Isolation of the Killer Yeasts and Its Characteristics

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Killer 효모의 분리 및 특성

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ABSTRACT: Ten strains out of about 1,000 yeast strains isolated from byproducts of alcoholic industries, milk products, fruits, greens, food-related industries and soils of nature, revealed the killer activities. Two strains which have excellent killer activities among them were isolated and identified with *Saccharomyces cerevisiae* B 15-1 and *Hansenula anomala* Y 33 by investigation of the morphological, cultural and physiological properties. The optimal conditions on these strains for the production of killer toxin were investigated. The strain B 15-1 showed the highest killer toxin activities when it was cultured up to the log phase of 48 hr in YPD medium (pH 4.7) at 25°C. On the other hand, the strain Y 33 revealed the highest activities when it was cultured up to the stationary phase of 60 hr in YPD medium (pH 4.0) at 20°C. The sensitive strain Kyokai 7 was found to be killed entirely by the killer toxin produced from the wild killer yeast B 15-1 when B 15-1 was cocultured with the same cell concentration (10^6 cells/m*l*) of Kyokai 7 after cultivation of 36 hr, and with large concentration (9×10^7 cells/m*l*) after 48 hr.

KEY WORDS □ Killer yeast, Killer toxin.

A certain group of the yeast *Saccharomyces cerevisiae* is known to secrete a protein toxin (Bostian *et al.*, 1980) which is lethal to other strains of the same or other species. The killer strains inhibited the growth of the sensitive lawn, where a seeded lawn of a sensitive strain was streaked with a killer strain. All wild-type killer strains are resistant to the effects of the toxin they produce (Bostian *et al.*, 1980). This killer character in *S. cerevisiae* was first reported by Bevan and Makower (1963).

The contamination by wild yeasts of *S. cerevisiae* is one of the major problems in the brewing industry, because it results in a slow fermentation and an inferior quality of the product. Most such wild yeasts are sensitive to the killer toxin. When killer yeasts invade the brewing mash, they cause more serious damage than the sensitive strains do, because they kill the culture yeast which has been seeded in the mash, and consequently fermentation becomes abnormal (Imamura *et al.*, 1974). A killer strain which can produce the fine quality alcoholic beverages will be useful for brewing, since it can prevent not only the contamina-

tion by sensitive wild yeasts but also serious damage by the invasion of killer wild yeasts.

In order to breed the new strains by the protoplast fusion in this work, we isolated about 1,000 yeast strains from nature and others, selected 2 strains out of them, identified, and then examined the culture conditions for the production of killer toxin.

MATERIALS AND METHODS

Yeast strains

The killer yeast strains used in this work were *Saccharomyces cerevisiae* B 15-1 and *Hansenula anomala* Y 33 isolated from alcoholic industries and rinds of grapes, respectively (Bang *et al.*, 1987). The killer sensitive strain was *S. cerevisiae* Kyokai 7 (Shimoda *et al.*, 1984; Ouchi *et al.*, 1979).

Media

YPD medium (1% yeast extract, 2% peptone, 2% dextrose) and YM medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone, 1% dextrose) were used for yeast culture. YPD-MB agar (YPD containing 0.003% methylene blue and 2% agar) buffered at pH 4.7 with 0.05 M citrate and phosphate was used for the assay of killer activity (Somers and Bevan, 1968). TTC (2, 3, 5-triphenyltetrazolium chloride) medium (lower layer; 1% dextrose, 0.2% peptone, 0.15% yeast extract, 0.1% KH_2PO_4, 0.04% $MgSO_4 \cdot 7H_2O$, 2% agar, upper layer; 0.5% dextrose, 0.05% TTC, 1.3% agar) was used for the colorization test of yeast colonies.

Isolation of wild killer yeasts

The wild yeasts were isolated from byproducts of alcoholic industries, milk products, fruits, greens, food-related industries and soils of nature. Each of these strains was inoculated on YPD-MB agar plate (pH 4.7) with a lawn of killer sensitive strain *S. cerevisiae* Kyokai 7 (0.5 m*l* of 10^6 cells/m*l* suspension spread on each plate and allowed to dry). After being incubated at 25°C for 2 days, the wild killer yeasts were detected by the formation of the clear zone in the sensitive lawn and then were isolated (Fig. 1-A).

Identification of isolates

According to the procedures and identification keys of Lodder (1970), the morphological, cultural and physiological characteristics of isolates were examined and identified.

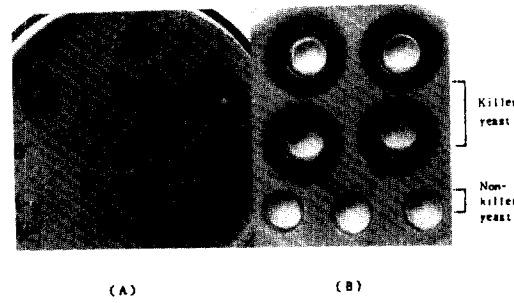


Fig. 1. *Screening of the killer yeast (A) and well test for determination of the killer activity (B) on YPD-MB agar plate (pH 4.7).*

Assay of killer activity

The killer activity was assayed by well test (Somers and Bevan, 1968). The cells cultured without shaking in YPD liquid medium were centrifuged and then discarded. Then 0.2 m*l* aliquots of the supernatant were put into the well bored using small cylinder 10 mm in diameter on the YPD-MB sensitive lawn. After 3 days of incubation at 25°C, the killer activity was expressed as a diameter of a clear zone on the sensitive lawns (Fig. 1-B).

Coculture of wild killer yeast and sensitive strain

The lethal extent of sensitive strain *S. cerevisiae* Kyokai 7 on the killer yeast was determined by coculture between 2 strains. The wild killer yeast was cocultured with the sensitive strain in YPD medium (buffered at pH 4.7 with 0.05 M citrate and phosphate) at 25°C without shaking. Then viabilities of each cell were determined by TTC colorization test and by replica on YPD-MB sensitive lawns in the course of time.

RESULTS

Isolation of killer yeasts

Ten strains out of about 1,000 yeast strains isolated from nature showed the killer activities. Among them, the strain B 15-1 and Y 33 isolated from alcoholic industries and rinds of grapes, respectively, were showed the excellent killer activities (Table 1).

Identification of isolates

The morphological, cultural and physiological characteristics of the isolated strains are shown in Fig. 2 and Table 2 and 3. The strain B 15-1 was

Table 1. *Screening of the wild type killer yeast.*

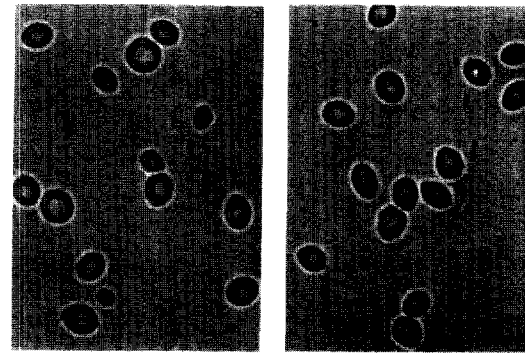
Strain	Clear zone (mm)
A 19-2	1
B 15-1	6
B 19	1
B 37	1
Q 57	2
Q 72	2
Y 33	4
Y 40	2
Z 18	1.5
Z 29	1.5

The strain was cultured on the YPD-MB lawn for 2 days at 25 °C. Killer activities were expressed as diameter of clear zone on the YPD-MB lawn by the well test.

Table 2. *Morphological, cultural and physiological characteristics of the isolated strains.*

Classification	Strain	
	B15-1	Y33
Cell shape	ellipsoidal	oval
Cell size (μm)	4.6–5.7 × 6.1–7.2	4.5–5.1 × 5.6–6.4
Vegetative reproduction	budding	budding
Ascospore	present (1-4)	present (1-4)
Pseudomycelium	absent	absent
True mycelium	absent	absent
Culture in YM		
Pellicle	absent	present
Ring	absent	present
Growth on YM agar		
Form	regular	regular
Edge	entire	entire
Elevation	raised	raised
Surface	smooth	smooth
Color	creamy	white creamy
KNO_3 assimilation	–	+
Splitting	–	+
Vitamin requirement	–	–
Growth at 37 °C	+	+
TTC colorization test	red	red-white
Acid production	+	–

+ or – means positive or negative, respectively.



(A) (B)

Fig. 2. *Microscopic photopraphs of the isolated strains.*
The strain B15-1 (A) and Y33 (B) were cultured to the stationary phase in YPD medium at 30 °C with shaking. Magnification: (× 1,300).

Table 3. *Assimilability and fermentability of carbon sources by the isolated strains.*

Carbon source	Strain		Carbon source	Strain	
	B15-1	Y33		B15-1	Y33
Assimilation					
Glucose	+	+	Raffinose	±	–
Galactose	+	+	Melezitose	±	+
Maltose	±	+	Starch	–	+
Sucrose	+	+	Xylose	–	+
Cellobiose	–	+	Ribose	–	+
Trehalose	±	+	Ethanol	–	+
Lactose	–	–	Citrate	–	+
Melibiose	–	–			
Fermentation					
Glucose	+	+	Raffinose	±	–
Galactose	+	–	Melezitose	±	–
Sucrose	+	+	Lactose	–	–
Maltose	±	+	Starch	–	–

+; good, ±; weak, and –; not assimilable or fermentable.

ellipsoidal shape and reproduced by budding. The strain was able to produce acid, and formed ascospore, but did not formed pseudomycelium and true mycelium. These results indicate that the strain could be classified as genus *Saccharomyces*. In addition, the strain was able to ferment glucose, galactose, sucrose, maltose and raffinose, but could not split arbutin, and did not require vitamin as

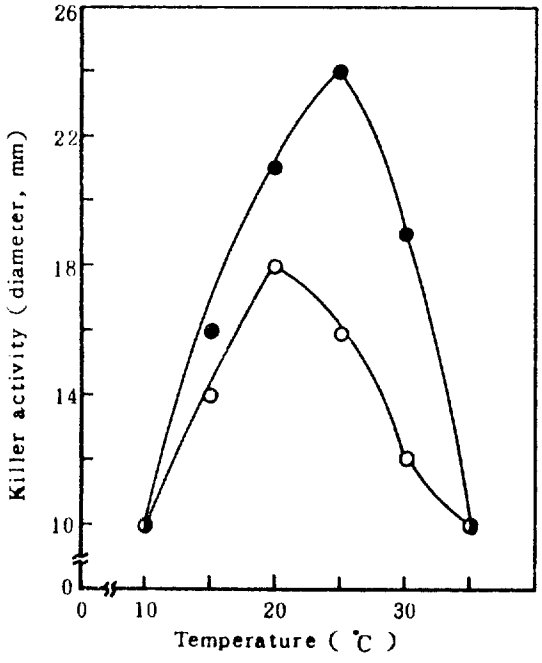


Fig. 3. *Effect of cultural temperature on the production of killer toxin.*
The strain B15-1 (●-●) and Y33(○-○) were grown in YPD medium for 48 hours at the indicated temperatures for the production of killer toxin. The killer toxin activities were expressed as diameter of clear zone on the YPD-MB lawn (pH 4.7) incubated for 48 hours at 25 ℃ by well test.

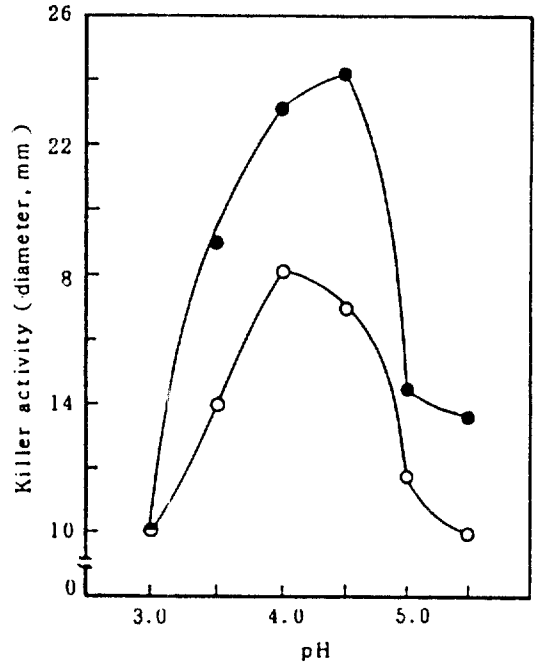


Fig. 4. *Effect of cultural pH on the production of killer toxin.*
The strain B15-1 (● - ●) and Y33 (○ - ○) were grown in YPD media (adjusted the pH from pH 3.5 to pH 5.5 with 0.05M citrate-phosphate buffer) for 48 hours at 25 ℃ and 20 ℃, respectively, at the indicated pH for the production of killer toxin. The determination of killer toxin activities are described in Fig. 3 and the 'Materials and Methods'.

growth factor. Also, colonies of the strain turned to red by TTC colorization test. From these results, the strain B 15-1 was identified as *Saccharomyces cerevisiae.*

The strain Y 33 was oval shape and reproduced by budding. The strain was able to form ascospore, pellicle and ring, and could assimilate potassium nitrate, ethanol, starch, xylose and citrate, but did not produce acid. These results indicate that the strain could be classified as genus *Hansenula.* In addition, the strain was able to ferment glucose, sucrose and maltose, and could split arbutin, but did not require vitamin. Also, colony of the strain was white-creamy. From these results, the strain Y 33 was identified as *Hansenula anomala.*

Culture conditions for production of killer toxin

Of the factors influencing the production of killer toxin by the killer yeasts, effects of culture temperature, pH and time course were investigated. In order to determine the optimum temperature for the production of killer toxin, the isolated killer yeasts were grown in YPD medium for 48 hr at the indicated temperatures without shaking. The killer activity of the culture filtrates were expressed as diameter of clear zone on the YPD-MB lawn (pH 4.7) incubated for 48 hr at 25°C by well test. As shown in Fig. 3, the optimum temperatures for the production of killer toxin from B 15-1 and Y 33 were 25 and 20°C, respectively. The killer yeasts B 15-1 and Y 33 secreted the killer toxin to some extent at 30°C, but did not at all at 35°C. The optimum pH was determined using 0.05 M citrate-phosphate buffer. As appeared in Fig. 4, the killer toxin from the strain B 15-1 and Y 33 reached the highest degree at pH 4.7 and 4.0, respectively, but did not be appeared below pH 3.0. The strain B 15-1 showed a little of killer activities at pH 5.5, but Y 33 did not at all. The

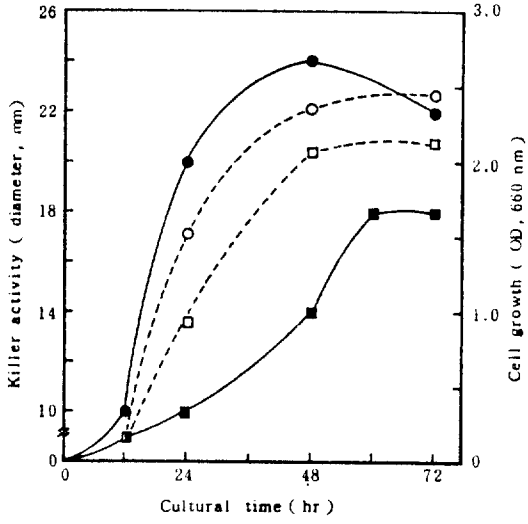


Fig. 5. *Time courses on the production of killer toxin.* The strain B15-1 (●, ○) and Y33 (■,□) were grown in YPD media (adjusted to pH 4.5 and pH 4.0 with 0.05M citrate-phosphate buffer, respectively) for the indicated times at 25 °C and 20 °C, respectively, for the production of killer toxin. The determination of killer toxin activities (—) are described in Fig. 3 and the 'Materials and Methods'. The cell growth (···) was expressed as OD at 660 nm.

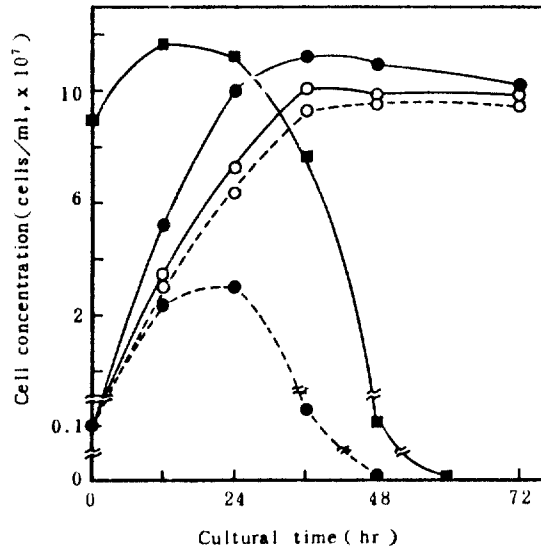


Fig. 6. *Effect of killer toxin from wild killer strain B15-1 on the sensitive strain Kyokai 7.* The wild killer strain B15-1 (○—○) was cocultured with the sensitive strain Kyokai 7 (●—●) in YPD medium (adjusted pH 4.7 with 50 mM citrate-phosphate buffer) by standing-culture for the indicated times at 25 °C. ●—● and ○—○ means growth curves of Kyokai 7 and B15-1, respectively. ···means cocultivation with Kyokai 7 and B15-1. ■—■ means lethal curve of Kyokai 7 inoculated to 9×10^7 cells/m*l* with wild killer B 15-1.

killer toxin from B 15-1 reached 14 mm of diameter within 48 hr and then was decreased gradually after 48 hr. In the case of Y 33, the killer toxin reached 8 mm in 60 hr (Fig. 5).

Effect of killer toxin on sensitive strain

Fig. 6 shows the effect of killer toxin from the wild killer strain B 15-1 on the sensitive strain Kyokai 7. The strain B 15-1 was cocultured with Kyokai 7 at the same cell concentration (1×10^6 cells/m*l*) or at the varied state (1×10^6 cells /m*l* of B 15-1 and 9×10^7 cells/m*l* of Kyokai 7). Regardless of cell concentration, two strains grew at the same rate upto 12 hr. The sensitive cells which began to decrease gradually after 24 hr were killed completely by the killer toxin from B 15-1 after 36 hr in the case of the same cell concentration and after 48 hr in the case of the varied state.

DISCUSSION

The wild killer yeasts were isolated from nature, and were identified as *Saccharomyces cerevisiae* B 15-1 and *Hansenula anomala* Y 33.

Young and Philliskirk (1977) reported that killer activity was in proportion to natural logarithms on the amounts of extracellular killer toxin. According to Pfeiffer and Radler *et al.* (Pfeiffer and Radler, 1982; Kotani *et al.*, 1977; Young and Philliskirk, 1975, 1977; Palfree and Bussey, 1979), the extracellular killer toxin was inactivated under the culture conditions over pH 5.0 and 20°C, and the production of killer toxin was inhibited by shaking culture. The killer toxin became known to be stabilized by the addition of ammonium sulfate rather than gelatin, glycerol, propylene glycol, inositol, sucrose and mannitol in the medium (Kotani *et al.*, 1977; Ouchi *et al.*, 1978; Young and Philliskirk, 1975, 1977). Thereupon, the optimal conditions for the production of killer toxin from these strains were established. The killer toxin from B 15-1 and Y 33 reached 14 mm of diameter in the log phase of 48 hr at 25°C in YPD (pH 4.7),

in the stationary phase of 60 hr at 20°C in YPD (pH 4.0), respectively (Fig. 4-6). These results are similar to those of Pfeiffer and Radler *et al.* (Pfeiffer and Radler, 1982; Kotani *et al.*, 1977; Young and Phillisкirk, 1975, 1977; Palfree and Bussey, 1979). Also, it is reported that optimal formation conditions of the killer toxin are similar to the optimal reaction conditions which can kill the sensitive strain (Wood and Bevan, 1968; Palfree and Bussey, 1979; Middelbeek *et al.*, 1979; Pfeiffer and Radler, 1982). According to Young and Phillisкirk (1977), these conditions could be changed a little because of the structural differences of killer factors and whether the component of killer toxin is protein or glycoprotein. The protein K_1 toxin had the optimum activity and maximum productivity in the range pH 4.0-4.6 below 22°C. The glycoprotein toxin had the same results in the range pH 4.6-4.8 below 25°C except the conditions over pH 5.0 and 30°C. From those results, we could assume that the component of the killer toxin from B 15-1 and Y 33 are glycoprotein and protein, respectively. Meanwhile, the killer toxin had a little or no activity at 30 or 35°C, respectively. Those results showed that the killer toxin which was unstable over 30°C inactivated as soon as producing, and M dsRNA known as the gene which dominate the formation and secretion of the killer toxin was cured or mutated by the elevated temperature above 30°C, therefore, the killer yeast converted to the non killer-sensitive strain, and then could not form the toxin (Wickner, 1974; Young and Yagiu, 1978). On the culture time, the B 15-1 toxin began to be shown the activity in log phase and reached the maximal state in late log phase. The Y 33 toxin began to be shown the activity in late log phase and reached maximum in stationary phase. From those result, we could find out that the B 15-1 and Y 33 toxin began to form in early log phase and log phase, respectively. According to Palfree and Bussey (1979), the killer toxin was formed the highest state in log phase and a little in stationary phase. The strain B 15-1 was similar to those results except Y 33, this seems that the strains and toxin component differ from those. In general, the killer activity was expressed as diameter of clear zone. Toh-E and Wickner (1978, 1980) reported that the killer yeast which have clear zone over 10 mm of diameter is called the "superkiller" yeast. In the case of B 15-1, the clear zone formed under the optimal conditions reached above 14 mm of diameter. Therefore, we could term that B 15-1 is the "superkiller" yeast by the classification of those. The sensitive yeast Kyokai 7 was killed completely by the killer toxin from B 15-1 after 36-48 hr. This result was similar to those of strain K_2-M-111 (Yamamoto *et al.*, 1984) cocultured with 10^6 cells/m*l* of sensitive strain, but was different from those of strain KL 88 (Shimoda *et al.*, 1984) cocultured with 10^5 cells/m*l* for 24 hr, and with 10^7 cells/m*l* for 40 hr (Seki *et al.*, 1985). The reason of those results seems to be the differences of sensitive cells concentration and culture conditions.

적 요

Killer system을 이용한 효모균주 개발의 일환으로 야생의 killer 효모를 분리, 동정하고 killer toxin 생성에 대한 최적조건을 검토하였다. 자연계에서 분리한 1,000여종의 효모 중 10 균주가 killer 활성을 나타내었는데, 이 중 killer 활성이 뛰어난 두 균주를 선별하여, 이들 균주의 형태학적, 배양학적, 생리학적 특성 등을 검토한 결과, *Saccharomyces cerevisiae* B 15-1 및 *Hansenula anomala* Y 33으로 동정하였다. 또한 이들 균주의 killer toxin 생성에 미치는 최적조건을 조사한 결과, B 15-1주는 pH 4.7로 조절한 YPD 배지에서 25℃, 48시간 배양한 균체의 대수 증식기에서, Y 33주는 pH 4.0으로 20℃에서 60시간 배양한 정지기 세포에서 각각 killer toxin 생성이 최대에 달했다. 또한 감수성 주에 미치는 killer toxin의 영향을 검토하기 위하여 B 15-1을 감수성 주와 동량 접종하여 혼합배양한 결과 36시간 이후에, 감수성 주를 다량 접종한 경우 48시간 이후에 감수성 주가 완전히 사멸되었다.

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