SELECTION AND IMMOBILIZATION OF ISOLATED ACETIC ACID BACTERIA ON THE EFFICIENCY OF PRODUCING ACID IN INDONESIA

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ABSTRACTS

Screening of AAB(Acetic acid bacteria) isolates from cane sugar juice in Indonesia for acid and alcohol tolerance was performed. Among the isolates tested, strain INT-7 was observed to grow to as high as 9% acetic acid and 15% alcohol. Based on the screening study, INT-7 is belong to *Acetobacter pasteurianus* were chosen for vinegar fermentation. The pure culture of strain INT-7 on unpasteurized coconut sap was found to have high acidity (5.42%) compared to pasteurized sap (3.42%). Strain INT-7 exhibited higher acid production of 62.7g/L in 5% ethanol under static condition compared to reference strains JCM 7640 *Acetobacter aceti* and JCM 7641 *A. pasteurianus* with 52.3g/L and 30.8g/L acid, respectively. On other study, results revealed the efficiency (2X) of using fermentor for acid formation compared to samples fermented with shaking. In addition, the crude metabolites of INT-7 suggest inhibitory effect against the growth of *E.coli* and *Salmonella* sp. but not against *S. aureus*, while strain of INT-17 metabolites inhibited the growth of all pathogenic bacteria tested. The immobilization of *A.pasteurianus* strain INT-7 for improving the ethanol resistance was done in the alginate gel. The result showed that optimum condition of *A. pasteurianus* INT-7 cell entrapped (10^7CFU/mL) on 3% alginate and the ratio of cell number and alginate solution was 1:3 v/v. The optimum condition of acetic acid fermentation by immobilized cells were initial pH 6.0, ethanol concentration 7.5% (v/v), temperature at 30^o C for 7 days produced acetic acid (35.81 g/L) is higher than free cells (16.29 g/L). The efficiency of fermentation by immobilized cells and free cells were 36.73% and 16.71%, respectively.

Keywords: Acetic acid bacteria, selection, immobilization, producing higher acid.

INTRODUCTION

Vinegar is worldwide known condiment because of its significant roles in the food industries and medical purposes. It is used as a preservative in the processing of fruits, fish, meat and poultry products, as flavor enhancer and a requisite in many food preparations. Traditional fermented foods are known to be potential source of microorganisms for food fermentation and biological researches. Thus manufacture of traditional foods will rarely be produced and eventually results in the lost of substrate for isolation of potential microorganisms. To

improve the vinegar industry therefore the selection and evaluation on the efficiency of isolated acetic acid bacteria should be done. Strains identified as *Acetobacter pasteurianus* are dominant organisms in the traditional vinegar and mostly have ethanol resistance (Entani and Masai, 1984).

Immobilized of cells could increasing the ethanol resistancy. Accordingly, in this present study the immobilization of *A. pasteurianus* INT-7 on alginate gel was done.

MATERIALS AND METHODS

Screening of selected strains of acetic acid bacteria (AAB) for acid and alcohol tolerance.

Twenty seven (27) strains of AAB isolated from Indonesia's vinegar were tested for their tolerance to difference levels of acetic acid and ethanol. The cell suspensions were inoculated in AE broth containing 1-10% acetic acid at specified intervals and 5.0 to 20% ethanol and GYP agar medium in the same level of acid and ethanol. The tubes were incubated at 30^{o} C for 4-5 days and the results were recorded based on turbidity of the broth.

Effect of fermentation conditions on the acetic acid formation of selected isolates.

Among the AAB isolates tested, INT-7 (*A. pasteurianus*) was found to be the most resistant strain to high acid and alcohol concentrations. Thus, it was used for further studies to test efficiency on acetic acid formation with varying conditions during fermentation, namely medium containing different ethanol concentrations at static condition, medium containing 10% ethanol and incubated with shaking, and medium containing 10% ethanol using fermentor.

Strain INT-7 was grown on YPE broth as described by Entani et al (1985) for 7 days. Then, the same culture medium was prepared and distributed in E. flasks (duplicate) at 300 ml per flask and in 2-L cap. fermentor. The acid formation of INT-7 was compared with reference strains of *Acetobacter* sp.

Inhibitory effect of AAB against some spoilage microorganisms.

The selected isolates were cultured in YPE broth at 30^{o} C for 7 days. The broth was filtered (0.45um) to separate microbial cells and adjusted to neutral pH. These metabolite extracts were used for inhibitory the growth of spoilage microorganisms especially *E. coli, S. aureus,* and *Salmonella* sp.

Immobilization method of AAB strain INT-7 on alginate gel.

The ratio of cells number and alginate solution (1:2; 1:2.5; and 1:3 v/v), and alginate concentration are 2%, 3% and 4%, respectively. The initial pH media are 5.5; 6.0; and 6.5, with the variable ethanol concentration on 5%, 7.5% and 10% w/v and the temperature of fermentation are 30^{o} C, 35^{o} C and 40^{o} C and the fermentation period is 1 to 10 days.

RESULT AND DISCUSSION

Selection of alcohol tolerance for each isolates as shown on the table 1.

Table 1. Effect of the different alcohol concentrations on growth of selected isolates

Isolates	Ethanol Concent (%)						
	5.0	7.5	10.0	12.5	15.0	17.5	20.0
INT-7	++	++	++	+	+	-	-
INT-9	+	+	-	-	-	-	-
INT-12	+	+	-	-	-	-	-
INT-14	+	+	-	-	-	-	-
INT-17	++	+	-	-	-	-	-
ISN-2	+	+	+	-	-	-	-
ISN15	++	+	+	-	-	-	-
JCM7640	++	++	++	-	-	-	-
JCM7641	++	+	+	-	-	-	-

Note : ++ : abundant ; + : scanty growth - : no growth

The result showed that among the isolates tested, strain INT-7 was observed to grow as high as 9% acetic acid and 15 % alcohol. Based on these screening studies, INT-7 (*A. pateurianus*) was chosen for vinegar fermentation and Immobilization. Addition of INT-7 on unpasteurized coconut sap was found to have high acidity (5.42%) compared to pasteurized sap (0.42%) as shown on the table 2, respectively.

Table 2. Chemical analysis of coconut sap vinegar produced by using pure cultures of selected isolates

Treatment	Isolate No	pH	Total acidity(%)	Reducing sugar (%)
Coconut sap		5.3	0.28	15.10
1.Pasteurized, Inoculated AAB	INT-7	3.2	0.42	1.63
	INT-12	3.3	0.84	2.84
	INT-17	3.3	0.41	2.72
2. Pasteurized, inoculated *S. cerevisiae* (2days) and AAB	INT-7	3.15	3.65	0.84
	INT-12	3.3	3.07	1.58
	INT-17	3.2	3.35	1.24
3. Not pasteurized, Directly inoculated With AAB	INT-7	3.1	5.42	1.63
	INT-12	3.3	3.82	2.84
	INT-17	3.2	4.02	2.72

Strain INT-7 exhibited higher acid production of 62.7g/L in 5 % ethanol under static condition compared to reference strains JCM 7640 *A. aceti* and *A. pasteurianus* JCM 7641 with 52.3g/L and 30.8g/L acid, respectively. The highest yield of acetic acid was obtained on the 8th day of fermentation for 5.0% and 7.5% ethanol, while 7th day for 10% ethanol (Fig 1 and 2). All treatments exhibited decreased in acidity when fermentation time exceeded 7 to 8 days. When the INT-7 was likewise examined using a fermentor and another batch in flask incubated with shaking the production of acetic acid showed remarkable increased of efficiency about 2x.

Table 3. Inhibitory effect of isolated AAB metabolites on growth of some pathogenic bacteria

Incubation time(day)	Pathogenic bacteria	AAB strain					
		JCM 7640	JCM 7641	INT-7	INT-12	INT- 17	ISN-15
2	*E.coli*	-	-	*2.0 mm*	*1.0 mm*	*1.0 mm*	*1.0 mm*
	S. aureus	-	*0.5 mm*	-	-	-	-
	Salmonella	*0.5 mm*	-	*0.5 mm*	-	*1.0 mm*	*0.5 mm*
4	*E.coli*	-	-	*2.0 mm*	*1.5 mm*	*2.0 mm*	*1.0 mm*
	S. aureus	*1.0 mm*	*0.5 mm*	-	*1.0 mm*	*0.5 mm*	-
	Salmonella	*1.0 mm*	-	*0.5 mm*	-	*1.0 mm*	*0.5 mm*
5	*E.coli*	-	-	*2.5 mm*	*1.5 mm*	*2.0 mm*	*1.0 mm*
	S. aureus	*1.0 mm*	*0.5 mm*	-	*1.0 mm*	*0.5 mm*	-
	Salmonella	*1.0 mm*	-	*1.0 mm*	-	*1.0 mm*	*0.5 mm*
7	*E.coli*	-	-	*2.5 mm*	*1.5 mm*	*2.0 mm*	*1.0 mm*
	S. aureus	*0.5 mm*	*0.5 mm*	-	*1.0 mm*	*0.5 mm*	*1.5 mm*
	Salmonella	*1.0 mm*	-	*1.0 mm*	-	*1.0 mm*	*1.5 mm*

Interesting results were obtained on the inhibitory effect of INT-7 crude metabolites against the growth of *E. coli*, and *Salmonella* but not in *S. aureus*. The other strains also capable of inhibitory pathogenic bacteria. Strain of INT-7 showed very effective inhibited *E. coli.* Aside from lowering the acidity which contribute to the preservative effect of vinegar, this study revealed that other metabolites produced by AAB help in preventing the foods from microbial spoilage. It is therefore importance to identify the compounds of crude metabolites produced by AAB for its possible application in biotechnological research.

The growth of AAB is prevented by the acid produced during the fermentation and alcohol as a substrates. Each of AAB strain have ability to grow in the certain acetic acid and alcohol concentration. Immobilized of cells could increase the ethanol and acetic acid resistance during the fermentation.

The optimum condition of immobilization of cells was observed in the three concentration of alginate and the number of active cell in the alginate for the three days conditioning time. The result as shown in the Table 4. The optimum condition was found on 3% alginate and the ratio of cell number and alginate solution was 1:3 v/v. The number cell entrapped was 10^7CFU/mL and those condition revealed that the gel is not very tough. The most tough of gel was found in the concentration of 3% alginate and the ratio cel/alginate 1:3 v/v. The longer time of conditional the more released cell out from the gel.

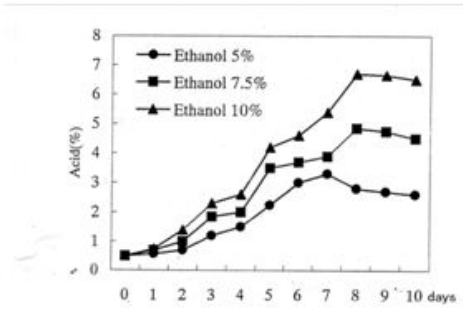

Fig. 1. Acetic acid formation of strain INT-7 in AE broth containing different ethanol concentrations under static condition

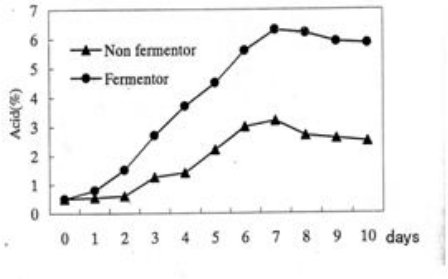

Fig. 2. Acetic acid formation of strain INT-7 in YPE broth containing 10% ethanol broth in flask (with shaking) and fermentor.

Table 4. The effect on variation of concentration of alginate and the number of cell ratio and the alginate during three days conditional time on the number of cell entrapped in the alginate gel

Alg (%)	cell/alg (v/v)	Number of cell during the conditional time (days) In the beads (CFU/g)				released from beads CFU/mL)			Texture of Gel
		0	1	2	3	1	2	3	
2	1 : 3	$1{,}9.10^7$	$2{,}8.10^8$	$4{,}0.10^7$	$2{,}7.10^7$	$1{,}3.10^8$	$2{,}2.10^7$	$5{,}6.10^6$	+++
	1 : 2,5	$1{,}0.10^7$	$3{,}5.10^8$	$9{,}9.10^7$	$2{,}9.10^7$	$1{,}1.10^8$	$1{,}6.10^7$	$3{,}7.10^6$	++
	1 : 2	$1{,}7.10^7$	$2{,}5.10^8$	$9{,}3.10^7$	$2{,}6.10^7$	$1{,}4.10^8$	$2{,}4.10^7$	$9{,}2.10^6$	+
3	1 : 3	$2{,}0.10^7$	**$2{,}9.10^8$**	$3{,}8.10^7$	$3{,}6.10^7$	**$2{,}7.10^8$**	$7{,}2.10^6$	$4{,}2.10^6$	+++
	1 : 2,5	$2{,}2.10^7$	$3{,}8.10^8$	$2{,}8.10^7$	$2{,}3.10^7$	$2{,}3.10^8$	$6{,}0.10^6$	$3{,}9.10^6$	++
	1 : 2	$1{,}0.10^7$	$2{,}3.10^8$	$1{,}1.10^8$	$3{,}6.10^7$	$1{,}5.10^8$	$3{,}5.10^6$	$3{,}6.10^6$	++
4	1 : 3	$1{,}4.10^7$	$3{,}5.10^7$	$9{,}2.10^7$	$4{,}1.10^7$	$8{,}3.10^7$	$3{,}8.10^6$	$8{,}1.10^6$	++
	1 : 2,5	$1{,}8.10^7$	$4{,}4.10^8$	$8{,}6.10^7$	$3{,}6.10^7$	$5{,}7.10^7$	$8{,}4.10^6$	$3{,}9.10^6$	+
	1 : 2	$1{,}9.10^7$	$2{,}5.10^8$	$1{,}0.10^8$	$4{,}6.10^7$	$2{,}7.10^7$	$1{,}7.10^6$	$5{,}5.10^7$	+

Note : +++ : very tough

The production of acetic acid from the immobilized cell and free cell was observed at the condition of initial pH at 6.0, ethanol concentration of 7.5% (v/v), and the temperature at 30^o C, during 7 days fermentation in batch culture, as shown in the Figure 3. In this condition the production of acetic acid 35.81 g/L in the immobilized cell higher than in the free cell (16.29 g/L). The immobilized cell potential of producing acetic acid in the certain condition because not affected by the environmental condition during the fermentation.

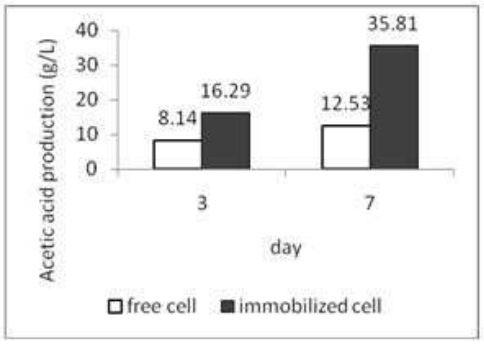

Fig 3. The production of acetic acid (g/L) of free cells and immobilezed cell of *A. pasteurianus* INT-7 in the condition initial pH 6.0, ethanol concentration 7.5% and temperature at 30° C

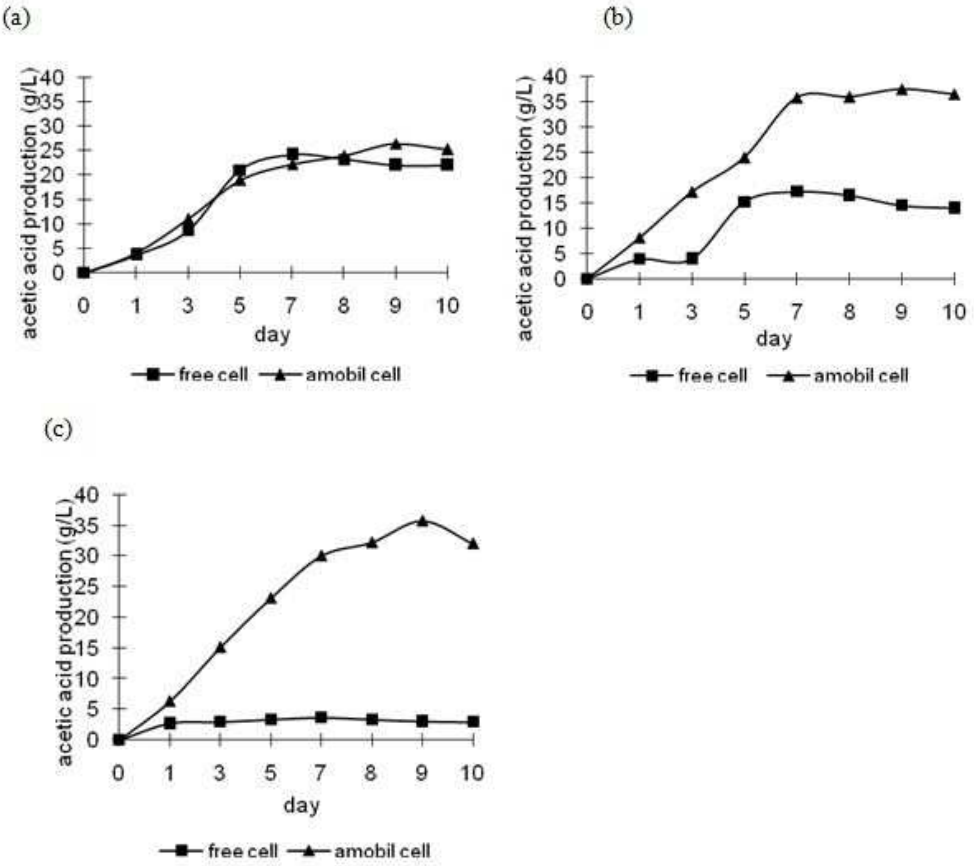

Fig. 4. The production of acetic acid (g/L) in the free cells and immobilized cells of *A.pasteurianus* INT-7 in the (a) 5% ethanol ; (b) 7.5 % ethanol and (c) 10% ethanol

A.pasteurianus INT-7 in the (a) 5% ethanol ; (b) 7.5 % ethanol and (c) 10% ethanol

The efficiency of fermentation by immobilized cells and free cells were 36.73% and 16.71%, respectively. Strain INT-7 showed the highest acid formation in 5% ethanol while lowest was obtained in 10% ethanol. The immobilized cell, therefore, producing higher acetic acid, because the entrapped cell in the alginate could increased the ethanol resistance. The differences between

free cells and immobilized cells was when the fermentation in the higher level of ethanol, free cell showed very low acid production, while the 10% ethanol medium produced higher acid during the fermentation (Fig.4).

CONCLUSSION

- Selected strain is *A. pasteurianus* INT-7 isolated from sugar cane juice has high tolerance in 15% ethanol and produced higher acetic acid compare to the strain of JCM 7640 and JCM 7641, respectively.
- The immobilized cells produced higher acetic acid compare to free cells.

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