



Survival of *Kluyveromyces marxianus* with stigmasterol as subjected to freezing stress

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***Kluyveromyces marxianus* is an aerobic yeast and is interested to be applied in many industries. This research was aimed to study the effect of the sterol alternative to ergosterol on the freezing stress of *K. marxianus* UBU1-11, a thermotolerant yeast. The 0–9 mgL⁻¹ stigmasterol were added to the YM broth and applied for culturing. The growth of all conditions were not interfered by the addition of stigmasterol. The intra-cellular sterol content was detected in the medium with 5 mgL⁻¹ stigmasterol and higher, where the maximum content was 0.32 mg g⁻¹ cell dry weight. After frozen and thawed, the cultures contained stigmasterol had significantly higher viability than those without. It was found that the amount of stigmasterol contained in cells did not affect the number of survival. The stigmasterol provided a significant protection to the yeast cell when subjected to slow freezing. It also increased the survival rate of the culture subjected to subzero temperature storage.**

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[Key words: *Kluyveromyces marxianus*; Thermotolerant yeast; Stigmasterol; Freezing stress; Survival rate]

Kluyveromyces marxianus is a yeast that has been classified as a generally recognized as safe (GRAS) culture, like *Saccharomyces cerevisiae* and *Kluyveromyces lactis* (1). It is interested to be applied in food industries. The culture has ability to grow in high temperature environment, Crabtree-negative, facultative fermentative and utilize wild variety of carbohydrates (2,3) and cannot grow in the anaerobic condition (1). Banat et al. (4) reported that the alcoholic production with *K. marxianus* was able to ferment in high temperature with high production rate and less contamination. Grba et al. (5) had applied *K. marxianus* for alcohol and biomass production on whey at 34°C with the production of biomass of 10 gL⁻¹ and ethanol about 7%. Anderson et al. (6) was able to produce up to 6% ethanol at 43°C from cane sugar and the fermentation time was 30 h with *K. marxianus*. *Kluyveromyces* spp. is also applied in the production of aroma compounds and enzymes, such as inulinase and β-galactosidase (7). A potential baker's yeast for frozen dough for the genus *Kluyveromyces* was reported (8). Maccaferri et al. (9) reported that the *K. marxianus* had behaviors to be a potential probiotic.

The application of freezing in preservation of a culture in a laboratory has been practiced for a long time. Freezing has also played an important role in food preservation nowadays. There is a growing interest in applying freezing in partially processed foods containing culture of microbes. Freezing dough have been extensively adopted in many food products such as in baking industries. It was reported that freezing of yeast cells caused a loss in the

number of viable cells (10). The most important problem encountered in these products is how to maintain the viability of the frozen yeasts. Many cryoprotective additives for yeasts were reported (11). They might be distinguished in categories as, those that penetrate both cell wall and cell membrane (glycerol and dimethyl sulfoxide), those that penetrate cell wall but not cell membrane (mono- and disaccharides and amino acids) and those that do not penetrate even cell wall (high molecular weight protein and polysaccharides) (12). Somehow, the addition of cryoprotective agents to foods may be restricted due to development of unsatisfactory physical and sensory qualities in the products.

Sterols are essential lipids in the membrane of yeasts. The cryopreserved yeast, when cells were enriched in sterols, demonstrated the increasing in survival (13). Sterols contribute to the membrane fluidity, membrane permeability and many of membrane activities (14). They also play roles in the resistance of thermal stress, both freezing and heating (15). Ergosterol is an essential sterol found in cell membranes of fungi and protozoa. Earlier studies with a yeast sterol auxotroph reported a positive correlation between ergosterol and tolerance to heat and ethanol (16). Dupont et al. (15) suggested a relationship between plasma membrane sterols and hydric stress resistance. Stigmasterol, a phytosterol, was reported to protect yeast plasma membrane from the effect of syringomycin when added in the culture medium (17). Stigmasterol has potential to be useful in medical applications as a precursor in the manufacture of semisynthetic progesterone and prevention of certain cancers. It was reported to be safe for use as a stabilizer in ready-to-freeze alcoholic cocktails (18).

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The study on the survival of freezing tolerance of yeasts other than *S. cerevisiae*, especially the thermotolerant species, has been scarcely reported. The aims of this study were to find the effect of the stigmasterol on the survival *K. marxianus* that subjected to freezing stress and on survival as stored under subzero temperature.

MATERIALS AND METHODS

K. marxianus UBU 1-11 was obtained from the laboratory of the Department of Biological Science, Ubon Ratchathani University. The culture was maintained on potato dextrose agar. It was transferred to fresh medium every 10 days and stored at 4°C. The active culture was prepared by culturing in the yeast malt extract broth (YM broth) for 22 h under room temperature.

Effect of stigmasterol on growth and sterol absorption The 1 mL of the active culture was transferred into each 250 mL flask contained 200 mL sterile YM broth with various amount of stigmasterol, from Sigma–Aldrich Co., Malaysia, at 0, 1, 3, 5, 7 and 9 mgL⁻¹. The cultures were left in room temperature for 22 h. The number of viable cells, unstained cells, in all conditions was determined under a microscope on a hemocytometer using methylene blue staining (MBS) followed Lee et al. (19). The sterol content in yeast cell was analyzed using a modified method described by Letter (20). Cell pellet was collected after centrifugation at 4°C for 10 min. They were washed for 2 times and resuspended with sterile saline water. Cells were broken by the addition of 10 g of 0.45 mm glass beads into the suspension and vortexed for 30 s. The suspension was then placed in an ice bath for 30 s. The vortexing and cooling were repeated 3 cycles. After the glass beads were removed, the cell suspension was centrifuged at 6000 rpm, 4°C, for 10 min. The pellet was collected and extracted by methanol, chloroform and water, following the method described by Bligh and Dryer (21). The analysis of cell sterol was performed using a HPLC, Agilent 1100 series; England, equipped with Eclipse XDB-C8 followed the procedure described by Saynajoki et al. (22). The mobile phase was a solution of 95% methanol and 5% H₂O with a flow rate of 10 mL min⁻¹. Sterol was detected by a diode-array detector, monitoring absorption at 210 nm. The area under the expected curve was transformed to concentration using standard curve, using stigmasterol as a standard material. The conditions that provide detectable intra-cellular sterol were selected for further study.

Effect of stigmasterol on cell survival after freezing *K. marxianus*, grown in the selected conditions, were washed 2 times and resuspended with sterile saline water. The 1.5 mL of cell suspension was filled in a 2 mL Eppendorf. They were frozen in an ethyl alcohol cooling bath. A circulating cooling bath, CBN 18-30, with a thermostat HMT 200, Heto-Holten A/S, Denmark was applied to control the temperature at -10°C, -20°C and -30°C. Then, they were thawed in a water bath until the temperature reached 37°C. The survival rate of the cultures were determined using MBS technique. The culture that had the highest survival rate was selected for further study.

Effect of freezing rate on survival of *K. marxianus* The *K. marxianus* suspensions of selected condition were prepared as described above. The 10 µL of cell suspensions, normal culture and culture contained stigmasterol, was pipetted into aluminum pans and subjected to freezing. The freezing conditions were controlled by a differential scanning calorimeter (PYRIS Diamond DSC, PerkinElmer Instruments, Shelton, CT, USA). The liquid nitrogen was used as a cooling medium to reduce the temperature of the pan. Freezing routine was initiated from 30°C to -20°C and returned to the starting temperature. The rate of cooling was programmed at 1°C, 7°C, 10°C, 20°C and 40°C min⁻¹. The thawing rate was set at 7°C min⁻¹. The number of viable cells, previous and after treatment, was counted by MBS technique. The freezing condition provided the highest survival number was selected for further study.

Effect of stigmasterol on cell survival after frozen storage The suspensions of *K. marxianus* contained stigmasterol and normal culture were prepared as described. Yeast suspension 1.5 mL was transferred into a 2 mL Eppendorf. Yeast suspension was frozen in a cooling bath, as described, to bring the temperature to -30°C at the optimal rate found in the prior experiment. The frozen suspensions were stored at -10 ± 2°C, -20 ± 2°C, -30 ± 2°C, -40 ± 1°C, -60 ± 1°C and -80 ± 1°C for 6 weeks. The number of viable cell, before freezing and after thawing, were determined.

To describe the reduction rate of viability during the frozen storage, the data was transformed using the first order kinetic equation (Eq. 1).

$$\ln N/N_0 = -k(t_2 - t_1) \quad (1)$$

where k is the reduction (death) rate constant, N_0 is initial number of cells (cells mL⁻¹), N is number of cells at any time of storage (cells mL⁻¹), and t is days.

Statistical analysis All experiments were designed using a completely randomized designed with 3 replications. Analysis of variance and Duncan's new

TABLE 1. The number of *K. marxianus* after 22 h incubated in the YM broth supplemented with stigmasterol, 0–9 mgL⁻¹, and intra-cellular sterol content.

Stigmasterol supplement (mg L ⁻¹)	Number of cells (log ₁₀ cells mL ⁻¹) ^{ns}	Total sterol content in cells (mg g ⁻¹ cell dry weight)
0	8.26 ± 0.10	0
1	8.28 ± 0.11	0
3	8.19 ± 0.09	0
5	8.16 ± 0.18	0.15 ^b ± 0.01
7	8.17 ± 0.21	0.32 ^a ± 0.06
9	8.24 ± 0.11	0.37 ^a ± 0.08

ns, not significant different ($p > 0.05$).

^{a, b} Means with different letters in the same column were differed significantly ($p \leq 0.05$).

multiple comparison range test were utilized with the application of IBM SPSS Statistics, version 17.0 (IBM Co., Armonk, NY, USA) at a confidence level of 95%.

RESULTS AND DISCUSSION

Effect of stigmasterol on growth and cell absorption The stigmasterol content in cell membrane and number of *K. marxianus* incubated in YM medium supplemented with stigmasterol are demonstrated in Table 1. The content of intra-cellular sterol was detected in the cultures grown in the medium with 5 mgL⁻¹ of stigmasterol and higher. The 0.32 mg g⁻¹ was the highest amount of stigmasterol absorbed by *K. marxianus* cell. There was no significant difference ($p > 0.05$) in number of cells for all cultures. This indicated that stigmasterol in the medium did not affect the growth of *K. marxianus*. The sterol that increased the growth of *K. marxianus* was ergosterol, which was reported by Martin (23). It is the only sterol that naturally found in the cell membrane and endoplasmic reticulum of *K. marxianus* (24).

Effect of stigmasterol content in the cell on cell survival after freezing The cultures, contained different amount of stigmasterol in the cells, were subjected to be frozen at 3 different temperatures. It was found that the cells with no stigmasterol content showed about 40% survival of the initial number, while other that contained stigmasterol showed almost double in survival rate. The different in the stigmasterol cell content, higher than 0.15 mg g⁻¹, did not show significant ($p > 0.05$) difference in survival rate (Fig. 1). Calcott and Anthony (13) reported the increasing of unsaturated fats and sterol to the cell membrane of yeast and bacteria might be a factor on freezing injury prevention of the cells. Sterols affect the membrane fluidity and permeability (25,26). Murakami et al. (27) studied the fat composition of *Torulasporea delbrueckii* D2-4, a freeze tolerant strain, and

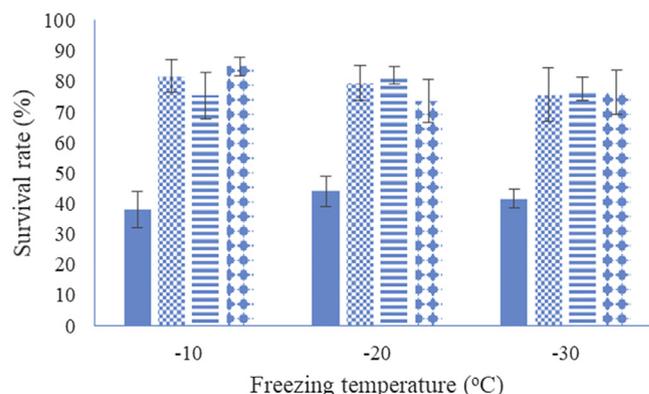


FIG. 1. The survival rate of the *K. marxianus* cells after frozen at -10°C to -30°C with various amount of stigmasterol content; 0 mg g⁻¹ (solid column), 0.15 mg g⁻¹ (small checker board column), 0.32 mg g⁻¹ (dark horizon column) and 0.37 mg g⁻¹ (solid diamond column).

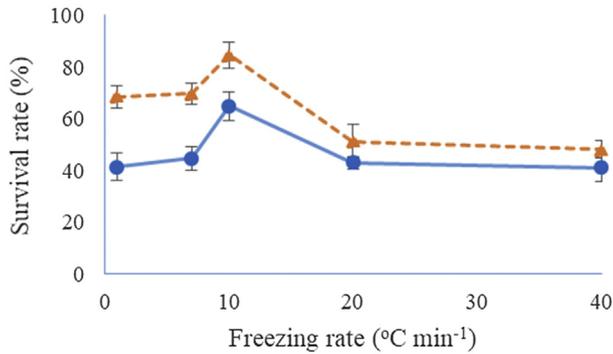


FIG. 2. Frozen-thawed *K. marxianus* viable cells, suspended in water, as a function of the cooling rate; no stigmasterol contained (solid line) and stigmasterol contained (dash line).

T. delbrueckii 60B3, a freeze-sensitive strain. They reported that both strains had similar in total lipid content but different in phospholipids and neutral lipids content.

The stigmasterol was found in cell membrane of the culture incubated in the medium added with stigmasterol. The function of sterol in protecting freezing injury was illustrated by Dawaliby et al. (28). According to their explanation, the absorbed sterol could interpolate between the phospholipids in the bilayer. At low temperature, sterol interfere the acyl-chain packing which prevent the stiffening of membrane. Somehow, we found that the different amount of stigmasterol contained in the cells had no effect on the survival of *K. marxianus*. For an economic reason, we chose a culture condition that contain the lowest amount of stigmasterol for further study.

Effect of stigmasterol on cell survival after freezing at various rate The survival of yeast cultures with stigmasterol were higher than those without stigmasterol when they were frozen at low freezing rate (Fig. 2). The low rate of freezing lead to increase intracellular concentration of solutes. The respond of yeast to subzero temperatures has been extensively reviewed (29,30). Each biological system has a specific optimal cooling rate, too low or too high cooling rate decreases the survival of cells (31). *K. marxianus* demonstrated the highest survival rate at the condition of 10°C min⁻¹ (Fig. 2). This result agreed with the study of Mazur (32) which reported that each cell had its own specific freezing rate depended on cell shape, structure, surface area-to-volume ratio and cell permeability, for yeast cells demonstrated the highest survival rate at the freezing rate of 7°C min⁻¹. Dumont et al. (33) reported that *S. cerevisiae* had the highest survival rate at 5°C min⁻¹. The stigmasterol did not alter the biological system of *K. marxianus*. Gerardo-Rodríguez et al. (34) reported the higher CO₂ production in quick dough frozen (1.75°C min⁻¹) than those in slow rate (0.14°C min⁻¹).

The survival of frozen-thawed cell depend on the physiological conditions (35). During the low cooling rate, the external medium is turned to ice while the liquid in cells generally remain unfrozen or in super-cooled at -10°C or -15°C (29). At this stage, the bulk of extracellular water crystallizes and leaves a highly concentrated solution. The ionic strength of intracellular solution is much lower than exterior of the cells. The cells are dehydrated to such extent to avoid ice crystallization. As a result, the cells have survived (36). The membrane permeability to liquid is a major factor to control the concentration of the cell solutes that it also affects the survival of cells. The data demonstrated the higher cell viability, almost 2 times, in the culture with stigmasterol at the low cooling velocity, 1–10°C min⁻¹. This result indicated that stigmasterol could be

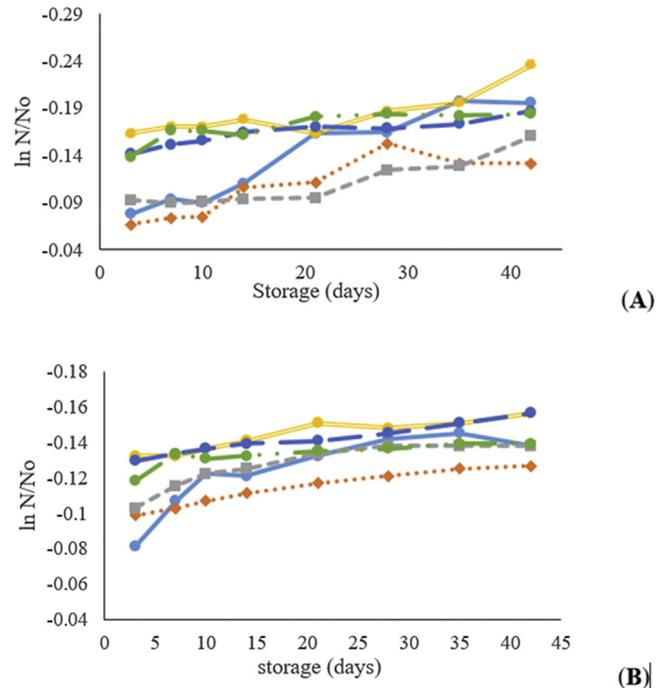


FIG. 3. The plot of viable cell reduction and storage time of *K. marxianus* cultures without stigmasterol contained (A) and with stigmasterol contained (B) under various subzero storage temperatures when stored under -10°C (solid line), -20°C (round dot line), -30°C (dash line), -40°C (double line), -60°C (long dash line) and -80°C (long dash dot line).

effected at the membrane of yeast and altered the membrane permeability to prevent the loss of cell viability.

There was no significant differences ($p > 0.05$) in survival rate among with and without stigmasterol cultures at the freezing rate higher than 10°C min⁻¹. At higher cooling rates than optimal condition, the intracellular dehydration may not be fast enough to prevent intracellular ice nucleation (37). Woelders et al. (38) suggested that the changes in size, shape and ultrastructure caused by the rapid efflux of water during fast cooling rate also contribute to the loss in viability.

Effect of stigmasterol on cell survival after frozen storage The number of living cells in suspension of yeasts decreased rapidly at first. After a period of storage, the reduction of living cells was slow (39). The reduction of *K. marxianus* viable cells demonstrated the same fashion. The relationship of the cell reduction and storage temperature is shown in Fig. 3. Under

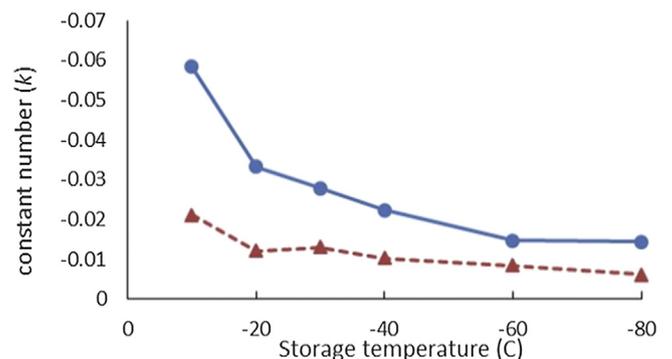


FIG. 4. The plot of reduction rate constant of *K. marxianus* viable cell as a function of subzero temperature, stored for 6 weeks; cells with no stigmasterol contained (solid line) and cells with stigmasterol contained (dash line).

subzero temperature storage, the high reduction rate of the viability was found in higher temperatures storage. The comparison of reduction rate (k) showed that stigmasterol had positive effects on the viability (Fig. 4). The cells with stigmasterol stored closer to zero Celsius, -20°C , showed the reduction rate of 0.012 week^{-1} , while those without stigmasterol stored at -80°C showed at 0.014 week^{-1} . The frozen storage temperature was reported to affect the viability of frozen cultures. Pejín et al. (40) reported the decrease of live yeast cells in dough to 50% after 28 days frozen storage. The commercial baker's yeast was reported a loss of 80% after 15 days under -20°C (41).

It was found that the rate of reduction in viability of the cell with stigmasterol stored at -20°C was closed to those without stigmasterol stored under -80°C . This demonstrated that stigmasterol provided a protection against freezing stress for a thermotolerant *K. marxianus*.

The absorbed polyunsaturated fatty acid, stigmasterol, by this thermotolerant yeast cell provided higher viability of the culture when subjected to the freezing stress. It showed a beneficial effect to the culture when cells were frozen at the low cooling rate and storage under subzero temperature. The survival rate of cells with stigmasterol stored in a commercial freezer (-20°C) was equal to those with no stigmasterol contained and stored in a deep freezing freezer (-60°C). This could be a beneficial practice for people in tropical temperature zone, where a deep freezing freezer consumes high energy and maintenance cost. It also could be an advantage for industries somewhere that ergosterol is found to be a controlled substance, like in Thailand. This practice could also increase the opportunities to apply the thermotolerant culture of *K. marxianus* in many frozen products.

In summary, the increase of *K. marxianus* cells viability, after subjected to freezing stress, was found in the culture grown in the medium added with stigmasterol, a plant sterol. Our study has shown that this practice increased the viability of the thermotolerant yeast cells at the commercial frozen storage temperature. They may be used in many food industries especially frozen dough where the live cells are required in the frozen products.

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