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The survival investigation of sonicated soy sauce fermentation yeast strains using a spectrophotometer

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Abstract

The present work demonstrated a method to determine the growing activities of typical soy sauce fermentation yeasts under sonication stimulation in stress and/or enriched conditions. The yeast activities were determined using a programmed spectrophotometer which automatically records the optical density (OD) of the growth media. The increasing OD values were directly proportionally to the amount of yeast cells within the growth media. Spectrometry method provides a typical sigmoidal curve with the lag time, maximum growth rate, and average growth rate of microorganisms. Activities of two industrial yeast strains (*Zygosaccharomyces* spp. and *Candida* spp.) under sonication stimulations (0.5 cycles, 60 amplitude) at different salt contents (1, 3, and 5%), glucose contents (5, 10, and 15%), sonication durations (10, 20, and 30 min), and incubation temperatures (30 and 37°C) were investigated in a 24-h growth cycle model system by one-factor-at-a-time (OFAT) design. Overall, sonication treatment in the presence of salt was not lethal to yeast cells. The maximum growth rate of both yeast strains was significantly reduced ($p < 0.05$) when the sample was treated with 5% salt content coupled with sonication. However, when the sample was further enriched with glucose at 10%, it yielded significantly highest growth rate (0.1310 ± 0.0107 OD/h). Finally, 10 min sonication and 30°C incubation temperature were found to be better as compared to the other treatment combinations. Sonication did not exert any detrimental effect on investigated yeasts, and stimulated the growth when enrichment was appropriately provided.

Introduction

Fermentation technology is widely applied on various foods. Fermentation involves complicated chains of reactions between microorganisms. To date, numerous species of moulds, yeasts, and bacteria from different soy sauce fermentation process have been isolated and characterised. Among the various yeasts species, *Zygosaccharomyces* and *Candida* are predominantly found during the *moromi* fermentation due to their high salt resistance (Wah *et al.,* 2013). Moreover, the interaction of microflora during the fermentation process is dynamic between the raw materials provided and the compositions of microflora, and this changes over time (Tanaka *et al.,* 2012; Yan *et al.,* 2013).

Soy sauce fermentation performed by the industries is a well-established technology. The basic process consists of two phases, namely a solid fermentation stage (*koji*), followed by mash fermentation (*moromi*). The fermentation is started by making *koji*, and followed by *moromi* until the desired maturation stage, which varies from months to years according to need (Yongmei *et al.,* 2009; Fidaleo *et al.,* 2012).

A culture strains can be evolved through laboratory selections to withstand stresses such as salt, which is the essential ingredient in soy sauce fermentation (Dhar *et al.,* 2011). Besides, modernised fermentation operations are more likely to have unique starter culture compositions evolved by natural selection over the generations; this variation also indirectly creates different regional types of soy sauce.

However, fermentation is time-consuming.

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The fermentation of a soy sauce can be as long as six months if a traditional fermentation method is employed (Fidaleo *et al.,* 2012). Research showed that a sonication stimulation was able to accelerate the fermentation of soy sauce in a laboratory scale (Goh *et al.,* 2017). Sonication technology is widely explored in the fermentation of milk products (Nguyen *et al.,* 2009; Gholamhosseinpour and Hashemi, 2019), soymilk (Ewe *et al.,* 2012a; 2012b), and apple cider (Al Daccache *et al.,* 2020). Ultrasound waves exerted by the sonication probe can increase the permeability of the cell walls, thus increasing the fermentation rate. Sonication can also be used if the inactivation of enzyme and/or microorganism is required (Gabriel, 2015). Therefore, it sounds logical to choose a stress-tolerant starting culture, which can survive and work throughout the fermentation process. However, a sonication stimulation during fermentation poses the question whether a suitable stimulation will accelerate or decelerate the fermentation process (Ojha *et al.,* 2017). Spectrometry method was chosen in the present work to provide a simple and straightforward method that allows the user to read the activities of soy sauce yeast growing pattern. Normally, a direct plating method was used to enumerate the number of viable microorganisms over the growing cycle, and produce a sigmoid growth curve by plotting the viable cell number (*y*-axis, usually in log CFU) versus the incubation time (*x*-axis, usually in h). This method allows the user to select stress-tolerant species by assessing its growth rate and viable cell number. However, direct plating and enumeration are labour-intensive, and could introduce human error. Alternatively, a spectrophotometer which is programmed to measure the absorbance (optical density, OD) of the growth medium at the desired interval is able to produce a typical sigmoidal growth curve (by plotting OD values versus time), whereby the lag time, maximum growth rate, and average growth rate of microorganism can be derived and presented in numerical data (OD/h) to evaluate the growth rate of the microorganism of interest (Madigan *et al.,* 2010).

In the present work, two commonly used industrial soy sauce yeast composites (dry form) were purchased from the industry, namely *Zygosaccharomyces* spp. and *Candida* spp. The yeast survival and activities under stressed (a common soy sauce fermentation condition with salt), enriched (yeasts were supplied with glucose in the growth medium), and sonication stimulations were observed by the spectrometry method using the one-factor-at-a-time (OFAT) experimental design. By manipulating only one factor at a time, the behaviour of these selected soy sauce yeasts could be observed. It was hypothesised that salt was a definite stress factor to the targeted yeast strains, whereas sonication and glucose levels could either act as enhancer or inhibitor of growth.

Materials and method

Materials

Two yeast composites were purchase from Angel Yeast Co. Ltd., China, namely *Zygosaccharomyces* spp. (Strain A) and *Candida* spp. (Strain B). The culture broth was made up of 1% yeast extract, 2% glucose, and 2% of peptone (YPD broth).

Activation

The yeast composites were inoculated into YPD broth at a 1:10 ratio, and incubated at 30°C for 24 h; then they were inoculated as slant culture on Potato Dextrose Agar (PDA), and kept as stock cultures at 4°C for up to two months. Multiple slants were prepared as replicates for the experiments. Prior to treatment and analysis, the culture was inoculated from the stock slant into 10 mL of YPD broth, and activated for 24 h (Qi *et al.,* 2013).

Determination of the standard growth curve of selected yeast

Activated yeast cells in 10 mL of YPD broth were diluted with 90 mL of YPD broth, the formula of which varied according to experimental factors. After that, the 100 mL YPD broth was subjected to treatment (sonication) if necessary. The determination of optical density (OD) was carried out using the Cary 60 spectrophotometer, with Agilent's WinCar software kinetics setting at 600 nm (OD_{600}), which automatically measured the OD at every 120 min interval for 1,440 min (24 h). A quartz rectangular cuvette with a 10 mm path length was filled with 3 mL of culture, and the temperature was controlled by the Peltier temperature control unit. A sterilised 2 mm magnetic stirrer bar was inserted into the cuvette to allow the spinning of the culture along the growth cycle. Upon the completion of a 24-h growth cycle, a standard sigmoid curve was generated, from which the lag phase durations, maximum growth rate (slope), and the average growth rate were calculated.

Sonication of yeast cells

Sonication was carried out by using the Sartorius Stedim biotech ultrasonic homogeniser (Labsonic P, Germany). The parameter used was adapted from a previous study (Goh *et al.,* 2017). The generator converts the supplied voltage of 230 V/50

Hz into longitudinal mechanical vibrations at 24 kHz through a 14 mm diameter titanium alloy standing probe. The ultrasonic probe was inserted at 25 mm depths to the targeted sample, and each was contained in a 100 mL volume laboratory bottle with treatment set at 0.5 cycles and 60% amplitude. The 0.5 cycles indicated that the sonication was exerted for 0.5 s for every 1 s duration, and 60% amplitude indicated that the sonication intensity was delivered to the medium at 60% through the 14 mm sonication probe surface.

Calculation of lag phase, slope, and average growth rate from the sigmoid curve

The standard sigmoidal curve obtained from spectrometry was plotted as the OD values as a function of time (min). The curve was further divided into the lag phase, log phase, and stationary phase. Lag time was measured by reading the intercept between the tangent line and the propagation of the lag phase across the *x*-axis (min). The maximum growth rate was represented by the log phase, which was calculated from the tangent line slope (OD/h). The average growth rate was calculated by dividing the difference between the initial and final OD by the total run time (Jomdecha and Prateepasen, 2011).

Experimental design

The growth curve of yeast strains was determined by spectrometry in standard YPD broth without the designed treatment at a growing temperature of 30°C as a control (standard YPD broth with 0% salt, 0% glucose enrichment, and 0 min sonication). Yeast cells were first treated by different salt contents (1, 3, and 5%), and then the next batch was combinations of salt and sonication treatment (0.5 cycles, 60% amplitude) for 10 min. Then, only one yeast strain was selected for further investigation based on their tolerance at 5% salt with sonication. After that, the glucose enrichment was given at 5, 10, and 15%, and the sonication durations were set for 0, 10, 20, and 30 min. Finally, the effect of temperature on yeast growth was studied at 30 and 37°C.

Statistical analysis

Minitab 16 was used for all statistical analyses. The significance among the results were obtained by analysis of variance (One-way ANOVA) with Tukey's test at a confidence level of 95% (α = 0.05).

Results and discussions

Effects of salt on soy sauce yeast growth rate

Salt is considered an essential ingredient in the fermentation of soy sauce because it provides organoleptic properties to the end product and control the growth of undesired microorganisms during fermentation (Song *et al.,* 2015). The reactions among the halophilic aromatic yeasts will create the unique flavour of typical soy sauce (Wah *et al.,* 2013). However, the presence of salt is also a stress factor which is inhibiting the microorganisms to grow at optimal level. Generally, the presence of salt increases the concentration of ionic (Na^+) solutes (Dakal *et al.,* 2014). The presence of Na^+ directly decreases the water activity and becomes the major stressor limiting the yeast growth (Morin-Sardin *et al.,* 2016). From our study, Table 1 shows that both soy sauce yeast activities were significantly reduced at 5% salt addition when compared with control. Although the lag phase duration was not significantly affected, the maximum growth rate and average growth rate were reduced. Moreover, we observed that both yeast strains were showing a very low increment in OD values at 10% salt or higher (Liu *et al.,* 2020), which was relatively insufficient to produce a standard sigmoid curve (therefore data not shown). However, we did not conclude that the growth of both yeast strains was inhibited by a 10%

Table 1. Activities of two industrial soy sauce fermentation yeast strains for control and after salt addition at 30°C for a 24-h growth cycle.

Salt content (%)	Lag phase duration (min)		Max. growth rate (OD/h)		Average growth rate (OD/h)	
	A	B	A	B	A	B
Control	205 ± 49^{aA}	160 ± 57^{aA}	0.3137 ± 0.0317^{aA}	0.1964 ± 0.0399^{abA}	0.0881 ± 0.0001^{aA}	0.0674 ± 0.0092^{aA}
1	180 ± 28^{aA}	145 ± 78^{aA}	0.2240 ± 0.0107^{bA}	0.2544 ± 0.0493^{aA}	0.0797 ± 0.0010^{bA}	0.0819 ± 0.0081^{aA}
3	240 ± 0^{aA}	185 ± 92^{aA}	0.1687 ± 0.0037^{bA}	0.1703 ± 0.0201^{abA}	$0.0700 \pm 0.0011b^{cA}$	0.0743 ± 0.0133^{aA}
5	250 ± 70^{aA}	190 ± 14^{aA}	0.07318 ± 0.0015^{cA}	0.0617 ± 0.0201^{bA}	0.0564 ± 0.0010^{dA}	0.0459 ± 0.0030^{aB}

Values are mean of one determination from two replicate experiments ± SD. Mean values with different superscript letters in the same column are significantly different at $p < 0.05$ by ANOVA and the Tukey's test. Mean $^{A-B}$ values of the same row for each parameter are significantly different at $p < 0.05$ by ANOVA and the Tukey's test. Strain A = *Zygosaccharomyces* spp. composite; and Strain B = *Candida* spp. composite.

salt solution. These yeasts were considered as osmotolerant and halotolerant (Dakal *et al.,* 2014). To date, most studies and reviews suggest that salt addition in soy sauce is necessary because it prevents microbial contamination, especially those of the pathogenic ones (Han *et al.,* 2004; Wei *et al.,* 2013). However, the presence of sodium chloride also reduces most of the enzymatic reactions, especially protease activity. Thus, higher salt contents prolong the soy sauce maturation (Su *et al.,* 2005). In our observations, salt was a stress factor that lowers and/or hinders the growth of the activities of both soy sauce yeasts used in the present work. Salt content above 10% was not assessed because the growth was extremely slow to match a typical sigmoidal curve within the growth duration observation (24 h).

Effects of sonication under stressed and enriched conditions

From Table 2, we can see that the sonication treatment did not significantly affect the growth pattern of both yeasts. Surprisingly, there was a slight increment in the maximum growth rate observed in strain B. From Table 3, sonication coupled with salt treatment led to decreased growth rates. At this stage, strain A was not favoured for further investigation because of its lower stress-tolerance when compared with strain B in both salt and/or sonication treatments. Moreover, overall observation showed that the lag phase of both yeast species were not significantly reduced under all treatment combinations at this point. The average growth rate exhibited relatively minor differences. Therefore, the maximum growth rate (slope in the exponential phase) was considered as the main parameter to determine the stress-tolerance of the yeasts because it directly reflects the multiplication speed of cells (Boulton and Quain, 2008). Thus, the maximum growth rate calculated served as a direct indication of the fermentation speed in our model system.

In soy sauce fermentation, quantities of soybeans and wheat grains are always abundant, and provide adequate nutrients for yeasts, and thus the enzyme activity and maximum cell number become the limiting factors in their growth. To simulate the real scenario, different levels of glucose enrichment were added to the growth medium, and the results are shown in Table 4. When the selected yeast was cultured in the enriched growth media followed by sonication stimulation, the highest growth rate was observed at 10% glucose enrichment instead of 5 or

Table 2. Activities of two industrial soy sauce fermentation yeast strains for control and after sonication treatment (0.5 cycles, 60% amplitude for 10 min) at 30°C for a 24-h growth cycle.

Treatment	Lag phase duration (min)		Max. growth rate (OD/h)		Average growth rate (OD/h)	
	A	B	A	B	A	B
Control	205 ± 49^{aA}	160 ± 56^{aA}	0.3137 ± 0.0317^{aA}	0.1964 ± 0.0399^{aA}	0.0881 ± 0.0001^{aA}	0.0674 ± 0.0092^{aA}
Sonicated	180 ± 14^{bA}	155 ± 64^{aA}	0.2719 ± 0.0010^{aA}	0.2644 ± 0.0044^{aA}	0.0828 ± 0.0058^{aA}	0.0850 ± 0.0042^{aA}

Values are mean of one determination from two replicate experiments ± SD. Mean values with different superscript letters in the same column are significantly different at $p < 0.05$ by ANOVA and the Tukey's test. Mean $^{A-B}$ values of the same row for each parameter are significantly different at $p < 0.05$ by ANOVA and the Tukey's test. Strain A = *Zygosaccharomyces* spp. composite; and Strain B = *Candida* spp. composite.

Table 3. Activities of two industrial soy sauce fermentation yeast strains under sonication treatment at 0.5 cycles, 60% amplitude for 10 min at 30°C for a 24-h growth cycle with different salt contents.

Salt content (%)	Lag phase duration (min)		Max. growth rate (OD/h)		Average growth rate (OD/h)	
	A	B	A	B	A	B
0	180 ± 14^{bA}	155 ± 64^{aA}	0.2719 ± 0.0010^{aA}	0.2644 ± 0.0044^{aA}	0.0828 ± 0.0058^{aA}	0.0850 ± 0.0042^{aA}
1	190 ± 14^{bA}	140 ± 57^{aA}	0.2344 ± 0.01847^{abA}	0.1576 ± 0.0088^{abB}	0.0822 ± 0.0049^{abA}	0.0689 ± 0.0019^{aA}
3	145 ± 35^{bA}	225 ± 35^{aA}	0.1674 ± 0.0367^{bA}	0.2551 ± 0.0605^{aA}	0.0662 ± 0.0254^{bA}	0.0726 ± 0.0077^{aA}
5	400 ± 57^{aA}	355 ± 64^{aA}	0.0506 ± 0.0006^{cA}	0.0973 ± 0.0173^{bA}	0.0455 ± 0.0010^{cA}	0.0625 ± 0.0098^{aA}

Values are mean of one determination from two replicate experiments ± SD. Mean values with different superscript letters in the same column are significantly different at $p < 0.05$ by ANOVA and the Tukey's test. Mean $^{A-B}$ values of the same row for each parameter are significantly different at $p < 0.05$ by ANOVA and the Tukey's test. Strain A = *Zygosaccharomyces* spp. composite; and Strain B = *Candida* spp. composite.

15%. When the glucose supply was abundant, the yeast cells showed an increase in the growth rate of 5 to 10%. Similar to salt, glucose at high levels exerted osmotic pressure, but glucose was considered to be much less toxic when compared with an equivalent amount of sodium. This explains why the activities of the selected yeast reached the highest at 0.1310 ± 0.0107 OD/h when glucose was enriched at 10% (Alonso *et al.,* 2015), whereas 10% salt retarded the yeast activity as previously mentioned. However, at 15% glucose enrichment, the solutes were too high for the yeast to grow in limited free water (Rojo *et al.,* 2014). Studies have revealed that the growth of *Zygosaccharomyces* (strain B) can be significantly controlled and limited by increasing the sugar content of the growth medium (Rojo *et al.,* 2014). From our observations, salt was an absolute stressor when present in the fermentation model system while glucose served as extra nutrients to sustain the growth of yeast when the concentration was appropriately supplied. In our case, 10% extra glucose was proven to be sufficient to recover the growth of selected yeast under stressed conditions.

Comparison of growth rate between sonicated and control at 10% glucose enrichment

Interestingly, when compared to the effects of sonicated and non-sonicated treatment to the growth pattern of the medium with 5% salt and 10% glucose enrichment, there were significantly different in maximum growth rate and average growth rate. The maximum growth rate of non-sonicated yeast was 0.0858 ± 0.0063 OD/h, and the sigmoidal growth curve shows that the activities reached their maximum number (OD = 0.9316) at 960 min (16th h during 24-h growth cycle) followed by a decreasing trend. The decreased in OD after 960 min suggests that the cells were dead, coagulated, or became sediments or flotation, and contributed to a very low average growth rate at 0.0266 ± 0.0042 OD/h. On the other hand, when the yeast cells were stimulated by sonication in the medium with 5% salt and 10% glucose, significantly ($p < 0.05$) higher maximum growth rate of 0.1310 ± 0.0107 OD/h and average growth rate of 0.0551 ± 0.0004 OD/h (100% higher as compared to non-sonicated yeast) were recorded. This finding was very crucial because it showed that, at proper enriched conditions, sonication reversed the effects of salt as a stressor. To date, ultrasound-assisted technology has proven to be effective in improving the fermentative ability of lactobacilli (Ewe *et al.,* 2012a; 2012b), bifidobacteria (Nguyen *et al.,* 2009), and bioethanol production (Nikolić *et al.,* 2010). We expect the improvement fermentative ability seen in these bacteria to agree with our findings, whereby sonication indeed increased the activities and survival rate of stressed yeast cells. Besides, studies of common yeast *Saccharomyces cerevisiae* under low-intensity sonication treatment also yielded positive effects such as enhancing fermentation strength and proteinase activity (Lanchun *et al.,* 2003a). Furthermore, pulsed (0.5 cycles) sonication which was performed in the present work was also applied in the study of *Saccharomyces* by another research group. Their results showed that pulsed sonication could reduce the lag time of *Saccharomyces* before entering the exponential phase (Jomdecha and Prateepasen, 2011), and pulsed sonication could accelerate the growth when applied just before the onset of maximum growth (exponential phase) (Lanchun *et al.,* 2003b).

Furthermore, sonication treatment can also induce the formation of tiny bubbles and vibration in the medium which brings about the effect called microstreaming (Cárcel *et al.,* 2012). Microstreaming causes extensive forces leading to the abrasion of the cell membrane (Zinoviadou *et al.,* 2015). This minor disruption of the microorganism cell wall might not be lethal but it can increase the membrane permeability (Ewe *et al.,* 2012b) and speed up the transfer of substrates (Nguyen *et al.,* 2009). This phenomenon also explains the trend mentioned earlier where the growth rate of the selected yeast was increased as glucose enrichment increased from 5 to 10%. On top of that, sonication plus 10% glucose enrichment exhibited a significantly higher ($p < 0.05$) growth rate as compared to the control. Therefore, we deduced

Table 4. Activities of strain B (*Candida* spp.) under sonication treatment (0.5 cycles, 60% amplitude for 10 min), 5% salt addition, and different levels of glucose enrichment at 30°C for a 24-h growth cycle.

Glucose enrichment (%)	Lag phase duration (min)	Max. growth rate (OD/h)	Average growth rate (OD/h)
5	540 ± 28^{a}	0.0994 ± 0.0090^{ab}	0.0590 ± 0.0044^{a}
10	400 ± 58^{a}	0.1310 ± 0.0107^{a}	0.0551 ± 0.0004^{ab}
15	430 ± 14^{a}	0.06882 ± 0.0137^{b}	0.0464 ± 0.0037^{b}

Values are mean of one determination from two replicate experiments ± SD. Mean values with different superscript letters in the same column are significantly different at $p < 0.05$ by ANOVA and the Tukey's test.

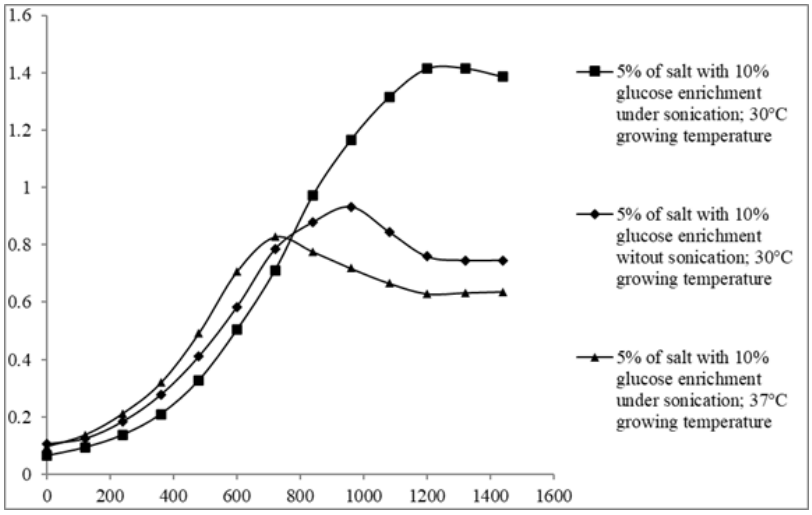

Figure 1. Comparison of sigmoid curves of selected yeast strain B (*Candida* spp.) among the different growing conditions; whereby the sigmoid curves of sonicated yeast in the medium of 5% salt, 10% glucose, and 30°C growing temperature was compared to non-sonicated yeast in the same medium, and the comparison was made between sonicated yeast at 30 and 37°C growing temperatures.

Table 5. Effects of sonication duration on the strain B (*Candida* spp.) under sonication treatment (0.5 cycles, 60% amplitude for 10 min in 5% salt and 10% glucose) at 30°C for a 24-h growth cycle.

Duration (min)	Lag phase duration (min)	Max. growth rate (OD/h)	Average growth rate (OD/h)
10	400 ± 57[a]	0.1310 ± 0.0107[a]	0.0551 ± 0.0004[a]
20	470 ± 70[a]	0.1157 ± 0.0147[a]	0.0500 ± 0.0032[a]
30	390 ± 42[a]	0.0984 ± 0.0097[a]	0.0295 ± 0.0072[b]

Values are mean of one determination from two replicate experiments ± SD. Mean values with different superscript letters in the same column are significantly different at $p < 0.05$ by ANOVA and the Tukey's test.

that, when the enriched nutrient is supplied at constant abundance, yeast number will be a limiting factor of the fermentation process. However, with precise sonication stimulation, increases in cell wall permeability can promote the mass transfer between substrates and the cells, thus promoting maximum growth rate.

Effects of sonication duration and growing temperature of Candida spp. under stressed conditions

From Table 5, it is clear that sonication did not significantly affect the maximum growth rate although there was a decreasing trend observed when sonication was prolonged to three times longer (30 min). This finding is supported by a previous study which suggested that total sonication duration had the least effects on yeast cells (Lanchun *et al.,* 2003b).

The targeted *Candida* cells can grow at 30 and 37°C. Generally, the higher the temperature, the faster the *Candida* cells can grow. However, we suggest that 30°C be used in the presence of stress. At 37°C, the *Candida* cells reached the stationary phase faster, and there was a decrease in absorbance value seen, which resulted from the coagulation of dead cells. Therefore, the growth at a higher temperature (37°C) caused early cell death with sonication stimulation. However, 30°C provided the optimum condition to the yeast cells to survive under stress and sonication stimulation.

Conclusion

Using an OFAT experimental design, the effects of selected factors were compared and optimised according to fermentation needs. Sonication (0.5 cycles, 60% amplitude) had no absolute detrimental effect on the selected soy sauce yeasts (*Zygosaccharomyces* spp. and *Candida* spp.) under all treatment combinations performed in the experiment. Instead, sonication stimulation reversed the effects of stressors in the growing medium. It can thus be concluded that the best conditions for the

Candida yeast strain was 10 min of sonication at 0.5 cycles, 60% amplitude, enriched by 10% in glucose in the medium, with a maximum tolerance of 5% salt at 30°C growing temperature.

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