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PRODUCTION OF ACETIC ACID FROM ETHANOL SOLUTION BY *Acetobactor acetigenum* AND EFFECT OF GAMMA-RAY IRRADIATION ON THE BACTERIA

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Jenny M. UMAR*, Shinpei MATSUHASHI and Shoji HASHIMOTO

日本原子力研究所
Japan Atomic Energy Research Institute

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Production of Acetic Acid from Ethanol Solution by *Acetobactor acetigenum* and Effect of Gamma-ray Irradiation on the Bacteria

Jenny M. UMAR*, Shinpei MATSUHASHI and Shoji HASHIMOTO

Department of Radiation Research for Environment and Resources
Takasaki Radiation Chemistry Research Establishment
Japan Atomic Energy Research Institute
Watanuki-chi, Takasaki-shi, Gunma-ken

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A preliminary study on fermentation of acetic acid by *S. cerevisiae* and *A. acetigenum* was carried out to obtain information to develop the effective utilization technology of agricultural liquid wastes. Aqueous solutions of glucose and/or ethanol were used as a model of agricultural liquid waste. The effect of gamma-ray irradiation on *A. acetigenum* for enhancement of the fermentation was also examined. In this study, irradiated *A. acetigenum* had activity to produce acetic acid even after loss the activity to grow.

Keywords: Acetic acid, Fermentation, Ethanol, Glucose, *S. cerevisiae*, *A. acetigenum*, Gamma-ray

* Center for Application of Isotopes and Radiation, National Atomic Energy Agency, Indonesia

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Acetobactor acetigenum によるエタノール溶液からの酢酸製造およびガンマ線照射のバクテリアに対する影響

日本原子力研究所高崎研究所環境・資源利用研究部
Jenny M. UMAR*・松橋　信平・橋本　昭司

（1996年2月2日受理）

農林産廃液の効果的な有効利用技術を開発するために必要な知見を得るために，*S. cerevisiae* と *A. acetigenum* を用いた酢酸発酵について，予備的な研究を行った。グルコース，およびエタノール，あるいはそれらの混合水溶液を農林産廃液のモデル溶液として使用した。また，*A. acetigenum* にガンマ線照射を行うことによる発酵の促進効果についても検討を加えた。この研究により，*A. acetigenum* は照射によりその増殖能を失った後でも，酢酸生産能は有することが明らかとなった。

高崎研究所：〒370－12　群馬県高崎市綿貫町1233
＊インドネシア原子力庁

Contents

目　　次

1. Introduction

The fermentation of acetic acid from alcoholic liquid with bacteria has been known from ancient time as long as the production of wine. Even now the acetic acid bacteria is still using for commercial production. The acetic acid bacteria is classified into two genera, *Gluconobacter* and *Acetobactor*. The first group oxidize ethanol to acetic acid. The another group is able to oxidize ethanol to acetic acid, and furthermore oxidize acetic acid to CO_2 and H_2O. Therefore, *Acetobactor* sp. is called peroxidizer. *Acetobactor* sp. shows the characteristics of gram-negative, acid tolerant, and peritrichously flagellated (or non motile). The microorganisms used for commercial acetic acid fermentation are *Acetobactor acetigenum*, *A. pasteurianus*, or *A. peroxidans*.

For the growth of *Acetobactor* sp. both acetic acid and ethanol are required. The ethanol concentration of below 0.2 vol.% gives an increase of death rate. On the other hand, too much supply of ethanol is critical for the bacteria, and the maximal concentration is 5 vol.% in conventional processes [1].

Recently, many trials have been developed for utilization of agricultural liquid wastes as the substrate of fermentation process, such as production of acetic acid from pineapple waste juice [2]. In the process of pineapple waste juice fermentation, yeast and *Acetobactor* sp. are used for alcohol and/or acetic acid fermentation.

On the other hand irradiation may give some influence to the microbe so that there is a possibility to increase the yield of its fermentation products. Irradiation could provide positive influence

if a correct dose is used. Irradiated yeast with a low dose (0.1 kGy) could stimulate microbes to increase its fermentation results [3]. Some efforts have been carried out by irradiated microorganisms to stimulate its enzymes activity [4] and alcohol production [5]. It was shown that irradiated *Lactobacillus plantarum* could increase acid production. The highest acid content was obtained at an irradiation dose of 0.5 kGy [6]. It was also reported that irradiated fungi showed a decrease of the colony formation, but enzyme fermentation activities were increased.

In this study, we examined the optimum fermentation condition and effect of gamma-irradiation on *A. acetigenum* for enhancement of acetic acid fermentation from model liquids of agricultural wastes.

2. Materials and Methods

2.1 Preparation of seed microorganisms

Saccharomyces cerevisiae was used for the production of alcohol from glucose. *S. cerevisiae* was inoculated on Potato-Dextrose (PD) agar slant medium and incubated for 48 hr. at 30°C. Ten ml of sterilized water was added to the incubated *S. cerevisiae* on the slant agar, mixed and used as seed suspension.

Acetobactor acetigenum was used for the production of acetic acid from ethanol. *A. acetigenum* was inoculated on Glucose-Peptone-Yeast extract (GPY) agar slant medium and incubated for 72 hr. at 30°C. The GPY agar medium was consisted of 3 wt.% of glucose, 0.3 wt.% of peptone, 0.5 wt.% of yeast extract and 2.0 wt.% of agar.

The first culture of *A. acetigenum* from slant agar was inoculated into GPY broth and incubated 72 hr. in the rotary shaker (100 rpm) at 30°C as second culture to increase total number of bacteria. The cells were harvested by centrifugation at 10,000 rpm for 10 min., then washed three times with sterilized distilled water, suspended into 70 ml of sterilized distilled water, and used as seed suspension.

2.2 Gamma-ray irradiation of *A. acetigenum*

A. acetigenum suspension (70 ml) was irradiated with Cobalt-60 gamma-ray at dose rate of 0.6 kGy/hr with and without aeration.

2.3 Fermentation

The model medium of 100 ml in flasks with porous silicon cap were used for the double stage and simultaneous fermentation with *S. cerevisiae* and *A. acetigenum*. The medium contained 5 wt.% of glucose, 0.3 wt.% of peptone and 0.5 wt.% of yeast extract.

The model medium used for the fermentation of acetic acid from ethanol with *A. acetigenum* contained 0.3 wt.% of peptone, 0.5 wt.% of yeast extract, and 5 vol.% of ethanol. The concentration of glucose was changed from 0 to 3%.

The fermentation was done at 30°C in the rotary shaker (0 or 100 rpm).

2.4 Enumeration of microorganisms

Four ml of the fermented liquids were sucked from the flask with sterilized syringe, put into a sterilized small bottles. After serial ten-fold dilution, 0.1 ml of the samples were spread on GPY agar

plates and incubated at 30°C for 3 days, then colonies of *A. acetigenum* and *S. cerevisiae* were counted.

2.5 Analysis of components in fermented liquid

Concentrations of acetic acid, glucose and ethanol in the fermented medium were measured using High Performance Liquid Chromatography (HPLC). The condition of HPLC was as follows; column: Shodex SUGAR SH1821, ϕ 8 x 300 mm, pre-column: Shodex SUGAR SH1011P, ϕ 6 x 50 mm, mobile phase: 0.001 N sulfuric acid, column temperature: 30°C, flow rate: 1.0 ml/min, chromatograph: IRICA Auto-sampler 01, detector: JASCO 830-RI Intelligent RI, attenuation: 1.6×10^{-5} RIU, recorder: SIC Chromatocoder 12. Sample liquid was filtered to remove the cells by Millipore Molcut UFP1 LGC, and 0.5 ml of filtrate was mixed with same volume of 4% propionic acid as an internal standard. The concentration of glucose, ethanol and acetic acid in samples were calculated from the ratio of the peaks to that of propionic acid.

3. Results and Discussion

3.1 Double stage fermentation by *S. cerevisiae* and *A. acetigenum*

Table 1 shows the retention times and peak area ratios obtained from chromatograms of standard liquid containing 1 vol.% of ethanol, acetic acid, propionic acid and 1 wt.% of glucose.

Table 1 Retention times and peak area ratios of standard substances

Peak No.	Component	Retention time (min)	Peak area ratio
1	Glucose	8.8	0.86
2	Acetic acid	11.6	0.38
3	Propionic acid	13.1	1.00
4	Ethanol	14.3	0.25

Figure 1 shows an example of chromatogram of the fermented substrate containing 1 wt.% of glucose after 6 days incubation by *A. acetigenum*. The initial count of the bacteria was 1.6×10^{7} cfu/ml. Four large peaks were observed in the chromatogram and identified to be glucose (1), acetic acid (2), propionic acid (3) and ethanol (4) from the retention times. Few unidentified small peaks with short retention time were also observed.

Figure 2 shows the amount of components and bacterial count during double-stage fermentation by *S. cerevisiae* and *A. acetigenum* without shaking. The first stage was fermented by *S. cerevisiae* and

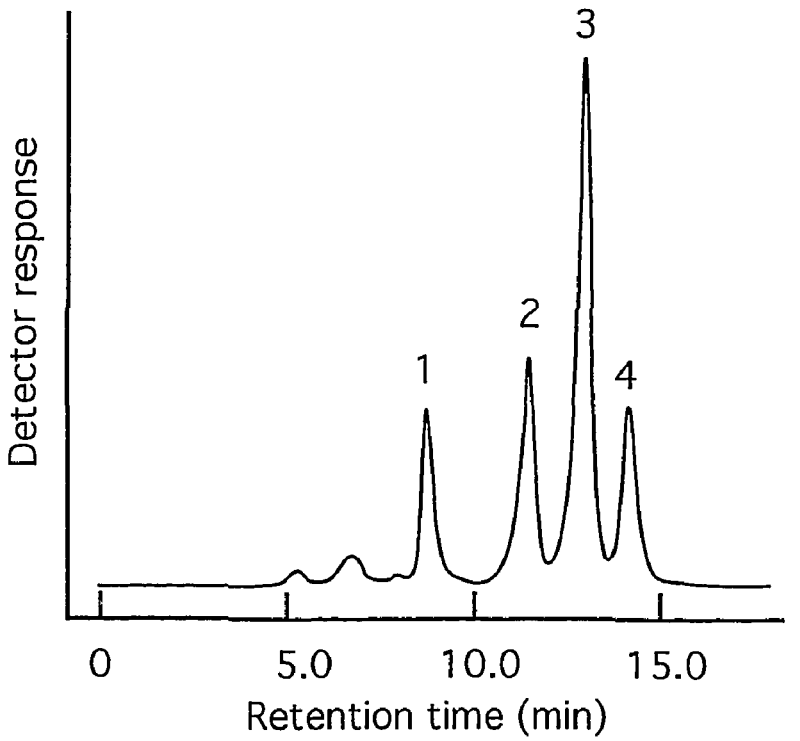

Fig. 1 HPLC chromatogram of fermented products by *A. acetigenum*

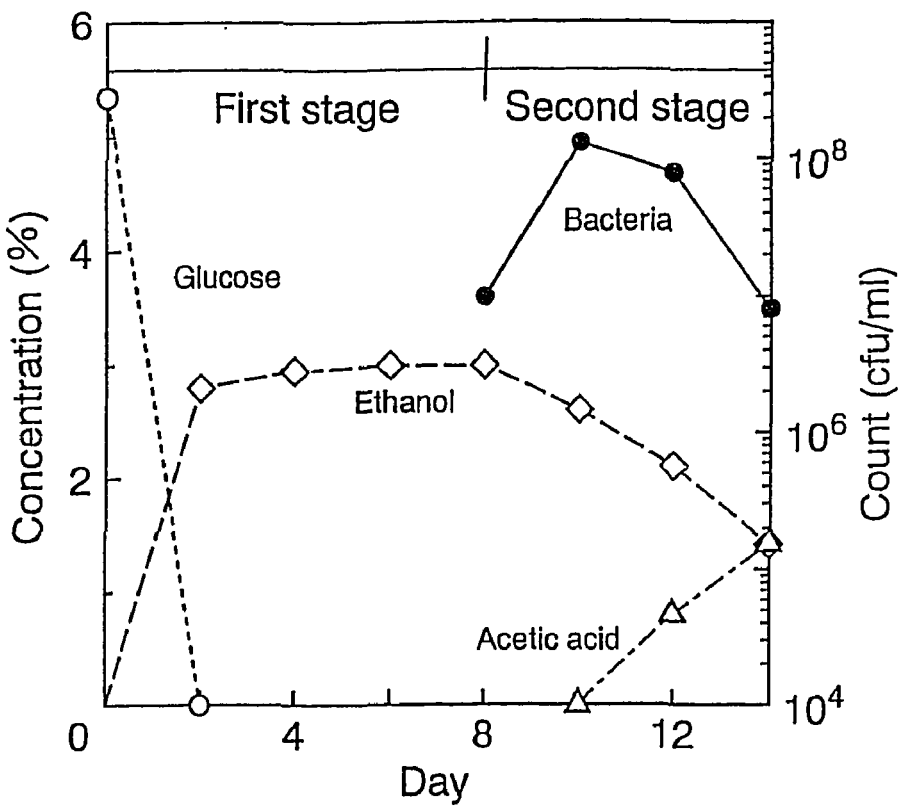

Fig. 2 The amount of component and bacterial count during *double-stage fiermentation without shaking incubation*

the second stage was started from 8th day by addition of *A. acetigenum*. Initial counts of *S. cerevisiae* and *A. acetigenum* were adjusted to be 4.5×10^5 and 1.0×10^7 cfu/ml, respectively. It was difficult to count the population of *S. cerevisiae* during the fermentation, because the yeast precipitated rapidly and could not get homogeneous suspension in the fermented liquid without shaking. At the first stage, concentration of glucose was decreased to zero after 2 days fermentation. While the ethanol concentration was increased from zero to 2.8% during 2 days fermentation, and attained to 3.0% after 8 days incubation. Glucose was completely converted to ethanol within 2 days fermentation by *S. cerevisiae*. At the second stage fermentation, ethanol was consumed and changed into acetic acid by *A. acetigenum*. The concentration of acetic acid did not increase 2 days incubation after the second stage fermentation, but increased to 1.4% at 6th day. The bacterial count of *A. acetigenum* increased from zero to 1.3×10^8 cfu/ml after 2 days incubation, while decreased to 8.0×10^6 cfu/ml after 6 days.

It is known theoretically that 1 mol (180 g) of glucose is converted to 2 mol (92 g) of ethanol and 2 mol (88 g) of carbon dioxide by fermentation (formula (1)).

$$C_6H_{12}O_6 \rightarrow 2\,C_2H_5OH + 2\,CO_2 \qquad (1)$$

Since 5 g of glucose was dissolved into 100 ml of the liquid in this study, produced amount of ethanol by fermentation should be 2.6 g (it means 3.3 vol.% in the liquid). From the result, maximum concentration of ethanol was obtained as 3.1%. Therefore, the efficiency of conversion in this fermentation was calculated as 94%, and this result was very reasonable.

Production of acetic acid from ethanol by fermentation was shown in formula (2). One mol (60 g) of acetic acid and 1 mol (18 g) of water should be produced from 1 mol (46 g) of ethanol and 1 mol (32 g) of oxygen .

$$C_2H_5OH + O_2 \rightarrow CH_3COOH + H_2O \qquad (2)$$

The reduction of ethanol concentration after the second stage fermentation was 1.6%. It was equal to 1.3 g in 100 ml of liquid. So, the calculated value of acetic acid was 1.7 g, and this was equal to 1.6%. The concentration of acetic acid was 1.5% and the conversion rate was calculated to be 94%. This result was also reasonable. In the case of shaking incubation, production of ethanol and acetic acid was smaller than no shaking incubation.

3.2 Simultaneous fermentation of glucose by *S. cerevisiae* and *A. acetigenum*

Figure 3 shows the amount of components and the cell number of *A. acetigenum* during the simultaneous fermentation by *S. cerevisiae* and *A. acetigenum* without shaking. The initial counts of *S. cerevisiae* and *A. acetigenum* were 2.9×10^5 and 3.0×10^6, respectively. The glucose concentration was decreased from 5.5% to 3.3% after 2 days incubation and kept same value until 6 days period. The ethanol concentration was increased up to 0.5 % after 2 days fermentation and then decreased to undetectable level after 4 days. Acetic acid was able to detect after 4 days and 0.9% of acetic acid was obtained by the fermentation. The cells number of *A. acetigenum* increased to 2.9×10^7 cfu/ml after 2 days and decreased to 2.5×10^6 cfu/ml after 6 days incubation. Ethanol produced by *S. cerevisiae*

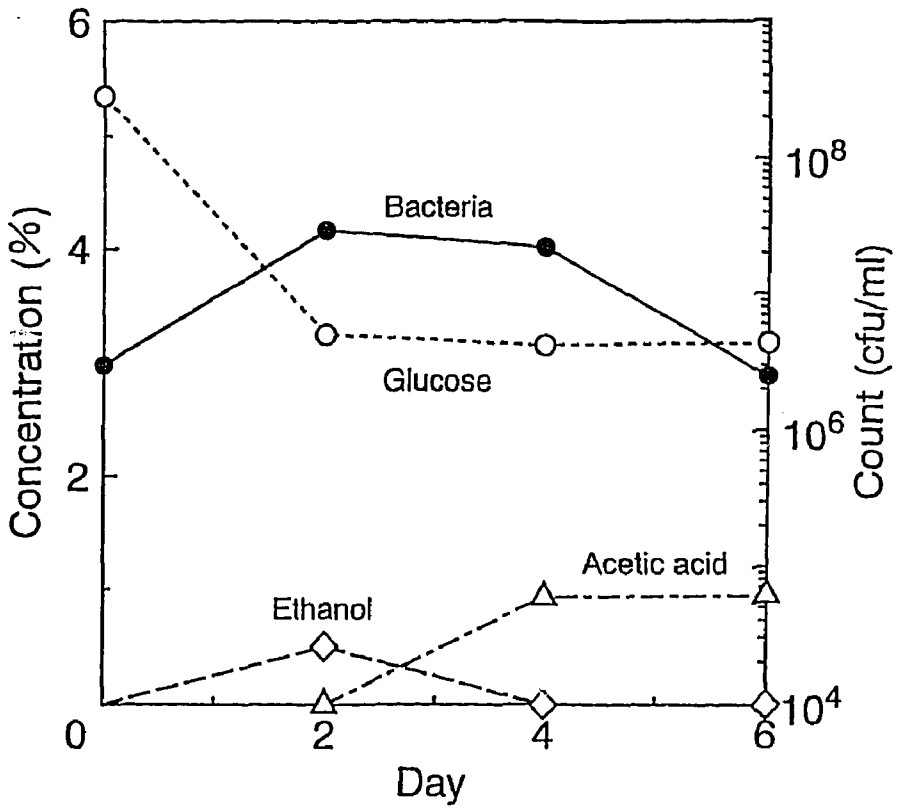

Fig. 3 The amount of component and bacterial counts during simultaneous fermentation

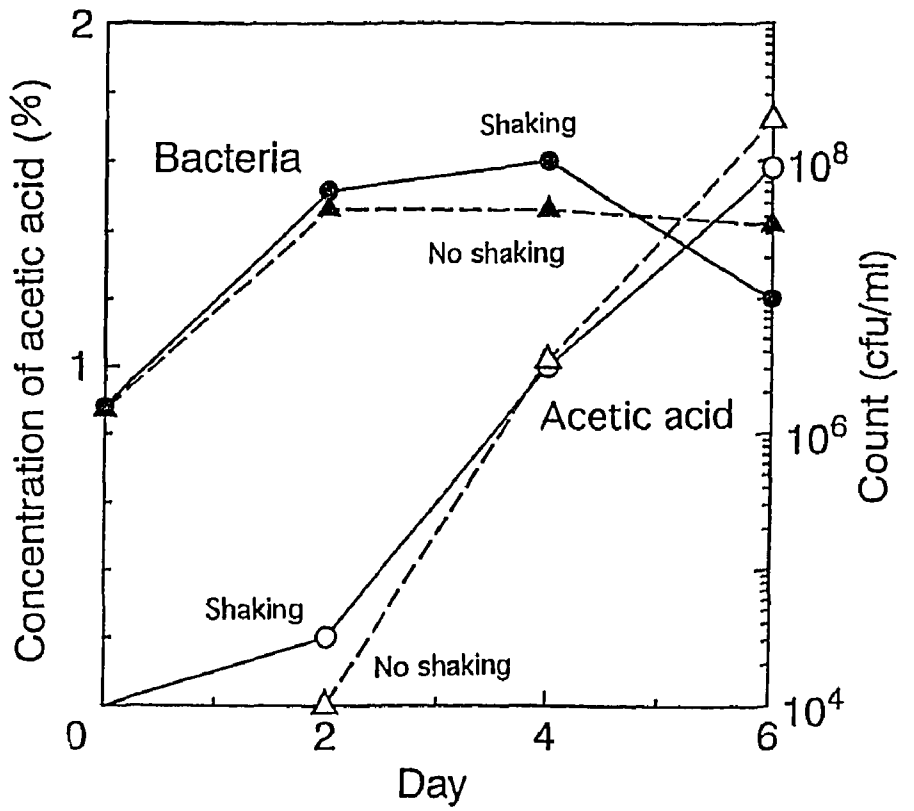

Fig. 4 Production of acetic acid with and without shaking incubation

was converted to acetic acid by *A. acetigenum*. The acetic acid production from glucose by mixture of *S. cerevisiae* and *A. acetigenum* (Simultaneous fermentation) was smaller than that of the double stage fermentation because of decrease of pH during the fermentation. According to the formula (2), 0.5% of ethanol is converted 0.65% of acetic acid. However, the amount of acetic acid in this result was 1.4 times of the theoretical value. This may be from experimental error.

3.3 Fermentation of ethanol by *A. acetigenum*

The effect of acetic acid production by *A. acetigenum* was examined. For this purpose, the liquid containing 5 % of ethanol was used.

Fig. 4 shows the production of acetic acid with and without shaking. With shaking, the concentration of acetic acid did not increase so much after 2 days but the marked increase of the concentration was observed after 4 days incubation. However, almost the same result was obtained on acetic acid production and growth of the bacteria in both cases with and without shaking. Fig. 5 and 6 show effect of initial count of A. acetigenum and addition of glucose on the increase of bacterial counts and production of acetic acid. Initial counts of the bacteria were adjusted by dilution of the original bacterial suspension with sterilized water and the values were ranged from 10^2 to 10^7 cfu/ml. In the case of low initial bacterial counts (Fig. 5), the bacterial counts increased very rapidly, especially with addition of glucose and was saturated after 4 days incubation. The production of acetic acid was observed clearly after

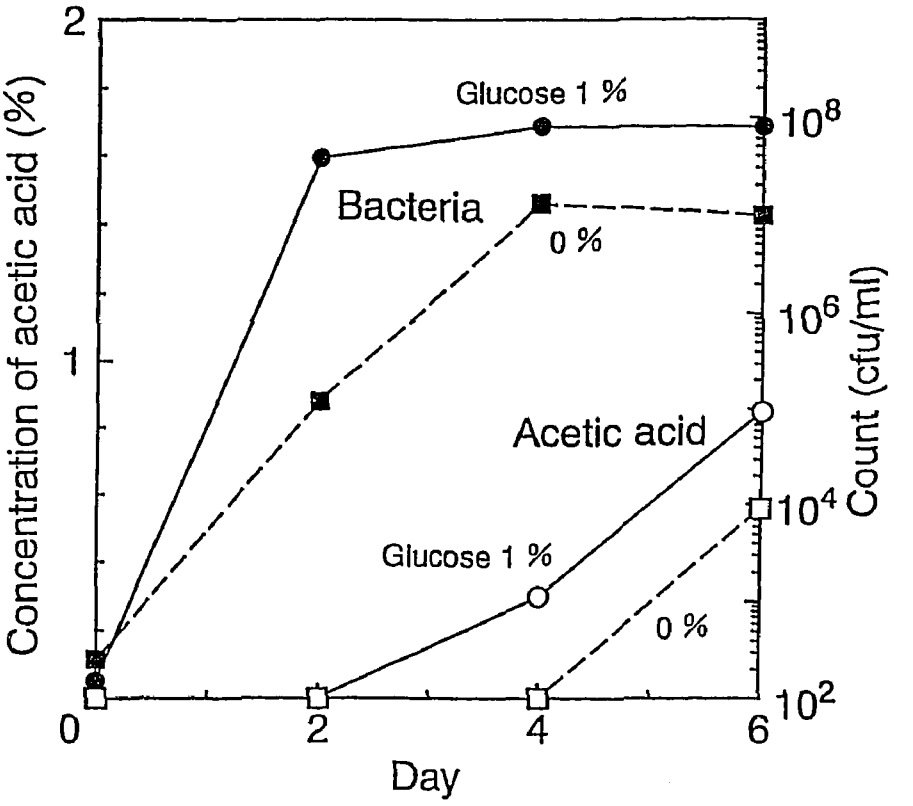

Fig. 5 Acetic acid production with low initial bacterial count

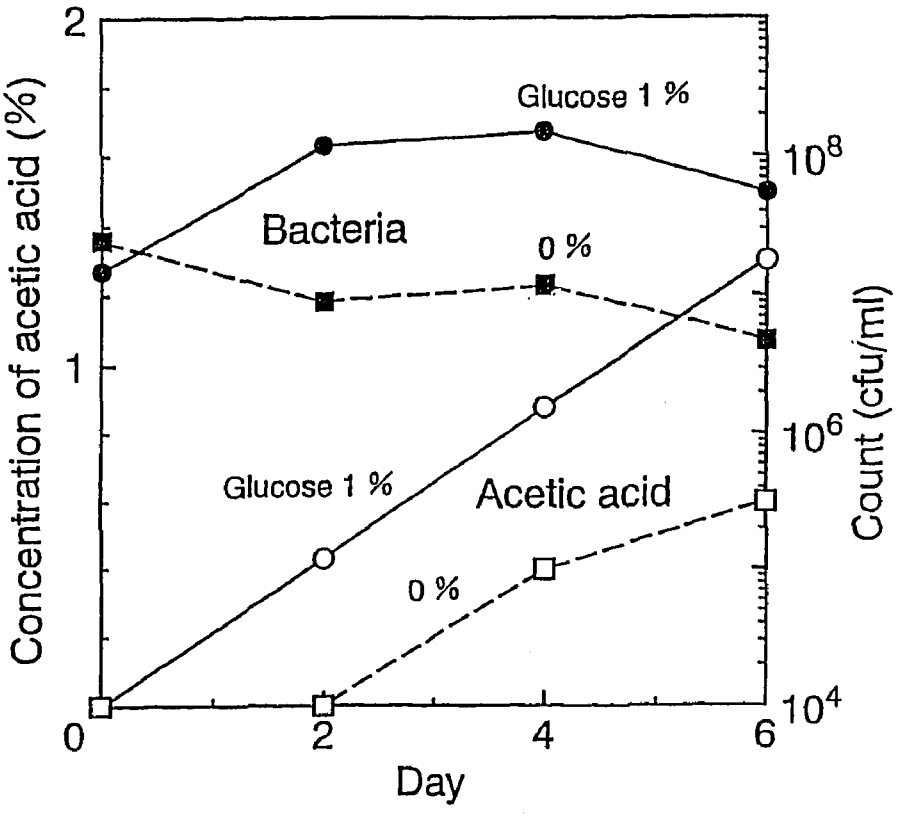

Fig. 6 Acetic acid production with high initial bacterial count

2 days with glucose and 4 days without glucose. In the case of high initial counts (Fig. 6), production of acetic acid was observed clearly just after starting of incubation when incubated with glucose.

3.4 Effect of gamma-ray irradiation on *A. acetigenum*

Surviving curves of *A. acetigenum* irradiated by gamma-ray with an without air bubbling are shown in Fig.7. The bacterial counts decreased with increasing dose and the D_{10} value (the necessary dose for one log cycle decrease) was obtained as 0.11 kGy for air bubbling and 0.54 kGy for without bubbling.

3.5 Growth of bacteria during fermentation

The growth of irradiated bacteria at 0.4 and 0.6 kGy and unirradiated bacteria during the fermentation with and without glucose are shown in Fig. 8. In the case of unirradiated bacteria, initial bacterial counts were adjusted by dilution of the original bacterial suspension with sterilized water. The diluted bacteria started to grow immediately after starting the incubation. On the other hand, the irradiated bacteria could not grow even after 2 days incubation and became the same counts as the growth of unirradiated bacteria after 4 days for 0.4 kGy and 6 days for 0.6 kGy respectively.

3.6 Fermentation of ethanol by irradiated *A. acetigenum*

Fig. 9 shows the relation between initial bacterial counts and acetic acid concentration and bacterial counts after 2 days incubation. The diluted bacteria could not produce acetic acid when the bacterial count was less than 10^6 cfu/ml. On the other hand, the

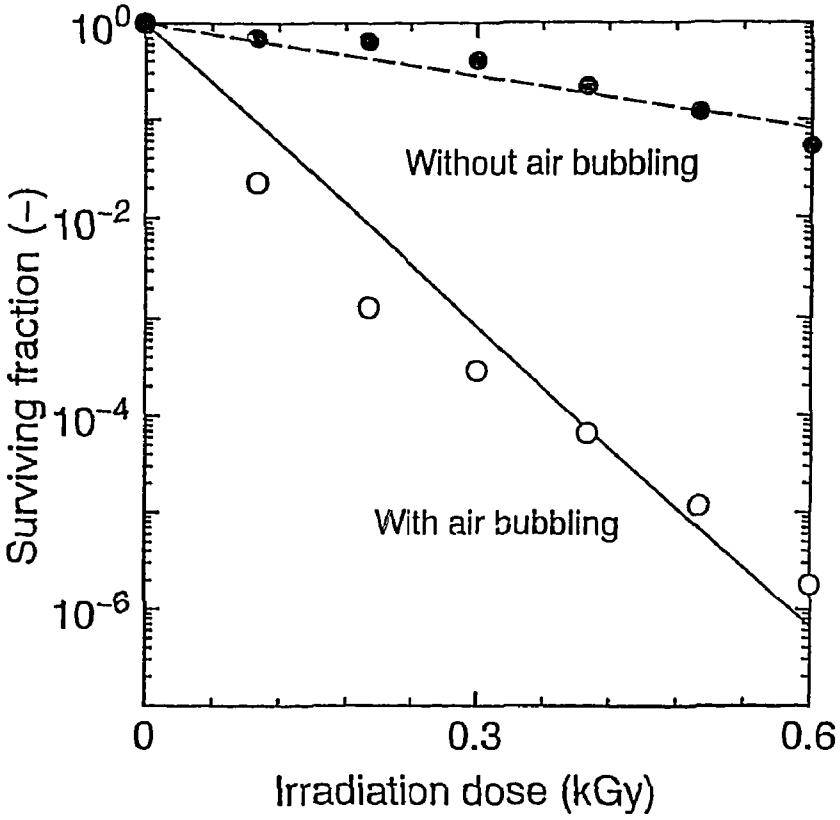

Fig. 7 Surviving curves of *A. acetigenum* by gamma-ray irradiation with and without air bubbling

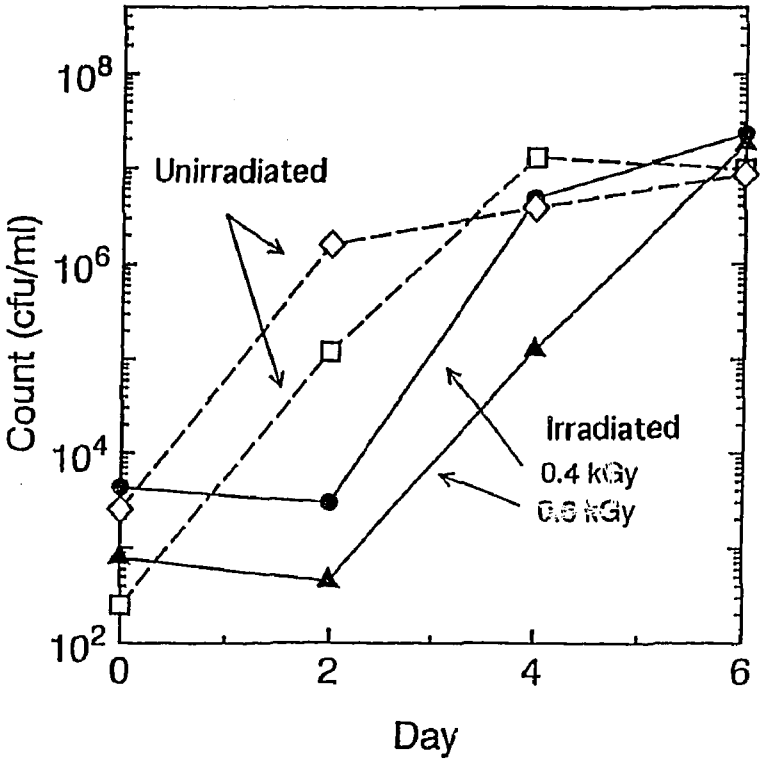

Fig. 8 Growth of *A. acetigenum* during ethanol fermentation with glucose

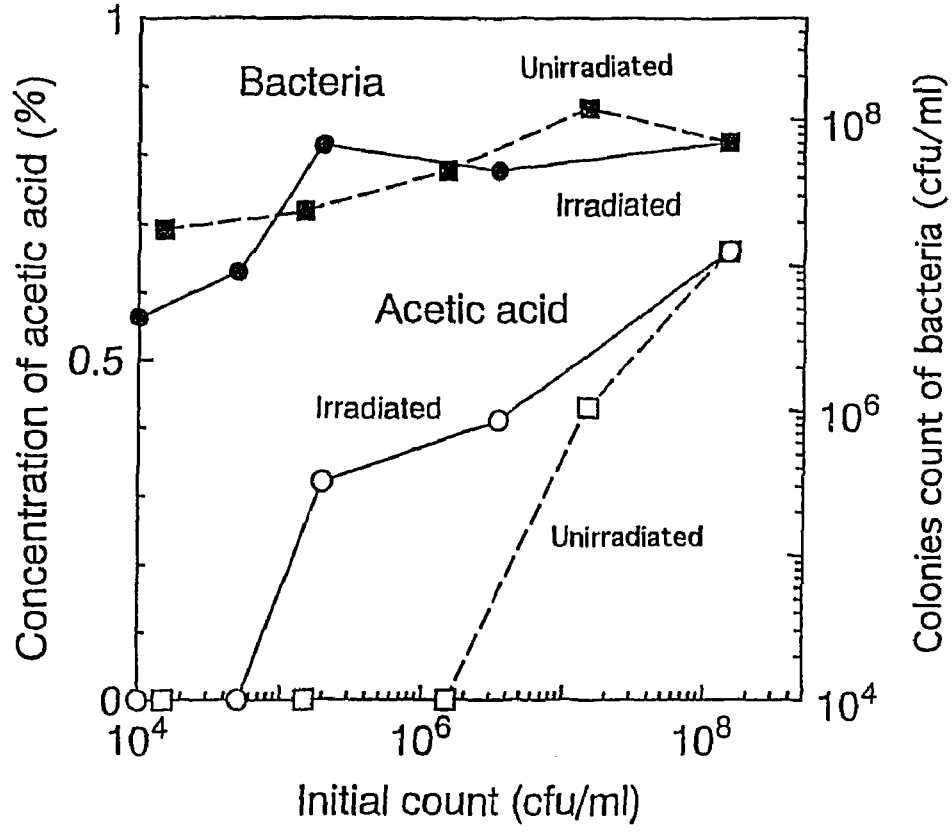

Fig. 9 Production of acetic acid and cells number of *A. acetigenum* after 2 days fermentation with glucose

irradiated bacteria produced acetic acid even the bacterial count was less than 10^5 cfu/ml (0.3 kGy). In this study, survived bacteria were counted from the number of colonies formation on agar plates after incubation for 3 day at 30°C. So, the bacteria can not count if they lose the ability to grow. But, it is known that even the bacteria lost the ability to grow, some part of the bacteria still surviving and often have the activity of fermentation. In this case, the bacterial count before irradiation was 10^8 cfu/ml and high enough to produce acetic acid even after lose the activity to grow when the irradiation dose was less than 0.3 kGy.

4. Conclusion

1) The acetic acid was produce from ethanol effectively without shake in double stage fermentation by *S. cerevisiae* and *A. acetigenum*. But no production of acetic acid was observed with shaking.

2) Bacterial counts of *A. acetigenum* suspended in distilled water decreased exponentially with increasing dose and D_{10} values were 0.11 kGy and 0.54 kGy with air bubbling and without air bubbling.

3) The irradiated bacteria was possible to produce larger amount of acetic acid compared with unirrdiated bacteria at the same initial counts.

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国際単位系 (SI) と換算表

表1　SI基本単位および補助単位

量	名称	記号
長さ	メートル	m
質量	キログラム	kg
時間	秒	s
電流	アンペア	A
熱力学温度	ケルビン	K
物質量	モル	mol
光度	カンデラ	cd
平面角	ラジアン	rad
立体角	ステラジアン	sr

表2　SIと併用される単位

名称	記号
分，時，日	min，h，d
度，分，秒	°，′，″
リットル	l，L
トン	t
電子ボルト	eV
原子質量単位	u

1 eV=1.60218×10^{-19}J

1 u=1.66054×10^{-27} kg

表3　固有の名称をもつSI組立単位

量	名称	記号	他のSI単位による表現
周波数	ヘルツ	Hz	s^{-1}
力	ニュートン	N	$m\cdot kg/s^2$
圧力，応力	パスカル	Pa	N/m^2
エネルギー，仕事，熱量	ジュール	J	N·m
工率，放射束	ワット	W	J/s
電気量，電荷	クーロン	C	A·s
電位，電圧，起電力	ボルト	V	W/A
静電容量	ファラド	F	C/V
電気抵抗	オーム	Ω	V/A
コンダクタンス	ジーメンス	S	A/V
磁束	ウェーバ	Wb	V·s
磁束密度	テスラ	T	Wb/m^2
インダクタンス	ヘンリー	H	Wb/A
セルシウス温度	セルシウス度	℃	
光束	ルーメン	lm	cd·sr
照度	ルクス	lx	lm/m^2
放射能	ベクレル	Bq	s^{-1}
吸収線量	グレイ	Gy	J/kg
線量当量	シーベルト	Sv	J/kg

表4　SIと共に暫定的に維持される単位

名称	記号
オングストローム	Å
バーン	b
バール	bar
ガル	Gal
キュリー	Ci
レントゲン	R
ラド	rad
レム	rem

1 Å= 0.1 nm=10^{-10}m

1 b=100 fm^2=10^{-28} m^2

1 bar=0.1 MPa=10^{5}Pa

1 Gal=1 cm/s^2=10^{-2}m/s^2

1 Ci=3.7×10^{10}Bq

1 R=2.58×10^{-4}C/kg

1 rad=1 cGy=10^{-2}Gy

1 rem=1 cSv=10^{-2}Sv

表5　SI接頭語

倍数	接頭語	記号
10^{18}	エクサ	E
10^{15}	ペタ	P
10^{12}	テラ	T
10^9	ギガ	G
10^6	メガ	M
10^3	キロ	k
10^2	ヘクト	h
10^1	デカ	da
10^{-1}	デシ	d
10^{-2}	センチ	c
10^{-3}	ミリ	m
10^{-6}	マイクロ	μ
10^{-9}	ナノ	n
10^{-12}	ピコ	p
10^{-15}	フェムト	f
10^{-18}	アト	a

(注)

1. 表1－5は「国際単位系」第5版，国際度量衡局 1985年刊行による。ただし，1 eVおよび1 uの値はCODATAの1986年推奨値によった。
2. 表4には海里，ノット，アール，ヘクタールも含まれているが日常の単位なのでここでは省略した。
3. barは，JISでは流体の圧力を表わす場合に限り表2のカテゴリーに分類されている。
4. EC閣僚理事会指令ではbar，barnおよび「血圧の単位」mmHgを表2のカテゴリーに入れている。

換算表

力	N(=10^{5}dyn)	kgf	lbf
	1	0.101972	0.224809
	9.80665	1	2.20462
	4.44822	0.453592	1

粘度　1 Pa·s(N·s/m^2)=10 P(ポアズ)(g/(cm·s))

動粘度　1 m^2/s=10^{4}St(ストークス)(cm^2/s)

圧力	MPa(=10 bar)	kgf/cm^2	atm	mmHg(Torr)	lbf/in^2(psi)
	1	10.1972	9.86923	7.50062×10^3	145.038
	0.0980665	1	0.967841	735.559	14.2233
	0.101325	1.03323	1	760	14.6959
	1.33322×10^{-4}	1.35951×10^{-3}	1.31579×10^{-3}	1	1.93368×10^{-2}
	6.89476×10^{-3}	7.03070×10^{-2}	6.80460×10^{-2}	51.7149	1

エネルギー・仕事・熱量	J(=10^{7}erg)	kgf·m	kW·h	cal(計量法)	Btu	ft·lbf	eV
	1	0.101972	2.77778×10^{-7}	0.238889	9.47813×10^{-4}	0.737562	6.24150×10^{18}
	9.80665	1	2.72407×10^{-6}	2.34270	9.29487×10^{-3}	7.23301	6.12082×10^{19}
	3.6×10^6	3.67098×10^5	1	8.59999×10^5	3412.13	2.65522×10^6	2.24694×10^{25}
	4.18605	0.426858	1.16279×10^{-6}	1	3.96759×10^{-3}	3.08747	2.61272×10^{19}
	1055.06	107.586	2.93072×10^{-4}	252.042	1	778.172	6.58515×10^{21}
	1.35582	0.138255	3.76616×10^{-7}	0.323890	1.28506×10^{-3}	1	8.46233×10^{18}
	1.60218×10^{-19}	1.63377×10^{-20}	4.45050×10^{-26}	3.82743×10^{-20}	1.51857×10^{-22}	1.18171×10^{-19}	1

1 cal = 4.18605 J (計量法)

= 4.184 J (熱化学)

= 4.1855 J (15 °C)

= 4.1868 J (国際蒸気表)

仕事率　1 PS (仏馬力)

= 75 kgf·m/s

= 735.499 W

放射能	Bq	Ci
	1	2.70270×10^{-11}
	3.7×10^{10}	1

吸収線量	Gy	rad
	1	100
	0.01	1

照射線量	C/kg	R
	1	3876
	2.58×10^{-4}	1

線量当量	Sv	rem
	1	100
	0.01	1

(86年12月26日現在)

PRODUCTION OF ACETIC ACID FROM ETHANOL SOLUTION BY *Acetobactor acetigenum* AND EFFECT OF GAMMA-RAY IRRADIATION ON THE BACTERIA