NUSANTARA BIOSCIENCE
Vol. 1, No. 3, Pp. 105-109
November 2009

ISSN: 2087-3940 (print)
ISSN: 2087-3956 (electronic)

Activity of *Zymomonas mobilis* on ethanol products made of cashew nut apple (*Anacardium occidentale)* with different sources of nitrogen

AKHMAD MUSTOFA[1,2,♥], SURANTO[2]

[1] University of Slamet Riyadi (UNISRI), Jl. Sumpah Pemuda, No. 18, Joglo, Surakarta. Central Java, Indonesia, Tel. +62-271-7013199, 856879, Fax.: +62-271-854670

[2] Bioscience Program, School of Graduates, Sebelas Maret University, Surakarta 57126, Central Java, Indonesia.

Manuscript received: 7 June 2009. Revision accepted: 6 August 2009.

Abstract. *Mustofa A, Suranto. 2009. Activity of* Zymomonas mobilis *on ethanol production made of cashew nut apple (*Anacardium occidentale*) with different sources of nitrogen. Nusantara Bioscience 1: 105-109.* This research is aimed at identifying *Zymomonas mobilis* in producing ethanol through batch fermentation process (in 24, 48 and 72 hours) using cashewnut apple extract (red, green and yellow variety) and urea, ammonium sulphate, extract of green peanut sprout and extract koro (*Mucuna pruriens*) as sources of nitrogen. The research showed that green cashewnut extract with ammonium sulphate in 24 hours of fermentation produced ethanol in optimum result. This treatment had pH of 5.87, 7.64 g/100 mL of sugar (with 48.44% of consumption), $8.0x10^7$ amount of bacterium ($\mu = 0.154$) and production of ethanol equal to 33.02 g/L ($Y_e = 90.19\%$).

Key words: *Zymomonas mobilis*, cashewnut apple extract, ethanol.

Abstrak. *Mustofa A, Suranto. 2011. Aktivitas* Zymomonas mobilis *pada produk etanol dari buah semu jambu mete (*Anacardium occidentale*) dengan variasi sumber nitrogen. Nusantara Bioscience 1: 105-109.* Penelitian ini bertujuan mengetahui kemampuan *Zymomonas mobilis* dalam memproduksi etanol melalui proses fermentasi batch (selama 24, 48 dan 72 jam), menggunakan sumber karbon sari buah jambu mete (varietas merah, hijau dan kuning) dan sumber nitrogen berupa urea, ammonium sulfat, ekstrak kecambah kacang hijau dan ekstrak kacang koro (*Mucuna pruriens*). Hasil penelitian menunjukkan bahwa varietas buah jambu mete hijau dengan sumber nitrogen ammonium sulfat dan lama fermentasi 24 jam memberikan hasil etanol yang paling optimal. Pada perlakuan tersebut diperoleh nilai pH 5,87, kadar gula reduksi 7,64 g/100 mL (tingkat konsumsi 48,44%), jumlah bakteri $8,0x10^7$ ($\mu = 0,154$) dan etanol sebesar 33,02 g/L ($Y_e = 90,19\%$).

Kata kunci: *Zymomonas mobilis*, sari buah jambu mete, etanol.

INTRODUCTION

There are 5,322 ha land in Yogyakarta used as an area to grow cashewnut apple and Gunung Kidul is the largest one (60.38%). Meanwhile in Central Java it covers 11,828.68 ha with Wonogiri covering equal to 7,059 ha. Both Gunung Kidul and Wonogiri produce 3,242.9 tons of nuts without its fruit (Darsono 2004). There are 200 nuts in 1 kg of the nuts of cashewnut apple, or 648.580.000 of nuts for 3,242.9 tons. It means there are about 194,574 tons of cashew fruit for that amount of nuts.

Cashew fruits are not widely utlilized in Indonesia . Meanwhile in Brazil from where it comes, People produce juices from the fruits. In India it is widely used to make alcoholic beverage called *feni* (Van Eijnatten 1991). Cashew fruit contains 16.3% of carbohidrat that can be fermented into ethanol (Thomas 1989; Van Eijnatten 1991). Some researches indicate that *Saccharomyces cereviseae* and *Zymomonas mobilis* ferment sugar from cashew fruit extract into ethanol (Hermawan *et al.* 2000; Sapariantin 2005). However, *Z. mobilis* is less frequently used than *S. cerevisiae* in ethanol products made of cashew. Pinheiro et al. (2008) and Neelakandan and Usharani (2009) indicate that cashew apple juice is a suitable substrate for *S. cerevisiae* yeast growth and ethanol production. Pacheco et al. (2009) and Rodrigues et al. (2009) showed that cashew apple bagasse is an efficient support for cell immobilization aimed at ethanol production. While, Karuppaiya et al. (2009) showed the ability of *Z. mobilis* for alcohol production from cashew apple bagasse.

Zymomonas mobilis, a gram-negative bacterium, is considered as an alternative organism in large-scale fuel ethanol production (Gunasekaran and Raj 2002). *Z. mobilis* has some good characteristics for producing ethanol namely its higher sugar uptake and ethanol yield, lower biomass production, higher ethanol tolerance, not require controlled addition of oxygen during fermentation and its amenability to generic manipulations (Wijono 1988; Doelle 1990; Hobley and Pamment 1994; Nowak 2000).

Cashew fruit contains only 4.6% of protein (Thomas 1989; Van Eijnatten 1991), resulting in less nitrogen in which it is used for growth and metabolism production. For those reasons, it has been researched on producing ethanol from cashew apple extract (red, green and yellow variety)

with *Z. mobilis* in 24, 48 and 72 hours of fermentation. Urea, ammonium sulfat, green bean (*Phaseolus radiates*) sprout extract and koro (*Mucuna pruriens*) extract were added as sources of nitrogen.

MATERIALS AND METHODS

Materials

Cashew apples with red, yellow and green variety that have been used as the source of carbon were taken from Ngadirojo sub district, Wonogiri district, Central Java. The added carbon source was glucose 7 g /100 mL of fermented media. The nitrogen sources were from urea, ammonium sulfat, *Phaseolus radiatus* (green bean) sprout extract and *Mucuna pruriens* (koro) extract. *Z. mobilis* was taken from Food and Nutrient Microbiology Laboratory of Gadjah Mada University Yogyakarta. The chemicals needed in this research are $MgSO_4.7H_2O$ 0.1% (b/v), anhydrate glucose, amonium molibdat, H_2SO_4, $Na_2H_2SO_4$, Na_2CO_3, KNa, $NaHCO_3$, Na_2SO_4, $CuSO_4.5H_2SO_4$, $MgSO_4$, and NaOH. The cashew extract was made from the blended juice and kept in a freezer (Hermawan *et al*. 2000).

Ethanol fermentation

The fermentation media (100 mL) consisted of cashew apple extract (red, yellow or green variety) with some amount of sugar about 7.44-7.82% (b/v), $MgSO_4.7H_2O$ 0.1% (b/v) and glucose 7 g. The sources of nitrogen were urea 0.2 g/L, ammonium sulfat 0.443 g/L, *Phaseolus radiatus* sprout extract 7.73 mL/L and *Mucuna pruriens* extract 9.25 mL/L. NaOH was added to keep the pH around 6. The fermentation media was sterilized at 121°C for 10 minutes. 6.10^8 cell/mL of *Z. mobilis* were used in this research.

The fermentation media consisted of cashew apple extract and different source of nitrogen was fermented for 24, 48 and 72 hours. The pH, consumption of sugar (Nelson-Somogyi) (Nelson 1944; Somogyi 1952), the amount of bacterium (Standard Plate Count) and ethanol production (Conway microdiffusion) (Conway and O'Malley 1942) were analysed during the fermentation process.

RESULT AND DISCUSSION

Ph level

The pH level determines the process of fermentation due to the characteristic of the enzyme that only works in certain pH interval. This research showed that pH level decreased along the fermentation. The pH was set at 6 at the beginning of this research, as Worden *et al.* (1983) said that *Z. mobilis* grows well at 5.6-7.5 of pH. The changes of pH at Table 1 below showed the decrease of pH in each treatment. The decrease of pH was caused by contamination of other bacteria especially lactose acid bacteria. Although fermentation media has been sterilized but this contamination can still posibly happen (Rahayu dan Rahayu 1988). The research showed insignificant decrease of pH; 0.19 (1.7%). It indicates that the acid level formed was not significant. Theoretically, the forming of ethanol by *Z. mobilis* will not produce another element in which 1 mol of glucose produce 2 mol of ethanol and 2 mol CO_2.

Table 1 showed that the pH from urea and ammonium sulphate was lower compared to pH from *P. radiatus* sprout extract and extract of *M. pruriens.* It is because the nitrogen source of ammonium (urea dan amonium sulfat) was produced NH_4^+ in the medium that then get into the cell as $R\text{-}NH_3^+$ so that H^+ remains in the media and reduce the pH. While in another nitrogen source namely nitrate or protein, the H^+ is taken from the media to form $R\text{-}NH_3^+$ so that the pH increase (Wang et al. 1979; Standbury and Whitaker 1984). The acid as the product of the fermentation will increase the pH in the medium with urea and ammonium sulphate compared to those in media with

Table 1. The data of pH changing in cashew fruit fermentation into ethanol by *Zymomonas mobilis*

Cashew variety	Nitrogen source				Fermentation time (hours)
	Urea	Amonium	Green bean sprout	Koro	
Red	5.90	5.88	5.95	5.94	24
	5.87	5.84	5.91	5.89	48
	5.84	5.80	5.87	5.86	72
Yellow	5.91	5.87	5.93	5.91	24
	5.88	5.85	5.89	5.88	48
	5.83	5.82	5.84	5.82	72
Green	5.89	5.87	5.92	5.90	24
	5.86	5.85	5.86	5.86	48
	5.83	5.81	5.81	5.84	72

Table 2. The data of sugar level (g/100 mL) changing in cashew fruit fermentation into ethanol by *Zymomonas mobilis*

Cashew variety	Nitrogen source				Fermentation time (hours)
	Urea	Amonium	Green bean sprout	Koro	
Red	9.29	8.33	9.50	9.42	24
	8.77	6.73	8.73	8.62	48
	8.11	5.39	8.41	8.31	72
Yellow	8.85	7.87	9.32	9.04	24
	8.17	6.61	9.07	8.90	48
	7.50	5.93	8.87	8.77	72
Green	8.61	7.64	9.61	9.06	24
	8.07	6.92	9.07	8.82	48
	7.10	5.17	8.93	8.81	72

extract of *P. radiatus* sprout and *M. pruriens* extract .

The statistic test with Tukey's method showed that the differences in the variety of cashew apples, nitrogen sources, and the duration of the fermentation influenced the pH of the media

The sugar level

The glucose from cashew fruit extract and the added sugar (7 g) were the sources of carbon for *Z. mobilis* that was then changed into ethanol and CO2. The amount of sugar before the fermentation was 7.44 g/100 mL for the red, 7.26 g/100 mL for the yellow and 7.82 g/100 mL for the green variety of cashew apple. Because of the lack of the sugar (under 10 g/100 mL) in cashew apple, 7 g of glucose was added to each treatment. Kirsop and Hilton (1981) suggested to get more economical fermentation by giving at least 10% of sugar fermentation media. The use of sugar by *Z. mobilis* was indicated in Table 2. The Table shows that there was a use of glucose as a carbon source. Up to the third day, hour 72, the highest use of sugar by *Z. mobilis* was in the treatment with ammonium sulphate. The high use was 65.12% for green variety, 62.67% for red and 58.42% for yellow.

The use of sugar was better compared to the similar research (The ethanol fermentation with cashew extract by *Z. mobilis*) by Sapariantin (2005) that only produced 33.83%, but compared to other research, the use of this sugar was low. Hermawan *et al.* (2000) reported of the use of sugar 80-90% in a research of ethanol fermentation with cashew fruit extract by *Saccharomyces cerevisiae.* Another research with only glucose media was reported to use 98.6% of *Z. mobilis* (Nowak 2000). The low usage of reduced sugar by *Z. mobilis* was because of the usage of standard bacteria (as used by Sapariantin (2005), while Nowak (2000) used modified *Z. mobilisi* namely strain 3881 dan 3883. It was also a possibility of a contamination by other bacteria consuming the glucose.

Table 2 shows that the use of glucose by *Z. mobilis* was optimum by using nitrogen of ammonium sulphate, 65.12% for green variety. Torres and Barrati (1988) stated that the best nitrogen source for *Z. mobilisi* is khamir extract, ammonium sulphate and the mixture of them. It produces different amount for those using *Saccharomyces cerevisiae* for ethanol fermentation. For this microbia, the best nitrogen source is urea compared to ammonium sulphate (Hermawan *et al.* 2000). Table 2 also shows that the highest decrease of the sugar occurred in 0-24 hours, while after 24 hours the decrease was not high. Doelle (1990) stated that the optimum time of ethanol fermentation for *Z. mobilis* occurs in 24 to 34 hours.

The nitrogen source of extract *P. radiatus* sprout and extract of *M. pruriens* contain carbohydrate (4.1 g and 55 g respectively out of 100 g) meaning that both contribute sugar as carbon source for the bacteria. The big amount of the leftover of sugar from the media with extract of *P. radiatus* sprout and extract of *M. pruriens* could be caused by the bigger amount of the sugar in them compared to other media with different sources of nitrogen.

The statistic test of sugar changing with Tukey's method shows that the differences of the cashew variety, nitrogen sources, and the duration of the fermentation impact the sugar level to the result of the fermentation

The amount of bacteria

The amount of bacteria gives huge impact of the success of a fermentation. The more amount of bacteria, the better fermentation will get. The amount of bacteria is also to cover up with the time needed fot the adaptation toward the new media.

This research used *Z. mobilis* FNCC 056 with 6.10^8 cell/mL for each treatment injected to 100 mL media to get 6.10^6 cell/mL bacteria. The changing of the amount of bacteria is shown in Table 3. Table 3 shows that the use of ammonium sulphate influenced the growth of the bacteria. The average amount of the bacteria with ammonium sulphate as the source of nitrogen showed the highest compared to other sources.

Table 3. The data of the amount of bacteria (x 10^7) changing in cashew fruit fermentation into ethanol by *Zymomonas mobilis*

Cashew variety	Nitrogen source				Fermentation time (hours)
	Urea	Amonium	*P. radiatus* sprout	*M. pruriens*	
Red	6.60	7.30	6.40	6.50	24
	26.7	34.7	26.7	27.3	48
	46.0	69.3	44.3	45.0	72
Yellow	6.80	7.70	6.50	6.70	24
	28.7	35.3	25.7	26.3	48
	49.7	63.0	42.3	42.7	72
Green	7.10	8.00	6.30	6.70	24
	29.0	33.7	25.7	26.3	48
	52.7	72.3	42.0	42.3	72

Table 4. The growth of *Zymomonas mobilis* on media for each variety of cashew

Cashew variety	Nitrogen source				Fermentation time (hours)
	Urea	Amonium	*P. radiatus* sprout	*M. pruriens*	
Red	0.146	0.150	0.144	0.145	24
	0.102	0.107	0.102	0.102	48
	0.076	0.081	0.075	0.075	72
Yellow	0.147	0.152	0.145	0.146	24
	0.103	0.108	0.101	0.102	48
	0.077	0.080	0.074	0.075	72
Green	0.149	0.154	0.144	0.146	24
	0.104	0.107	0.101	0.102	48
	0.077	0.082	0.074	0.074	72

This research showed that the growth rate of specific μ indicating the speed of changing of a microorganism growth. The bigger μ value, the faster a microorganism grows. Counted every 24 hours from hour 0 to 72 the μ value was got as it is shown in Table 4. The highest growth of bacteria was reached in the fermentation with amonium sulphate as the nitrogen source and cashew of green variety. It showed μ of 0.082/hour, and then red variety with 0.081 and yellow with 0.08 with the same nitrogen source. The Table also showed that eventhough there was always increase of the amount of bacteria at every hour but it also showed the decrease of μ meaning that eventhough there was an increase of bacteria but there was also a decrease of the growth. This decrease was caused by the limited media so that in certain step the bacteria were competing in gaining nutrition causing the decrease of the bacterias growth.

The μ in this research was higher than that in the similar research by Sapariantin (2005). In her research, the μ is 0.062 after hour 72. It is also higher than that of Gunasekaran and Raj (2002), namely 0.03 after hour 12. That was in fermentation of 12 hours that may change in the next period. The statistic test shows that there was a significant impact causing by different variety, nitrogen sources, and the duration of the fermentation toward the amount of bacteria.

Ethanol level

The different nitrogen sources gave different impact toward the ethanol produced by the fermentation of cashew fruit extract with *Z. mobilis*. This research showed that the highest ethanol level gained in fermentation with ammonium sulphate as the nitrogen source and green variety namely 46.31 g/L and then red 43.24 g/L, yellow 38.57 g/L with the same nitrogen source. The result can be seen in Table 5.

The other nitrogen source producing high ethanol level is urea, and then *M. pruriens* extract and green bean sprout extract. It is similar with what is stated by Torres and Barrati (1990) that the best nitrogen source for *Z. mobilis* is khamir extract, amonium sulfat or the mixture of them.

The green variety showed a good performance in gaining ethanol in this research. It was caused by the usage of sugar of the bacteria. It was shown by the sweetness of the green variety compared with other varieties shown in the reduced sugar level (5.1% higher than the red and 7.7% higher than the yellow)

Theoretically, the ethanol produced by *Z. mobilis* is 0.51 g for each gram of glucosa given (Gunasekaran and Raj 2000). Therefore, if it uses 9.65 g/100 mL (Table 1), for the green with ammonium sulphate as the nitrogen source, it will produce 4.922 g/100 mL or 49.22 g/L ethanol. If it produces the highest, 46.31 g/L, it will gain yield ethanol 94.09%. It can be seen in Table 5. Another research showed ethanol level to 98% (Gunasekaran and Raj 2000), while Nowak (2000) reported that fermentation by *Z. mobilis* with *batch fermentation* got 96% and 94,5% of yield ethanol with *continuous fermentation*.

Table 5 shows that during hour 0 to 24 the increase of ethanol level was higher than between hour 24 to 48 or 72. As it is stated by Doelle (1990) that the time needed to produce ethanol by *Z. mobilis* is between 24 and 34 hours to gain optimum production. Therefore. it is advisable to conduct only 24 hours to produce ethanol from cashew fruit extract. The statistic test with Tukey showed that the ethanol from green variety was much different with other variety. while those of red and yellow variety were not significantly different. The usage of nitrogen source of ammonium sulphate gave significant impact to the ethanol production than those of other sources. While the production of ethanol by green bean sprout extract is not much different with those by *M. pruriens* extract. The duration of the fermentation also gave significant impact to the production. This research showed that though the production of ethanol with natural resources (*Phaseolus radiatus* sprout and *Mucuna pruriens*) was not as high as those with urea and ammonium sulphate but they are potential to be used for its easy sources and the less residu and eco friendliness.

CONCLUSION

The green variety of cashew apple produced the highest ethanol than those of red and yellow variety. It also gave optimum production with ammonium sulphate in 24 hours. This treatment gave 33.02 g/L of ethanol or in other words gave 90.19% of ethanol's yield.

Table 5. The data of ethanol level (g/L) and yield ethanol (%) changing in cashew fruit fermentation into ethanol by *Z. mobilis.*

Cashew variety	Nitrogen source and yield ethanol (Ye)								Fermentation time (hour)
	Urea	Ye	Amonium	Ye	*P. radiatus* sprout	Ye	*M. pruriens*	Ye	
Red	23.05	87.77	28.11	90.27	21.64	85.97	22.31	87.11	24
	25.78	89.13	36.50	92.73	25.50	87.62	26.15	88.07	48
	29.03	89.86	43.24	93.70	26.83	87.21	27.32	87.40	72
Yellow	24.16	87.51	29.66	91.04	21.53	85.47	22.98	86.28	24
	27.92	89.85	35.74	91.63	22.95	86.49	23.86	87.22	48
	30.56	88.60	38.57	90.77	23.56	85.66	25.05	89.49	72
Green	27.70	87.42	33.02	90.19	22.90	86.21	25.45	86.71	24
	31.03	90.11	37.70	93.62	26.00	88.69	27.26	89.09	48
	35.44	90.04	46.31	94.09	27.12	90.21	27.70	90.42	72

REFERENCES

Conway EJ, O'Malley E. 1942. Microdiffusion methods: ammonia and urea using buffered absorbents (revised methods for ranges greater than 10μg. N). Biochem J 36 (7-9): 655-661.

Darsono. 2004. Cashew Economy. Andi Offset. Yogyakarta. [Indonesia]

Doelle HW. 1990. *Zymomonas* ethanol process laboratory to commercial evaluation. In: Fermentation technology industrial aplication. Elsevier. New York.

Gunasekaran P, Raj CK. 2002. Ethanol fermentation technology-*Zymomonas mobilis*. Curr Sci 77 (1): 56-58

Hermawan DRWA, Utami T, Cahyanto MN. 2000. Ethanol fermentation from cashew pseudo-fruit (*Anacardium occidentale* L.) by *S. cereviseae* FNCC 3015 using ammonium sulfate and urea as nitrogen source. Agritech 20 (2): 93-98. [Indonesia]

Hobley TJ, Pamment NB. 1994. Differences in response of *Zymomonas mobilis* and *Saccharomyces cereviseae* to change in extracellular ethanol concentration. Biotech Bioeng 43 (2): 155-158.

Karuppaiya M, Sasikumar E, Viruthagiri T, Vijayagopal V. 2009. Optimization of process parameters using Response Surface methodology (RSM) for ethanol production from waste cashew apple juice using *Zymomonas mobilis.* Chem Eng Comm 196: 1-11.

Kirsop BH, Hilton MG. 1981. Research into utilization of food industry wastes. In Food industry wastes: disposal and recovery. Applied Science Publishers. London.

Neelakandan and Usharani. 2009. Optimization and production of bioethanol from cashew apple juice using immobilized yeast cells by *Saccharomyces cerevisiae.* Am Eur J Sci Res 4 (2): 85-88.

Nelson N. 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J Biol Chem. 153: 375-381.

Nowak J. 2000. Ethanol yield and productivity of *Zymomonas mobilis* in various fermentation methods. Electr J Polish Agric Univ 3 (2): #04. http://www.ejpau.media.pl/volume3/issue2/food/art-04.html

Pacheco AM, Gondim DR, Gonçalves LRB. 2009. Ethanol production by fermentation using immobilized cells of *Saccharomyces cerevisiae* in cashew apple bagasse. Appl Biochem Biotechnol 161 (1-8): 209-217.

Pinheiro ADT, Rocha MVP, Macedo GR, Gonçalves LRB. 2008. Evaluation of cashew apple juice for the production of fuel ethanol. Appl Biochem Biotechnol 148 (1-3): 227-234.

Rahayu ES dan Rahayu K. 1988. Alcoholic Beverage Processing Technology. Faculty of Agricultural Technology, Gadjah Mada University. Yogyakarta. [Indonesia]

Rodrigues THS, Rocha MVP, Macedo GR, Gonçalves LRB. 2009. Ethanol production from cashew apple bagasse: improvement of enzymatic hydrolysis by microwave-assisted alkali pretreatment. The 31st Symposium on Biotechnology for Fuels and Chemicals. Society for Industrial Microbiology. San Fransisco, May 3-6, 2009.

Sapariantin E. 2005. Ethanol fermentation of juice pseudo-fruit cashew (*Anacardium occidentale* L.) by *Zymomonas mobilis* by the addition of urea. [Thesis S1]. Faculty of Mathematics and Natural Sciences, Sebelas Maret University. Surakarta. [Indonesia]

Somogyi M. 1952. Notes on sugar determination. J Biol Chem 195: 19-23.

Thomas ANS. 1989. Traditional medicinal plants. Kanisius. Yogyakarta. [Indonesia]

Torres EF, Barrati J. 1988. Ethanol production from wheat flour by *Zymomonas mobilis*. J Ferment Tech 66 (2): 167-172.

Van Eijnatten CLM. 1991. *Anacardium occidentale* L. In: Verheij EWM, Coronel RE (eds). Plant Resources of South-East Asia, No. 2: Edible Fruits and Nuts. Pudoc/Prosea. Wageningen.

Wang DIC, Cooney CL, Demain AL, Dunnill P, Humprey AE, Lilly MD. 1979. Fermentation and enzyme technology. John Wiley and Sons. New York.

Wijono D. 1988. Evaluation of the kinetics process of ethanol fermentation by *Zymomonas mobilis* ZM 4. Inter-University Center for Food and Nutrition, Gadjah Mada University. Yogyakarta. [Indonesia]

Worden RM, Donaldson TL, Shumate SE, Stranberg GW. 1983. Kinetic study of ethanol productionby *Zymomonas mobilis.* http:// www.osti.gov/energycitations/product.biblio.isp?osti_id=538194