addition, the isolate Zym2 was produced 6.00 gL^{-1} ethanol at the same temperature (Fig. 4). All strains were grown at 25 and 40 ˚C with no ethanol production. To study the effect of time on ethanol production, RM broth was inoculated with active culture of Zym1- Zym6 then incubated static condition at 35 ˚C for 24, 48, 72 and 96 hours. The results revealed that the ethanol production was increased over time (Fig. 5). Ethanol production by Zym2 was raised due to increasing fermentation time from 24 to 72 hours whereas it was decreased after 96 hours (Table 2). More ethanol was produced by Zym6 after 48 hours and Zym2 after 72 hours incubation with 5.74 gL^{-1} and 6.28 gL^{-1} ethanol, respectively.

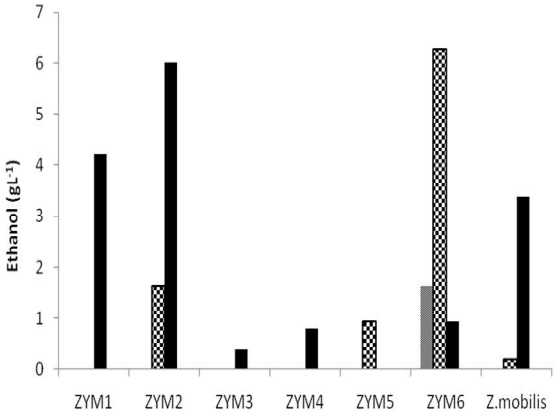

Fig 4. Effect of temperature on producing ethanol by isolates. (▩) 25 °C, (▩) 30 °C, (■) 35 °C and **(▩)** 40 °C.

Results from table 1 indicating agitation was played an important role in ethanol production. Ethanol production and biomass concentration was strongly improved by increasing agitation. The biomass was increased with raising agitation speed from 50 to 200 rpm. These results were correlated with ethanol production rate when agitation increased from 50-150 rpm (Fig. 6). Maximum biomass concentration was achieved after 48-72 hours incubation at 50-100 rpm agitation, while maximum biomass was observed after 24-48 hours when agitated at 150 and 200 rpm. The maximum biomass level of the culture Zym1 was 6.05 gL^{-1}, agitated at 150 rpm. Both Zym2 and Zym3 have been able to produce ethanol in all level of agitation. It was no doubt that agitation would strongly improve ethanol

concentration from the results achieved from table 1.

To study the effect of carbon and nitrogen sources on ethanol production, RM broth was supplemented with different carbon and nitrogen sources then incubated static condition at 35 ˚C for 48 hours. The results in Table 3 showed that the best carbon source for most isolates was xylose.

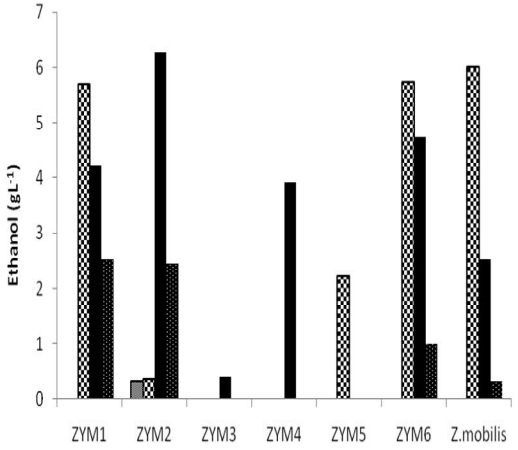

Fig 5. Effect of time on ethanol production by isolates. (▩) 24 Hour (▩) 48 Hour (■) 72 Hour **(■)** 96 hour.

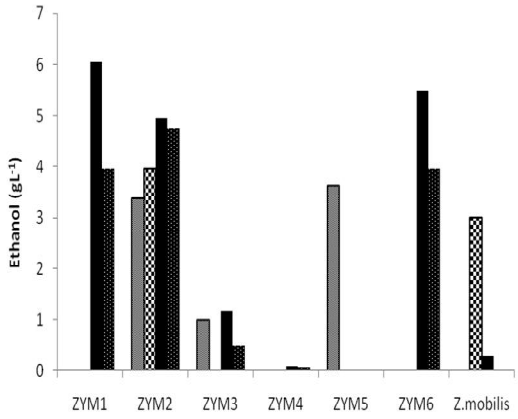

Fig 6. Effect of agitation on ethanol production by isolates. (▩) 50 rpm **(▩)** 100 rpm (■) 150 rpm **(■)** 200 rpm.

Among the isolates, Zym3, Zym5 and Zym6 were exhibited highest ethanol yield 11.01 gL^{-1}, 19.52 gL^{-1} and 15.00 gL^{-1} ethanol with xylose, respectively. In addition, the results revealed that most isolates were able to produce high ethanol when consumed five different carbon sources (Table 3).

Also, the results of nitrogen source obtained from Table 4 demonstrated that the high ethanol was produced by Zym6 (18.75 gL^{-1}) with tryptophan.

Discussion and Conclusion

For industrial ethanol production, several properties of the fermenting organism are very important in order to minimize the costs involved. New microbial isolates are always needed to meet the biotechnologists' requirements. The probability to isolate different species from the samples increases irrespective to their relative presence. In this study, the number of bacteria was isolated that able to grow in low-cost-row carbon source xylose with increase produces high ethanol. Morphological examination of the isolates revealed Gram negative, plump rod cells with distinct rounded ends. No endospores were observed. Such characteristics were reported by earlier workers (Sobana et al., 2012). The same characteristics were also observed in the reference organism, *Z. mobilis* PTCC 1718. In the present study, the isolated bacteria were able to grow at 15% sugar concentration of RM medium. Production of high ethanol at the initial stage of isolation was promising. Ethanol producing bacteria are characterized by the ability to oxidize sugars incompletely, and a common feature to most of them is the ability to produce ethanol.

Temperature optimization is essential for any biotechnological process because over temperature effect on bacterial deactivation and growth. This deactivation is attributed to the essential enzyme denaturation, membrane damage that causes cellular constituent scattering and the organism becoming more sensitive to the toxic effect of acetic acid (Panesar *et al.,* 2000). Thus, *Z. mobilis* showed maximum ethanol production and sugar utilization at 30 °C. It was also observed that the decrease in ethanol production was less between 30- 35 °C, in contrast to sharp decrease between 35- 40 °C (Panesar *et al.,* 2001). The decrease in the cell viability and final ethanol concentration with the increased in temperature from 30 to 40 °C in batch culture has also been found in *Z. mobilis* ATCC10988 (Lee *et al.,* 1981). In another study, *Z.mobilis* CP4 has shown optimal ethanol production from sugarcane molasses at 34 °C (Takeshi et al., 2012).

Table 3. Effect of different carbon sources on ethanol production by isolates.

Strain	Carbon	Ethanol (gL^{-1})	Strain	Carbon	Ethanol (gL^{-1})
ZYM1	Fructose	2.48	**ZYM4**	Fructose	0
	Sucrose	2.59		Sucrose	0
	Glucose	4.21		Glucose	0
	Maltose	0.39		Maltose	0
	Arabinose	1.12		Arabinose	0
	Xylose	0.75		Xylose	3.08
	Mannose	0		Mannose	0
	Ribose	5.99		Ribose	2.03
ZYM2	Fructose	0	**ZYM5**	Fructose	0
	Sucrose	0		Sucrose	0
	Glucose	6.28		Glucose	0
	Maltose	0		Maltose	0
	Arabinose	0		Arabinose	0
	Xylose	4.07		Xylose	19.52
	Mannose	0		Mannose	2.57
	Ribose	3.58		Ribose	10.00
ZYM3	Fructose	0	**ZYM6**	Fructose	0
	Sucrose	0		Sucrose	0
	Glucose	0		Glucose	0
	Maltose	0		Maltose	0
	Arabinose	4.66		Arabinose	0
	Xylose	11.01		Xylose	15.00
	Mannose	1.95		Mannose	0
	Ribose	3.42		Ribose	1.34

Table 4. Effect of nitrogen sources on ethanol production by isolates.

Strain	Nitrogen	Ethanol (gL^{-1})	Strain	Nitrogen	Ethanol (gL^{-1})
ZYM1	Cysteine	0	**ZYM4**	Cysteine	0
	Alanine	0		Alanine	0
	Arginine	0		Arginine	0
	tryptophan	0		tryptophan	0.58
	Ammonium sulfate	4.21		Ammonium sulfate	0
	Peptone	3		Peptone	0.79
	Yeast extract	4.21		Yeast extract	0
ZYM2	Cysteine	0.07	**ZYM5**	Cysteine	0
	Alanine	9.29		Alanine	3.75
	Arginine	5.76		Arginine	11.41
	tryptophan	0		tryptophan	7.50
	Ammonium sulfate	4.74		Ammonium sulfate	0
	Peptone	5.53		Peptone	7.11
	Yeast extract	6.28		Yeast extract	0
ZYM3	Cysteine	1.46	**ZYM6**	Cysteine	0
	Alanine	0.86		Alanine	0
	Arginine	0		Arginine	0
	tryptophan	1.96		tryptophan	18.75
	Ammonium sulfate	0		Ammonium sulfate	0
	Peptone	2.37		Peptone	7.9
	Yeast extract	0		Yeast extract	0

In our study, the optimum growth temperature was found to be 35 °C (Tables 2). It is clear from the observation recorded during this course of study that the isolated bacteria had optimal production of ethanol at 30- 35 °C. Therefore, with increasing temperature, the ethanol production was decreased.

The results of this research demonstrated that most isolates were able to produce high ethanol when consumed different carbon sources (Table

5). However, it was performed better on xylose as compared to glucose in terms of ethanol production, sugar utilization as well as ethanol and temperature tolerance. The xylose was demonstrated here to be enough to support the growth of isolated ethanol producing bacteria and ethanol production. Cheap materials, low-cost processing and high ethanol productivity are the main considerations for most ethanol fermentation (Tao *et al.,* 2005; Aggarwal *et al.,* 2001).

Fermentation with high concentration of substrates is desirable for the purpose of increasing the ethanol yield. In this study, the fermentation efficiency with high concentration of sugar (15-20 gL^{-1}) was achieved over 90%.

Acid-tolerant strains of *Z. mobilis* have been selected and used in ethanol fermentation with unsterile substrate (Tao *et al.,* 2005). Lower pH in the media is regarded to minimize the occurrences of contamination. Rogers *et al.* in 2007 showed that the growth optimal pH for ethanol producing bacteria was 6. Finding from present study, the optimum pH for both growth and ethanol production was a wider range 6-8. This result was confirmed with previous studies (Tao *et al*., 2005). However, the tolerance to low pH is strongly dependent on other parameters such as ethanol concentration and oxygen availability (Rogers *et al*., 2007).

The results of this research demonstrated that most isolates were able to produce high ethanol when consumed agitation (Table 2).The agitation was demonstrated here to be enough to support the growth of bacteria and ethanol production. Agitation could be beneficial to the growth and performance of the microorganism cells by improving the mass transfer characteristics with respect to substrates, products/byproducts and oxygen (Joao Paulo *et al.,* 2010).

In conclusion, these isolates can produce high ethanol. Owing to its low cost and no inhibition to ethanol production, the xylose is a feasible feedstock for ethanol fermentation with high efficiency using these isolates. Therefore, these organisms are projected as potential ethanol producer candidate for further commercial exploitation in industry to produce bioethanol and biofuel.

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