Mutagenic breeding of *Quambalaria cyanescens* strain for the production of milk-clotting enzymes

Chengzhong Wang[1], Hongyan Rao[1], Zhongkai Zhou[2*] and Yan Zhang[2]
[1]College of Food and Bioengineering, Qilu University of Technology Jinan, China
[2]Key Laboratory of Food Nutrition and Safety, Ministry of Education, Tianjin University of Science and Technology, Tianjin, China

Abstract: A strain for producing rennet was screened from red kojic rice and identified as *Quambalaria cyanescens*. In the current work, various mutagenic treatments, such as UV, diethyl sulfate and their combinations were applied for improving the production of milk-clotting enzymes. The mutagenic conditions were optimized based on the fatality rate of the strain. A significant increasing in the productivity of the fungal rennet produced from the strain *Quambalaria cyanescens* was achieved and its milk-clotting activity (MCA) was increased from 57 to 117 (SU mL^{-1}) through the mutagenic breeding. Further study showed that MCA was greatly inhibited ($P<0.0001$) by pepstain A, indicating it belongs to an aspartic acid protease, but absence of serine protease, metalloproteinase and cysteine protease. The mutated strain with the highest activity of milk-clotting enzymes showed a stable capacity to produce rennet through the test of its heritability. This is the first report of the breeding study of *Quambalaria cyanescens* for its capacity to produce microbial rennet.

Keywords: *Quambalaria cyanescens*, rennet, mutagenic breeding, milk-clotting activity.

INTRODUCTION

Milk coagulation is one of the key steps in cheese manufacturing (Bonfatti *et al.*, 2013). Calf rennet, the conventional milk-clotting enzyme obtained from the fourth stomach of suckling calves (Brown 1999) is the most widely used as coagulant in cheese making all over the world. However, the reduction in the supply of calf rennet and the ever increase of cheese production and consumption have stimulated the research for the alternatives of milk-clotting enzyme (MCE) (Areces *et al.*, 1992; Arima *et al.*, 1970; Escobar *et al.*, 1993; Lopes *et al.*, 1998), including microbial and plant rennet (Cavalcanti *et al.*, 2004). It was found that most of rennet from plant extractions was proved not to be well-satisfied because they impart a bitter taste to the cheese (Raposo *et al.*, 2008). Rennet obtained from microbita fermentation appears to be more promising compared to the rennet extracted from plants due to its cost effectiveness for the production, greater biochemical diversity, and easier genetic modification (Ageitos *et al.*, 2007). Microorganisms are known to be highly versatile in producing a wide range of enzymes, with varied patterns of activity. Although it has been known that many species of microorganisms have the capacity to produce milk-clotting enzymes (Arima *et al.*, 1970), only the MCEs produced by strains of *Rhizomucor miehei*, *Rhizomucor pusillus*var. *Lindt, Aspergillus oryzae* and *Enthothia parasitica* are widely used (Birkkjaer *et al.*, 1985; Crawford 1985; Thakur *et al.*, 1990). It has been reported that *Bacillus subtilis* (natto) Takahash was applied to produce milk-clotting enzymes (Chwen-Jen *et al.*, 2009); the factors affecting the enzymatic activity from the strain were also investigated (Chwen-Jen *et al.*, 2009), and its milk-clotting activity (MCA) and MCA/proteolytic activity (PA) ratio of the crude enzyme was comparable with those of Pfizer microbial rennin and Mucorrennin. Meanwhile, the crude enzyme showed an excellent pH value and thermal stability (Chwen-Jen *et al.*, 2009). Similarly, a milk-clotting enzyme named YS-1 was purified from a *Bacillus subtilis* (*B. subtilis)* YB-3, which was isolated from Tibetan Plateau, Gansu, China (Yang *et al.*, 2012). Results showed that YS-1 was stable over a wide range of temperatures and pH values. The purified enzyme exhibited a high specificity to substrate β-casein and yak milk casein and it led to a 75% more rapid coagulation of yak milk than cowmilk, which might be due to the higher β-casein content in yak milk than in cow milk (Yang *et al.*, 2012). *Bacillus subtilis* B1 was also found to have the ability to produce milk-clotting enzymes, characterized with low proteolytic activity (Ding *et al.*, 2011). Kurutahalli *et al.* (2010) studied 16 fungal strains and identified *Aspergillusoryzae* MTCC 5341 could be used to produce a high activity of milk-clotting enzymes. A new kind of milk-clotting enzyme was also found to exist in a vegetable source of *Jacaratiacorumbensis O. kuntze* (Duarte *et al.*, 2009). After fractional precipitation with ammonium sulphate and ion exchange chromatography purification, the enzyme properties were studied and found that its activity was inhibited by iodoacetic acid, which suggested that this enzyme was a cysteine protease. Response surface methodology was applied for extracting milk-clotting enzymes from *Solanum aethiopicum* seeds to optimize the operation conditions. The results showed that salt concentration, extraction time and pH value were the

**Corresponding author:* e-mail: zkzhou@tust.edu.cn

most important parameters of influencing the extraction efficiency of MCE from *S. aethiopicum* seeds (Guiama *et al.*, 2010).

In the present study, a strain *Quambalaria cyanescens* was isolated from red kojic rice and found it produced a certain activity of milk-clotting enzymes. Thus, this paper used *Quambalaria cyanescens* as an original strain and the improvement of activity of milk-clotting enzymes was determined through various mutagenic breeding conditions. After the strain purified, the time for the germination of spores was determined by microscope observation. The relationships among the strain death rate, UV radiation doses and diethyl sulfate (DES) acting time were established and a strain with the capacity to produce a higher MCA and a higher ratio MCA/PA was obtained. To the best of our knowledge, it is the first study of *Quambalaria cyanescens* for the production of rennet.

MATERIALS AND METHODS

Milk-clotting activity analysis

Milk clotting activity (MCA) was measured according to the procedure described by Arima *et al.* (1970) with slight modifications: before use, the culture medium was centrifuged at 20,200×*g* for 10min at 4°C. 10.0g of skim milk powder was dissolved in 100mL of solution containing 10mM $CaCl_2$. The clotting assay was carried out by mixing 10mL of the substrate with 1mL of culture sample. Clotting activity was expressed as Soxhelt Units (SU). One SU is defined as the amount of enzyme which clots 1mL of a solution containing 0.1g skim milk powder at 35°C for 40 min. Clotting activity was calculated using the following formula: SU=2400x D x 10/T, in which T is defined as coagulation time; D is the dilution multiple of the enzyme (He *et al.,* 2011; Srinivasan *et al.,* 1964).

Proteolytic activity analysis

Proteolytic activity (PA) was determined as followings: the reaction mixture was made up 1mL of 2% casein, 0.1mL of the sample, and 0.9mL of acetate buffer. The reaction was carried out at 35°C for 10 min, and then 2mL of 0.4M trichloroacetic acid (TCA) were added to terminate the reaction. Test tubes were centrifuged at 2, 300×g for 15min. 1mL of the supernatant was transferred into a new test tube, then 5mL of 0.4 M Na_2CO_3 was added and vortexed. The mixture was then incubated at 35°C for 20 min, and the absorbance was measured at 660 nm. A control was prepared, in which TCA was added before the addition of samples. One unit of PA is defined as 1mL of enzyme liquor that hydrolyzes casein and produces 1μg tyrosine at 35°C for one minute (pH 6.2). Enzymatic activity was calculated as follows: U=M x N/T, in which M is defined as amount of tyrosine produced during reaction (μg); T is reaction time, N is dilution multiple of the enzyme.

Determination of spore formation

In order to prepare a single spore suspension, the time for forming a spore has to be determined firstly. A glass container or glass slide is sterilized with 70% ethanol. It was quickly passed through the flame to burn off the alcohol. Melted medium was dipped on one side of glass container using an inoculation loop, and the fungi were inoculated on culture medium after solidification. The other three sides of the container were sealed with parafilm. A filter paper was immersed with glycerin and put into a sterilized culture dish, and some glass rods were set on the dish. The slides were put on glass rod and cultivated at 30°C, and examined under a microscope every day for the spore formation.

The preparation of spore suspension

The strains were activated and cultivated at 30°C on PDA to produce spores. 10mL of sterilized physiological saline was poured into PDA tube, which was covered with spores under a septic condition, and the spores were washed down through the gentle vibration. The solution was filtered by G3 glass funnel to get spore suspension, and the amount of spores were counted in blood counting chamber and it was diluted to the concentration of 10^6 cells mL^{-1}.

Ultraviolet (UV)-induced mutation

10mL of spore suspension prepared as above was transferred to a sterilized culture dish, put on a magnetic blender and transferred to an ultraviolet mutagenesis box. The sample was positioned at a vertical distance of 30cm from dish to the UV lamp (15 W). The spore suspension was mixed using a blender at a rate of 30 rpm, and set a different time intervals to induce spore suspension. After each mutation time, 1mL of UV-induced spore suspension was pipetted into sterilized test tube, and immediately cooled in ice water bath for 1h. Spores were transferred for culturing using a spread-plate technique under a red light and cultivated at 30°C for 72h in a dark place. All experiments were performed in triplicate. UV-induced mortality is calculated according to the number of colony and diluted times. The control was also prepared, which was subjected to the same procedure but without UV mutation. The survival curve was illustrated and the time of exposure was selected for optimizing the mutation process (Ikram-ul-Haq *et al.,* 2001).

Diethyl sulfate (DES)-induced mutation

The chemical mutagenesis was made using DES. Spore suspension was prepared as described at Section 2.4 with slight differences: 10mL of phosphate buffer (pH7.2) was used instead of sterilized physiological saline. 5mL aliquot from the prepared spore suspension and 2% DES phosphate buffer (prepared fresh daily) were pipetted respectively into a test tube, vortexed at different time intervals, then 0.5mL of $Na_2S_2O_3$ solution (2%, w/v) was added into the mixture for terminating the reaction. 1mL of the treated spore suspension was transferred into

sterilized test tubes and the following operations were same as described above. A control was also prepared, which was subjected to the same procedure but without DES addition. The survival curve was prepared and time of treatment was selected for optimizing the mutation process (Mohamed *et al.,* 2011).

Mutation combination

In order to achieve the optimum mutagenic results, a combination of mutagenesis of UV and DES were also used in this study. There were two schemes used for the mutation of the strains: the suspension was firstly induced by UV mutation, and followed by DES treatment; alternatively, the mutation sequence of the two treatments was swapped, eg. DES treated first, and followed by UV-induced mutation. After the treatments, the suspensions were kept in darkness at 30°C for 48h. The spores were transferred to the culture by a spread-plate technique under a red light and cultivated at 30°C for 72 h without lights. The complex UV/DES-induced mutations and the microbiota fatality rate (survivability) were examined under the different treating conditions.

The screening of the mutated strains

The strains from the culture medium plate were randomly selected, and two loops of the strains were inoculated into 50mL of PDA medium under aseptic conditions. The inoculum was allowed to grow at 30°C on a rotary shaker (160 rpm) for 4 days. The strains without mutation treatment were used as the control. Strains with milk-clotting activity being 10% higher than the control were harvested. The above fungal population was used for secondary screening. Briefly, spore suspension was prepared and the concentration of spores is adjusted to 10^7 cells mL^{-1}, and then 1% of spore suspension was inoculated into the fermentation liquid and cultivated at 30°C on a rotary shaker (160 rpm) for 4 days.

Effect of protease inhibitors on the activity of milk-clotting enzymes

Inhibitors of metallo proteases (ethylenediaminetetracetic acid, EDTA), cysteine proteases (transepoxy-succinylleucyl-amido-(4-guanidino)-butane; E-64), serine proteases (phenylmethylsulfonyl fluoride, PMSF), and aspartic proteases (pepstatin A) were added into precipitated protein fraction (1ml, 32mg of protein, PP) and the mixture was incubated at 37°C for 30 min. Subsequently, the incubated mixtures were evaluated for the milk-clotting activities. Inhibition percentages were calculated as follows: % inhibition = 100-[100×(residual activity/activity in control without inhibitor)].

Assay of genetic stability

The selected mutant was inoculated on the PDA slant for a spreading experiment. The curd enzyme activity was determined through the fermentation of the wave bottle up to a total of five generations of spreading. The influence of spread times on enzyme production was analyzed for expressing the strain's genetic stability.

STATISTICAL ANALYSIS

Each experiment was performed three times, and results were expressed as mean ±SD. Experimental data were subjected to the analysis of variance using Genstat 5 (Clarendon, NY). The statistical differences between the treatment and control were determined by Independent-Samples t-test *P<0.05.

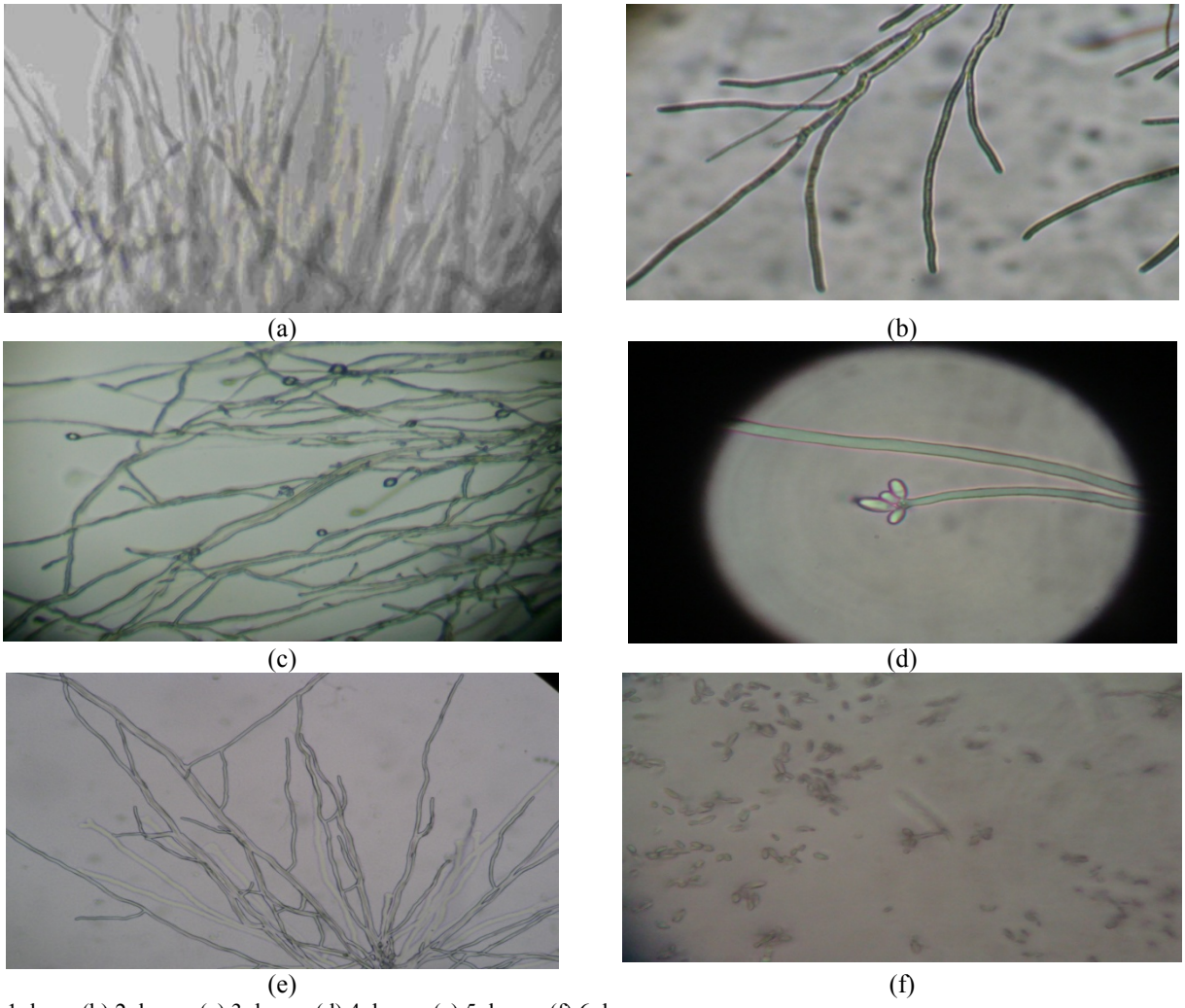
RESULTS

The germination of spore

The germination of spore at different time intervals from 1 to 6 days was presented in Figure 1, which showed that the spore germinated and mycelia has grown up at day 1 (Figure 1a). On the second day, mycelia stretched continually, and the branches were noticeable on the top of the mycelia. The top of the mycelia appeared along with the culturing time (Figure1b). There were no distinct changes on the fourth day for the morphology of the strains except that the primary conidia increased. Conidiole started to appear and increased on the numbers on the fifth day. From the sixth day, the amount of spores increased quickly, and gradually began to fall off from the mycelia. At that stage, a large number of spores appeared around mycelia. Considering the maturation of the spores at different stages, spores cultivated for 6 days were selected for the preparation of the spore suspension (Figure 1f).

Effect of UV mutation time on the fatality rate of the strain

The relationship between UV-induced fatality and mutation time was shown in Figure 2. UV radiation is a popular physical mutation-induction method, since the absorption spectrum of microbial nucleic acid is consistent with ultraviolet, and the DNA can be influenced and mutated. According to our previous experience of UV breeding (data not shown), the positive mutation on the microorganisms often appears within a low dose range. In general, an ideal effect from UV-induced mutation could be achieved when the fatality ranges from 70% to 80%. In contrast, the reversion mutation is usually very high when the fatality of UV mutation reaches up to 95%-99%. Burger *et al.* (2002) also found that UV irradiation of *E. coli* produces photoproducts in the DNA genome. Thus, some bacteria lose viability (colony-forming ability) or remain viable as mutant cells. However, the end-points of viability inactivation (lethality) or mutation are determined by cellular processes that act on the UV-damaged DNA. It was also seen from Figure 2 that the spores were almost dead when the mutation time was up to 5 min and even afterwards. Thus, the results from Figure 1 indicated that the mutation time with 70%-80% of fatality was 4 min. Considering a suitable mortality and positive mutation effectiveness, the UV-induced mutation time was chosen to be 4 min.



(a) (b) (c) (d) (e) (f)

(a) 1 day (b) 2 days (c) 3 days (d) 4 days (e) 5 days (f) 6 days

Fig. 1: The germination of spores visualized using an oil microscope at different time intervals.

Table 1: The change in MCA level before and after UV-induced mutation for the primary screening

Strain	MCA ($SUmL^{-1}$)	Amplification (%)	Strain	MCA ($SUmL^{-1}$)	Amplification (%)
UV1	46.42	-2.13	UV17	90.15	90.07
UV2	58.03	22.35	UV18	38.15	-19.57
UV3	46.03	-2.95	UV19	42.10	-11.24
UV4	44.07	-7.08	UV20	46.62	-1.71
UV5	64.36	35.69	UV21	40.43	-14.76
UV6	37.88	-20.13	UV22	48.33	1.90
UV7	42.45	-10.50	UV23	40.27	-15.10
UV8	37.88	-20.13	UV24	34.88	-26.46
UV9	42.62	-10.14	UV25	50.95	7.42
UV10	41.16	-13.22	UV26	48.65	2.57
UV11	47.13	-0.63	UV27	35.40	-25.36
UV12	46.82	-1.29	UV28	38.22	-19.42
UV13	45.65	-3.75	UV29	44.04	-7.15
UV14	43.73	-7.80	UV30	45.05	-5.02
UV15	45.94	-3.14	Control	47.43	0.00
UV16	46.13	-2.74%			

Table 2: The change in MCA level before and after DES-induced mutation for the primary screening

Strain	MCA ($SUmL^{-1}$)	Amplification (%)	Strain	MCA ($SUmL^{-1}$)	Amplification (%)
DES1	47.80	-8.38	DES17	48.14	-7.72
DES2	50.19	-3.80	DES18	57.56	10.33
DES3	52.12	-0.10	DES19	51.83	-0.65
DES4	45.87	-12.08	DES20	36.34	-30.34
DES5	49.15	-5.79	DES21	80.81	54.90
DES6	35.71	-31.55	DES22	56.85	8.97
DES7	48.06	-7.88	DES23	45.32	-13.13
DES8	50.55	-3.11	DES24	37.91	-27.33
DES9	51.34	-1.59	DES25	43.06	-17.46
DES10	35.37	-32.20	DES26	36.00	-30.99
DES11	50.00	-4.16	DES27	51.43	-1.42
DES12	64.34	23.33	DES28	41.74	-19.99
DES13	55.92	7.19	DES29	34.09	-34.66
DES14	49.55	-5.02	DES30	50.65	-2.91
DES15	36.44	-30.15	Control	52.17	0.00
DES16	47.03	-9.85			

Table 3: The change in MCA level before and after UV-DES mutation for the primary screening

Strain	MCA ($SU\ mL^{-1}$)	Amplification (%)	Strain	MCA ($SUmL^{-1}$)	Amplification (%)
UD1	87.59	58.71	UD17	54.54	-1.18
UD2	49.06	-11.11	UD18	99.18	79.71
UD3	56.59	2.54	UD19	53.50	-3.06
UD4	44.56	-19.26	UD20	50.00	-9.40
UD5	53.35	-3.33	UD21	46.80	-15.20
UD6	55.33	0.25	UD22	35.33	-35.98
UD7	66.42	20.35	UD23	54.39	-1.45
UD8	51.70	-6.32	UD24	51.95	-5.87
UD9	84.68	53.43	UD25	33.14	-39.95
UD10	44.88	-18.68	UD26	53.34	-3.35
UD11	51.94	-5.89	UD27	50.65	-8.23
UD12	42.28	-23.39	UD28	42.46	-23.07
UD13	36.33	-34.17	UD29	43.59	-21.02
UD14	54.79	-0.72	UD30	40.23	-27.11
UD15	51.93	-5.91	Control	55.19	0.00
UD16	41.08	-25.57			

Effect of diethyl sulfate (DES)-induced mutation on the fatality rate of the strain

At a certain dosage of DES used in the suspension, the influence of exposure time of DES on the microbiota survivability was investigated, and the relationship between DES-induced fatality and mutation time was demonstrated in Figure 3. This study clearly showed that the mortality rate was enhanced gradually with the increment of the mutation time from 0 to 40 min. The mortality was increased at a slower rate while the exposure time was up to 40 min. The concentration of mutagen and the time of mutation are crucial to mutation efficiency. If the concentration is higher and the time is longer, the harm would be greater and the chance of screening would be lost. However, if the concentration is lower and the time is shorter, variability would be less and the chance of screening would be reduced as well. For controlling an appropriate fatality rate for *Quambalaria cyanescens* with a range from 70 to 80%, the treatment time was selected to be 40 min.

Screening of mutant strains

The large single colonies were chosen randomly from above mutant strains and they were labeled as following descriptions: UV1~30 (UV-induced mutants); DES1~30 (DES-induced mutation); UD1~30 (UV mutation first, and then DES mutation); DU1~30 (DES mutation first, and then UV induced mutation). Transferred the single colony on the PDA culture and cultivated for 6 days, then stored in refrigerator at 4°C for the primary screening.

Table 4: The change in MCA level before and after DES-UV mutation for the primary screening

Strain	MCA (SU mL^{-1})	Amplification (%)	Strain	MCA (SU mL^{-1})	Amplification (%)
DU1	53.66	-1.11	DU17	52.43	-3.37
DU2	49.41	-8.94	DU18	49.98	-7.89
DU3	54.18	-0.15	DU19	48.09	-11.37
DU4	43.02	-20.72	DU20	50.51	-6.91
DU5	46.12	-15.00	DU21	43.4	-20.01
DU6	49.42	-8.92	DU22	81.8	50.76
DU7	54.05	-0.39	DU23	72.33	33.30
DU8	49.61	-8.57	DU24	69.99	28.99
DU9	51.55	-4.99	DU25	44.95	-17.16
DU10	81.97	51.07	DU26	47.31	-12.81
DU11	46.08	-15.08	DU27	35.16	-35.20
DU12	91.66	68.93	DU28	38.96	-28.20
DU13	53.36	-1.66	DU29	51.83	-4.48
DU14	50.46	-7.00	DU30	39.18	-27.79
DU15	54.9	1.18	Control	54.26	0.00
DU16	55.23	1.79			

Table 5: The production of MCA of different strains after the second screening

Strain	MCA (SU mL^{-1})	Amplification (%)	Strain	MCA (SU mL^{-1})	Amplification (%)
UV2	63.92	12.10	UD18	69.46	21.82
UV5	54.67	-3.65	DU10	116.98	105.16
UV17	52.25	-7.92	DU12	77.92	36.65
DES12	54.81	-3.40	DU22	52.7	-7.58
DES21	65.81	15.99	DU23	48.59	-14.78
UD1	54.75	-3.98	DU24	37.82	-33.67
UD7	49.94	-12.42	Control	57.02	0.00
UD9	53.99	-5.31			

Table 6: Different effect of protease inhibitors on the activity of milk-clotting enzymes

Inhibitors	The target of protease	Concentration (mM)	Milk-clotting activity (%)
Control		0	100 ± 0.38^{a}
PMSF	Serine protease	10	99 ± 2.4^{a}
EDTA	Metallo proteinases	10	100 ± 2.5^{a}
pepstain A	Aspartic acid protease	0.02	0 ± 0.53^{c}
E-64	Cysteine protease	0.01	98 ± 1.6^{a}

Different uppercase letters indicate significant differences at $p<0.05$. Control treatment corresponds to incubation of PP for 24 h at 28°C.

Primary screening of UV-induced mutants

The results of primary screening from UV-induced mutants are summarized in table 1. The level of MCA was increased for several mutants compared to the original strain. For example, the relative clotting milk activity of UV2, UV5 and UV17 was enhanced to be more than 110% than the original. Thus, these three mutants, e.g. UV2, UV5 and UV17, were chosen for the secondary screening.

Primary screening of DES-induced mutants

The effect of DES mutation on MCA level was determined and results were listed in table 2. After the mutation, DES12, DES13, DES18, DES21 and DES22 showed an increasing of MCA compared to the original strains. Among them, DES12 and DES21 had the highest MCA levels, and their relative activity of MCA was more than 110% than that from the original strain. Therefore, DES12 and DES21 would be the most potential mutated strains for the secondary screening.

Primary screening of strains mutated by UV first and then DES

The effect of the combined mutation treatments on MCA was illustrated in table 3. The overall effectiveness on the increasing of MCA level was improved from the combined mutation treatments compared to the single mutagenesis ($P<0.05$). After the mutation, strains UD1, UD7, UD9 and UD18 were probably the positive mutated strains with more than 110% increase in MCA level

compared to the original strains. Thus, the four mutated strains were transferred onto the PDA medium for the secondary screening.

Primary screening of strains mutated by DES first and then UV

Similar to the results at table 3, the combined mutation treatment significantly changed the MCA levels ($P<0.001$) and the influence of the combined mutation treatments on MCA was presented in table 4. In contrast to the sole mutagenesis, the effectiveness on the enhancement of MCA level was achieved through the combined mutation treatments ($P<0.05$). From table 4, it could be concluded that the clotting-milk activity of DU10, DU12, DU22, DU23 and DU24 were more than 110% increased. Thus, they were treated for the secondary screening.

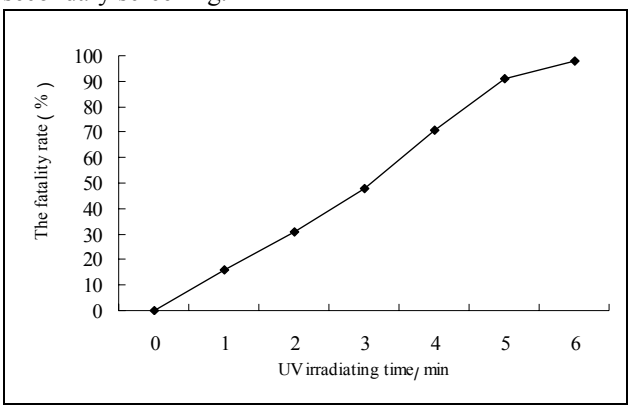


Fig. 2: The relationship between the UV-induced fatality and mutation time

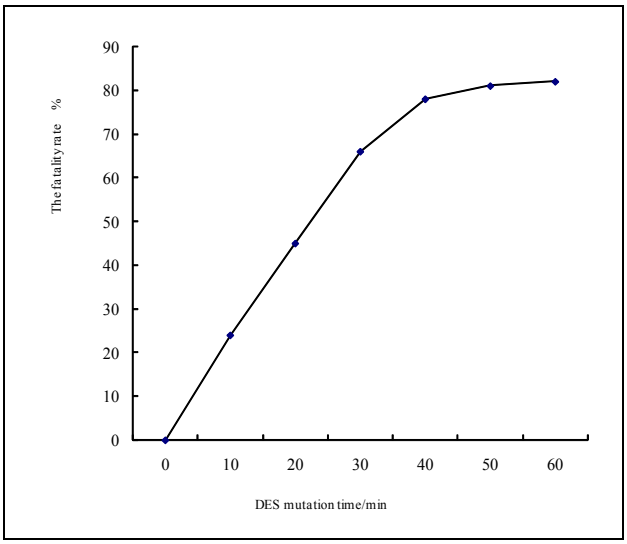


Fig. 3: Effect of the DES mutation time on the fatality

Secondary screening of the strains for a higher production of MCA

After the secondary screening, the MCA from each strain was measured and the results are shown in table 5. The MCA of UV2 and DES21 strains increased 12.10% and 15.99% respectively. However, the MCA of UD18, DU10 and DU12 strains increased 21.82%, 105.16% and 36.65% respectively. The MCA of DU10 was shown to be the highest among all the tested strain. Thus, after the mutagenic breeding of *Quambalaria cyanescens*, the selected strain (i.e., DU10) will be used for the study of its fermentation properties.

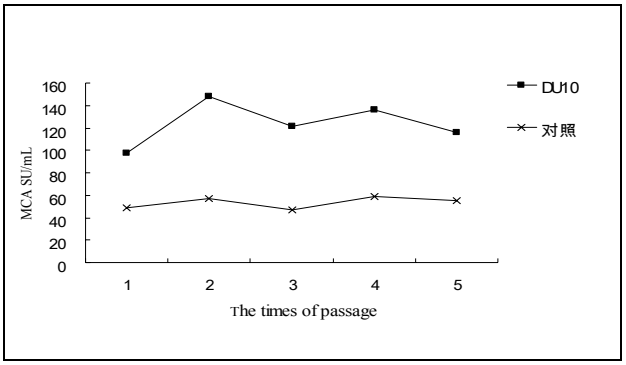


Fig. 4: The heritability of DU10

Effect of protease inhibitors on the activity of milk-clotting enzymes

The different inhibiting effectiveness of the selected inhibitors on the activity of milk-clotting enzymes is presented in table 6. The result shows that PMSF, EDTA and E-64 had no significantly inhibiting influence on the activity of *Quambalaria cyanescens* rennet, indicating rennet from *Quambalaria cyanescens* strain belongs to an aspartic acid protease. The absence of the inhibiting effectiveness on the activity of milk-clotting enzymes might suggest that serine protease, metalloproteinase and cysteine protease in the rennet from *Quambalaria cyanescens* are almost exclusive. Chymosin and milk-clotting enzymes from *C. cardunculus* flowers and *Strebus aspler* twigs are also reported to be aspartic proteases (Llorente *et al.*, 2004; Senthilkumar *et al.*, 2006).

The genetic stability measurement

Inoculated DU10 was used for the test of the heritability of the strain, and its enzymatic properties of potato glucose medium fermentation were measured for each passage and continued up to five generations. The result of experiment is shown in fig. 4, indicating that the enzyme properties and the heritability of the strain is stable in each generation, so the strain is a very useful one for the potential applications. The fermentation conditions for the production of milk-clotting enzymes and the enzymatic properties will be investigated in detailed in another study.

DISCUSSION

The determination of spore germination process is important for preparing a suitable spore suspension. The germination of spores was affected by many factors, and

the nutrient elements have been studied more extensively for some microorganisms (Aouadhi *et al.*, 2013). Nutrient-induced germination can be considered as the natural germination pathway, and this requires specific receptors. Germination is initiated when nutrient molecules activate the Ger receptors present in the spore's inner membrane (Paidhungat *et al.,* 2001). Afterwards, the release of Ca2þDPA and rapid efflux of monovalent cations (Hþ, Naþand Kþ) could be visualized, and the core is partly rehydrated. After that, the hydrolysis of the oppressing cortex layer, accomplished by cortex-lytic enzymes CwlJ and SleB, becomes crucial to provide more space to the expanding core. Following the cortex degradation, the fully rehydrated core allows the reactivation of enzymes and the synthesis of ATP from the 3-PGA (3-posphoglyceric acid) precursor. Degradation of the SASP proteins releases the spore's DNA, and the spore initiates RNA, protein, and DNA syntheses in the further outgrowth phase (Setlow, 2003).

DES is one of the mostly used mutagenic agents, and its mutagenesis has been recognized as a valuable supplement to the conventional breeding methods for crop improvement (Dhanayanth *et al.*, 2000). Many researchers have reported the role of chemical mutagens in enhancing genetic variability in higher plants (Ricardo *et al.,* 1998; Kumar *et al.*, 2009). It has been reported that the concentration of mutagen with lethality ranging from 65% to 75% was suitable for selecting the positive mutation strains (Liu *et al.,* 2011). Thus, in the current study, the treatment time was selected to be 40min so as to control an appropriate fatality rate for *Quambalaria cyanescens* to be within a range of 70 to 80%.

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After the mutagenic breeding, the MCA of strain DU10 was increased by 105.16%, indicating the MCA from strain DU10 was the highest among all the tested strains. Thus, after the mutagenic breeding of *Quambalaria cyanescens*, the selected strain (i.e., DU10) will be used for the study of its fermentation properties in the future because the operational and nutritional factors would greatly influence the production of MCA. For example, some microorganisms in sucrose (0.5%) were the most favorable carbon source for *Penicillium oxalicum* (Hashem 1999), *Aspergillus versicolor* (Abdel-Fattah *et al.,* 1979) and *B. subtilis* (natto) Takahashi (50g L^{-1}) (Chwen-Jen *et al*., 2009) to produce high yield of MCA. Glucose, on the other hand, was not favorable for *P. oxalicum* (Hashem, 1999), *M. baciliformis* (Areces *et al.*, 1992), and did not influence the enzyme production for *Nocardiopsis sp*. (Cavalcanti *et al*., 2005). However, sucrose and molasses were not considered as good carbon sources for *M. miehei* to produce MCA in solid-state and submerged fermentation, but glucose instead is a better choice of carbon source for *M. miehei* (Chazarra *et al.,* 2007; Silveira *et al.*, 2005). Thus, the next step of this study would optimize the fermentation properties of *Quambalaria cyanescens* for the production of higher level of MCA. Moreover, the genetic stability measurement showed that the enzyme properties and the heritability of the strain are stable, indicating the strain has a great potential application in the future.

CONCLUSION

The activity of milk-clotting enzymes produced from *Quambalaria cyanescens* was improved through mutagenic breeding. Considering the synergic effectiveness of the mutations from the combined mutagenic treatments (physical mutagen from UV, and chemical mutagen from DES), a strain of *Quambalaria cyanescens* with the highest yield of rennet was obtained through the breeding process. The inhibition study on the enzymes activity indicates that the rennet from *Quambalaria cyanescens* strain belongs to an aspartic acid protease, but exclusive of serine protease, metalloproteinase and cysteine protease. The targeted strain (DU10) was subcultured up to 5 generations showing a stable capacity to produce the enzymes.

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