ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Differential effect of interacting environmental factors on growth and human lysozyme production by a recombinant strain of *Pichia pastoris GS115/Mut*.*

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ABSTRACT

The abiotic factors influencing (a) growth and (b) human lysozyme production using buffering systems for a strain of *Pichia pastoris* in Yeast Peptone Dextrose broth (YPDB) and on agar-based media (YPDB, YPDA) were identified. Factorial designs compared solute stresses (glycerol vs KCl vs sorbitol); interaction with water activity (a_w; 0.98-0.92 a_w), temperature (15-37°C) and pH (4.6-7.6). Maximum cell yields of *P. pastoris* (3 x 10^3 CFUs ml^{-1}) was at 15°C with glycerol to modify a_w. Interactions between pH x a_w x solute type occurred at 30°C, with the non-ionic solute giving the highest cells numbers (2 x 10^3 CFUs ml^{-1}) at pH 7/0.97 a_w. A decrease in a_w and pH significantly reduced/delayed *P. pastoris* growth on YPDA. Statistically, a_w significantly affected *P. pastoris* growth ($P < 0.05$). Of the three factors, temperature had the most significant effects on human lysozyme production ($P < 0.05$). A maximum yield was 75.1 U mg^{-1} at pH 5.8, 20°C, 1.5% methanol concentration. This increased lysozyme by 2.3 times, compared to the controls. MES-buffered medium gave highest enzyme production (53.8 U mg^{-1}) at pH 6.8, 25°C and 0.97 a_w with methanol (0.6%). A_w was the important factor influencing growth, with temperature significant for lysozyme production.

Keywords: water activity, temperature, pH, growth, human lysozyme, induction, methanol

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ISSN: 0975-8585

INTRODUCTION

Lysozyme constitutes a family of enzymes which break down bacterial cell walls by cleaving 1-4 glycosidic linkage between N- acetylmuramic acid (NAM) and N- acetylglucosamine NAG) of peptidoglycan in the cell wall [1,2,3,4]. This enzyme occurs naturally in tears, human mucus, saliva and hen egg white. Its anti-bacterial properties have been beneficial for applications in both medical science and in the food industry. In the medical area, lysozyme has efficacy as an antiviral, immune modulatory, anti-inflammatory and an antitumor agent [5]. Although hen egg-white lysozyme is available commercially and used in various applications, several studies have revealed that hen egg-white lysozyme is allergenic. In addition, human lysozyme shows higher antimicrobial activity [6, 7, 8, 9].

In recent years, there has been considerable interest in pharmaceutical products from recombinant yeasts. *Pichia pastoris* has been used as a yeast expression system to produce recombinant human products. An understanding of different stress conditions on physiology of the *P. pastoris* strains has played a major role in the development of cell factories for the overexpression of foreign genes. There are two categories of stress: metabolic stress and environmental stress. Metabolic stress concerns the gene copy number and transcription process. On the other hand, environmental stress examines the cultivation conditions such as temperature, pH, osmolarity and oxygenation. However, few studies have examined the effect of interacting environmental conditions and how they may affect the physiology of the cell factory and how this would affect growth of *P. pastoris* and production of human lysozyme [10]. Previous studies by Parra *et al.* [11, 12] showed that such physiological manipulation of a genetically modified xerotolerant strain of *Aspergillus niger* resulted in a significant enhancement of lysozyme production.

The objectives of this study were to examine (a) growth and (b) lysozyme production by *P. pastoris* on a Yeast Peptone Dextrose agar (YPD) and in broth culture of the same medium in shake flask culture, respectively. The effect of different solute stress (glycerol vs KCl vs sorbitol) and the interaction with water activity (a_w; 0.98-0.92 a_w), temperature (15-37^{o}C) and pH (4.6-7.6) using three different buffered systems on growth and lysozyme production were also determined.

MATERIAL AND METHOD

Strain used in this study

A recombinant *P. pastoris* strain GS115/Mut$^+$ expressing human lysozyme under the control of the alcohol oxidase promoter was used in this study. Stock cultures were maintained on Yeast Peptone Dextrose agar (1% yeast extract, 2% peptone, 2% dextrose, 2% agar; YPDA) medium and suspended in 15% glycerol (v/v) and kept at –80 ^{o}C (Invitrogen Carlsbad, USA). The strain was kindly provided by Prof. D. Archer, Nottingham University, U.K.).

Effect of water activity, temperature and type of solute on growth of P. pastoris

The inoculum culture was prepared from a 3-day-old colony of *P. pastoris* grown on YPDA at 30°C which was then transferred into 20 ml of Yeast Peptone Dextrose Broth (YPDB) medium. The culture was incubated at 30°C in a shaker at 200 rpm for 24 hrs. The water activity (a_w) was modified by addition of the non-ionic solutes (glycerol, sorbitol) or an ionic solute (NaCl). The accuracy of the a_w treatments was checked with a a_w meter (AquaLab 4TE, Decagon Devices, Inc., USA). Media were spread plated with 100 µl of inoculum using a sterile glass spreader and kept in plastic polyethylene bags at the tested temperatures. Colonies were counted after 3-5 days. In order to identify which parameters significantly affected *P. pastoris* growth, a 4x5x3 factorial design was developed with MINITAB version 16 (Minitab, Inc., USA) shows the experimental variables and their levels used. All treatments were carried out in triplicate and repeated once **(Table 1a).**

Statistical analysis: The impact of a_w, temperature and type of solute on the colony forming units (CFUs) per millilitre of *P. pastoris* cells was compared with the MINITAB programme. Since the data was not normally distributed, a Kruskal-Wallis nonparametric ANOVA was conducted. A P-value of $P<0.05$ was considered significant. A nonparametric multiple comparison, Dunn's test with the Bonferroni correction was used to keep the Bonferroni individual alpha = 0.05.

ISSN: 0975-8585

Effect of interaction between water activity, pH and type of solute on growth of P. pastoris

A 2x2x5 factorial design was used to examine the a_w x pH x solute type three-way interacting factors **(Table 1.b).** The pH of the YPDA medium was modified by using a phosphate citrate buffer solution at each of the target a_w levels. The a_w was modified by addition of the non-ionic solute, glycerol, or the ionic solute NaCl. The media treatments were spread plated using 100 µl of inoculum as described previously. The inoculated plates were kept in sealed plastic polyethylene bags at the treatment temperature. The total viable CFUs were enumerated after 3-4 days. All treatments were carried out in triplicate and incubated at 30°C.

Statistical analysis: When assumptions of normality and equal variance were not met, significant differences were assessed using the Kruskal-Wallis nonparametric ANOVA at a statistical significant level of $P<0.05$. Post-hoc inter factor differences were calculated with Dunn's multiple comparison nonparametric tests with the MINITAB version 16 programme. Bonferroni correction was used to keep the Bonferroni individual alpha = 0.05. Mann-Whitney U test was adopted for statistical analyses between two samples with the significant level alpha = 0.05.

Effect of interacting abiotic factors on human lysozyme production by P. pastoris using a factorial design

The factorial design of 2^5 with repeated center points was initially used to identify which variables have a significant influence on the specific activity of lysozyme. The selected factors and their levels were chosen based on previous results. The variables were tested at two levels, a high (+1) and a low (-1) level and examined at the center point (0) level for quantitative variables. The range of the coded and actual levels which were used in this study is listed in **Table 1c.**

Table 1: Summary of the different experimental factors used in this study.

(a). Experimental factors and levels used in a 4x5x3 factorial design to evaluate effects of interacting factors of temperature and water activity on growth of *P. pastoris*.

Factor	Level				
water activity	0.99 (0.98) [a]	0.97 (0.96)	0.95 (0.94)	0.93 (0.92)	
temperature	15	20	25	30	37
types of solutes	NaCl	Glycerol	Sorbitol		

[a] The level in blanket was used for sorbitol.

(b). Experimental factors and their levels used in 2x2x5 factorial design to examine effects of pH, water activity and type of solute on growth of *P. pastoris*.

Factor	Level				
water activity	0.97	0.95			
type of solutes	NaCl	Glycerol			
pH	4.6	5	6	7	7.6

(c). Coded and actual values of independent variable used to identify optimum conditions for lysozyme production by the strain of *P. pastoris*.

Variable code	Variable	Low level -1	Center point 0	High level +1
X_1	water activity	No modification	-	0.97
X_2	pH	5.8	6	7
X_3	temperature	20	25	30
X_4	methanol	0.5	1	1.5
X_5	expression method	Type 1	-	Type 2

The inoculum media were prepared with phosphate citrate buffer at the different pH levels (pH 5.8-7.0) which included an unmodified (=0.995 a_w) treatment and that modified with glycerol to 0.97 a_w. An aliquot

of 5 ml of YPDB medium was placed in a 50 ml flask. One single colony (3 days old) from YPDA was transferred to the inoculum flask. Cultures were incubated for 18 hrs at 20, 25 and 30°C and agitated at 250 rpm. The inoculum culture was transferred to 45 ml of peptone yeast glycerol based medium (PYG; 2% peptone, 1% yeast extract, 1% glycerol) in a 0.1 M phosphate buffer with a pH of 5.8-7.0 at the target a_w levels (0.995 and 0.97) in 250 ml flasks. Then, the inoculated expression treatments and replicate media were cultivated at the three target temperatures for 100 hrs in a shaking incubator at 250 rpm. The methanol (100%) was added at a final concentration of 0.5, 1.0 or 1.5% every 24 hrs to maintain lysozyme secretion.

Measurement of lysozyme activity

The samples of the fermentation medium after 100 hrs were centrifuged for 5 min at 9000 g (Labofuge 400R, Thermo scientific). The supernatant was analyzed for lysozyme activity. This activity was determined using the method of Mackie *et al.* [13] with slight modifications. The solution of the free-cell supernatant (100µl) was added to 900 µl of a suspension of lyophilized *Micrococcus lysodeikticus* cells (Sigma-Aldrich, Inc., USA) in 0.1 M potassium phosphate buffer pH 7 (OD_{450}= 0.5-0.7). The change in absorbance at 450 nm was measured for 2 min using a spectrophotometer. A change in absorbance of 0.001 absorbance unit per minute was defined as 1 U enzyme activity. Lysozyme activity was calculated by the following equation:

$$Lysozyme\ activity\ \left(\frac{U}{ml}\right) = (\Delta abs/min) \times 10^4 \qquad \text{Equation 1}$$

In the equation, Δabs is the change in absorbance.

The total protein of the sample was quantified using the bicinchoninic acid (BCA) method using the Pierce® BCA protein assay kit applying bovine serum albumin as standard (Thermo Scientific, USA). The specific activity of lysozyme can be calculated from the following equation:

$$Specific\ activity\ (U/mg) = \frac{Unit\ of\ lysozyme\ per\ ml}{mg\ total\ protein\ per\ ml} \qquad \text{Equation 2}$$

Statistical analysis: The experimental design and statistical analyses were analyzed using Design Expert version 8 (Stat-Ease, Inc., USA). All non-normal distributed datasets of specific activity of lysozyme were transformed to fit normal distribution prior to performing statistical analysis. Johnson transformation in MINITAB software version 16.0 was applied. The transformed datasets were normally distributed and analysed using the Kolmogorov–Smirnov test with 95% confidence intervals. Significant differences were assessed with the analysis of variance (ANOVA) at the statistical significant level of P=0.05.

Influence of buffer systems on lysozyme production

Three buffer systems including MES, phosphate citrate (McIlvaine's buffer) and potassium phosphate were selected to examine the impact of buffer systems together with selected quantitative variables. The factorial experimental design consisted of 4 variables at 2 levels with a center point level included **(Table 2).**

Table 2: Experimental factors and their levels used to examine the effect of different pH buffer modifications to obtain the targeted values at different temperatures and a_w levels.

Variable	Low level	Centre point	High level
temperature (°C)	15	20	25
pH	5.6	6.2	6.8
a_w	0.97	0.98	0.99
methanol concentration	0.6	1.1	1.6

The inoculum media were prepared in the different buffer systems (MES, phosphate citrate, potassium phosphate buffer) at the target pH levels (pH 5.6-6.8) with a_w modified by adding glycerol to reach the target treatment levels. The medium components (YPD) were dissolved in these prepared solutions.

The inoculum media were prepared in a 50 ml flasks with 5 ml of prepared media as described previously. A single colony (3 days old) from YPDA was transferred to the inoculum flask. Cultures were incubated for 18 hrs at 15, 20 and 25°C at 250 rpm. The inoculum culture was transfer directly to 45 ml BMGY medium (2% peptone, 1% yeast extract, 100mM Potassium phosphate pH 6.0, 1.34% Yeast Nitrogen Base (w/o AA), 0.4µg/mL Biotin, 1.0% Glycerol. 1000mL water) in 250 ml flask. BMGY media were prepared as for YPD in terms of the buffer system at the different pH and a_w levels in 2. Inoculated expression media were cultivated at 15 to 25°C with agitation at 250 rpm for 100 hrs. The necessary methanol treatments were added every 24 hrs to maintain lysozyme expression. Samples of the fermentation medium were quantified for lysozyme activity as described previously.

Statistical analysis: The experimental design and statistical analyses were analyzed using MINITAB software version 16.0 as described previously. When assumptions of normality and equal variance were not met, significant differences were assessed using the Kruskal-Wallis nonparametric ANOVA at a significance level of $P<0.05$. Post-hoc inter-factor differences were calculated with the Dunn's multiple comparisons nonparametric test. Bonferroni correction was used at 0.99 to keep the Bonferroni individual alpha = 0.028, when the number of comparisons were 36 pairs.

RESULTS

Effect of water activity, temperature and types of solute on *P. pastoris* growth

The influence of a_w, temperature and types of solute on the lag phases prior to growth and on relative growth of *P. pastoris* were compared **(Figure 1).** The two significant factors were found to be a_w and temperature ($P<0.05$). *P. pastoris* was particularly sensitive to being cultured at 37°C ($P<0.05$). Growth was inhibited at 0.95 a_w with the ionic (NaCl) and non-ionic solute glycerol, but only at 0.94 a_w with sorbitol. Adding glycerol to YPDA medium supported growth, especially at the lowest temperature (15°C) examined with the maximum viable cell number of $3.1x10^3$ cfu/ml. The maximum cell yield in the control was 2.75×10^3 cfu/ml. In contrast, with glycerol as a solute, lower populations grew at elevated temperatures. Overall, the main effect of different types of solutes on *P. pastoris* populations are shown in the box plots and these were found to not be statistically significant **(Figure 2).** Growth at 15°C seemed to be better than at any other temperature. However, this involved longer initial lag phase durations (6 – 13 days) prior to growth (data not shown).

The highest populations of *P. pastoris* were found in the range 0.96-0.97 a_w. However, this was not significantly greater than when cultured in the range 0.98 - 0.99 a_w ($P<0.05$; see Figure 2). Water stress treatments not only influenced the multiplication of the yeast populations, but also changed the lag phases (in days) prior to growth. However, the result depended on the types of solute and incubation temperature. For example, at 0.97 a_w with the ionic solute NaCl, the shortest lag phase (2 days) was at 30°C**,** while this was 6 days at 25°C. Using glycerol to modify YPDA medium resulted in shorter lag phases. However, the populations of *P. pastoris* were lower than when using NaCl as a solute, especially at higher temperatures (data not shown).

Effect of interaction between a_w, pH and solutes on growth of *P. pastoris*

To evaluate the effect of several factors on growth we used a factorial design. NaCl and glycerol were selected as the solutes to test at 0.95 and 0.97 a_w based on previous results. This was combined with examining a range of pH values (4.6-7.6). **Figure 3** shows the box plot analyses of the effect of these three parameters. This revealed that a_w was the most significant factor influencing growth ($P<0.05$). *P. pastoris,* when cultivated on YPDA at 0.97 a_w, resulted in a significantly higher viable cell yield ($P< 0.05$) than at 0.95 a_w (see

Figure 33). Furthermore, using the non-ionic solute glycerol as the a_w depressor resulted in a higher viable cell yield than with the ionic solute NaCl. However, the main effect of solute type on yeast populations was not statistically significant. There were some differences in the populations of *P. pastoris* in the pH range of 4.6 to 7.6.

ISSN: 0975-8585

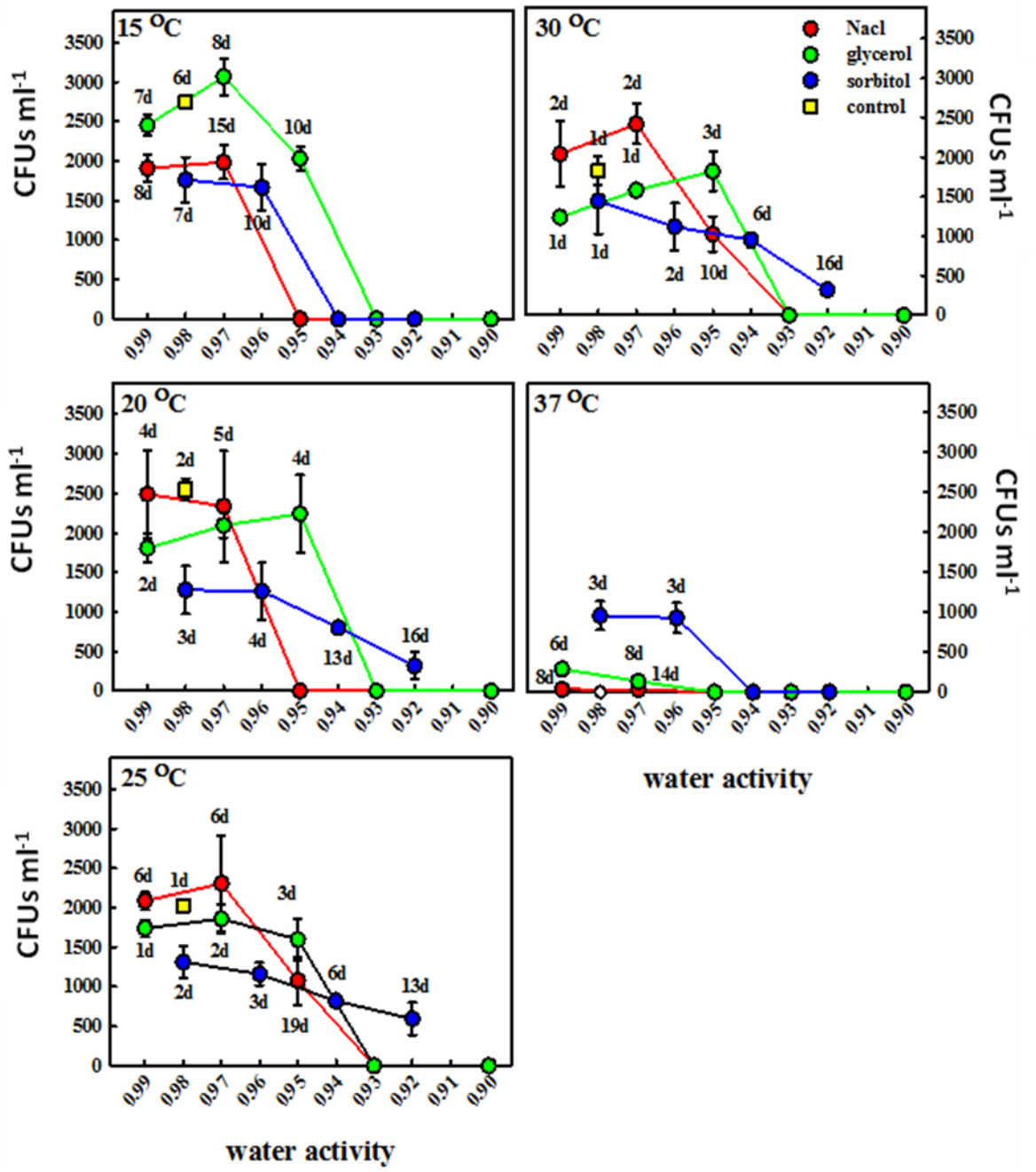

Figure 1: Effect of water activity (a_w), temperature and solute type on lag phase and growth of *P. pastoris* in media modified with NaCl, glycerol and sorbitol at 15, 20, 25, 30 and 37°C. The numbers of days for initiation of growth are indicated.

ISSN: 0975-8585

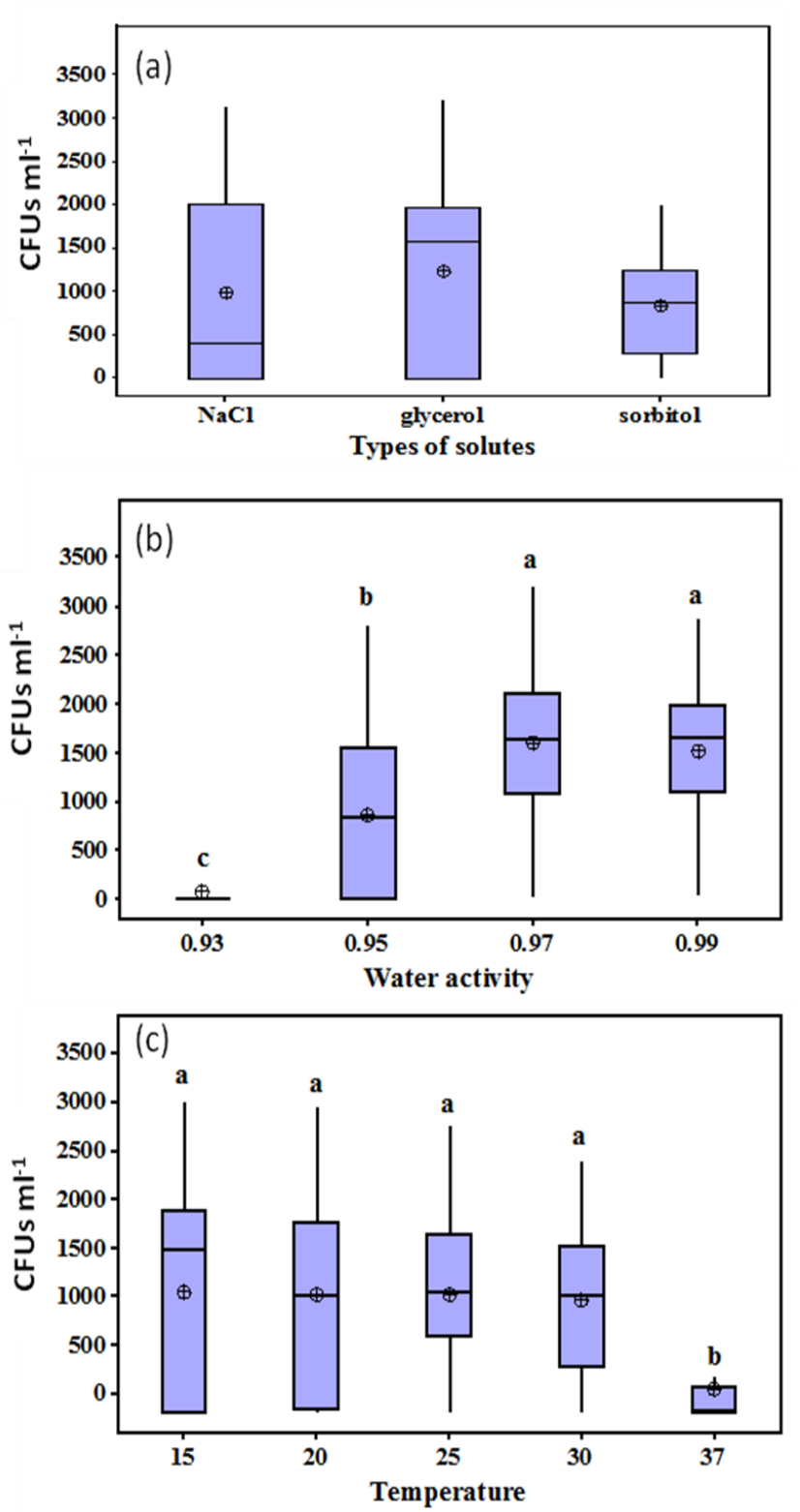

Figure 2: Differences in cell number of *P. pastoris* in response to (a) type of solute (b) water activity (a_w) and (c) temperature stress conditions. Box plots show the median (horizontal lines within each box), upper and lower quartiles (boxes). The circle inside the box is the mean. Boxes labelled with the different letter indicate significant median difference following Dunn's multiple comparisons test. The Bonferroni individual alpha for multiple comparisons was P=0.05.

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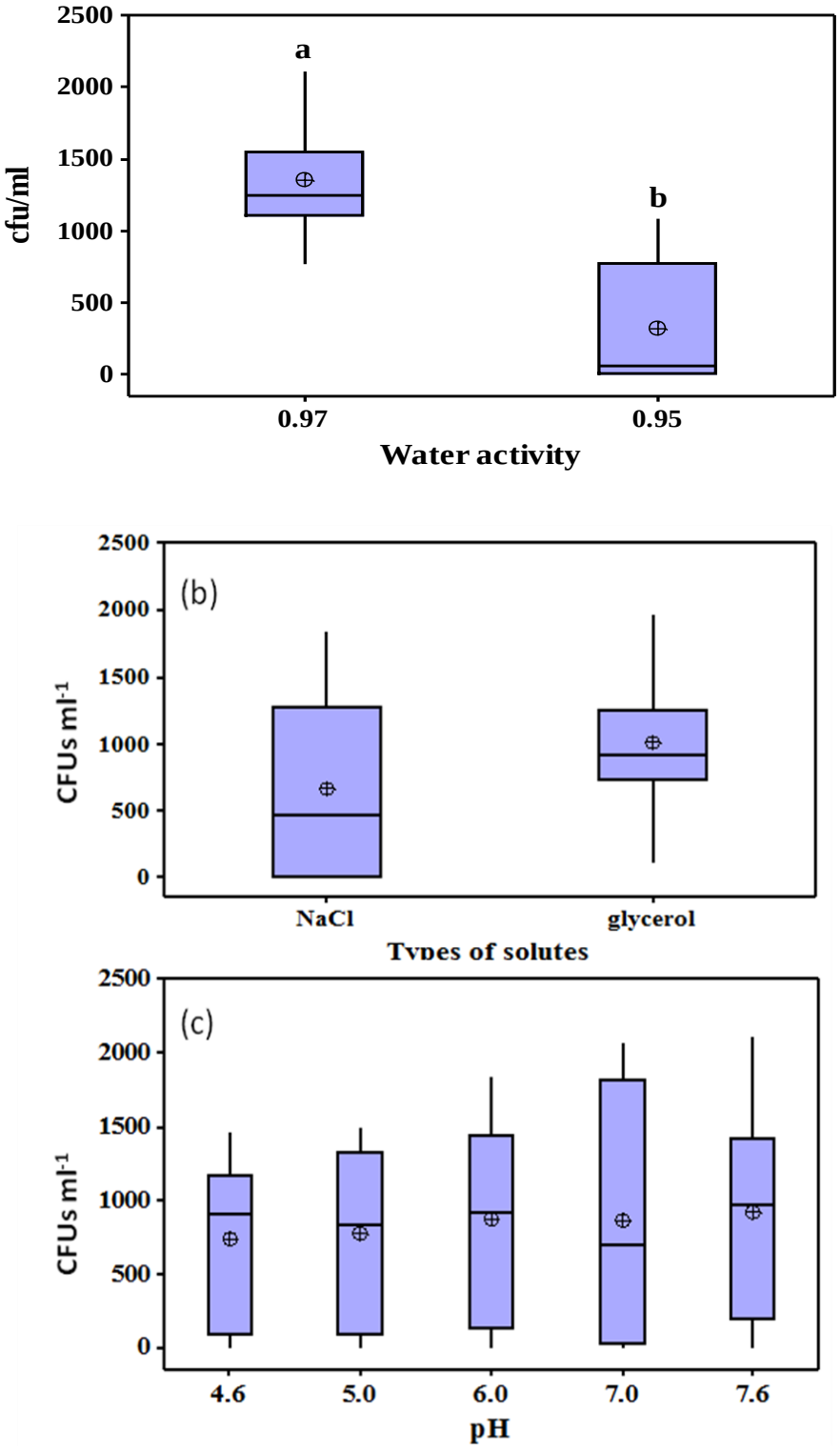

Figure 3: Box plots show the effect of the (a) water activity (a_w) (b) solute type and (c) pH modification on the population of *P. pastoris*. The line within the box indicates the median cell number of *P. pastoris*, the 25th and 75th percentile (lower and upper margin of the box) together with the minimum and maximum values (whiskers). The circle inside the box is the mean .Statistical differences between groups were determined using the non-parametric Man-Whitney test is indicated by the letter above columns. The different letters indicate statistic significant differences (P<0.05).

Based on the results obtained above on growth of *P. pastoris,* some of the environmental stress factors were selected for examining impacts on lysozyme production. By using a factorial design, the effect of several experimental factors and their interactions on specific activity of lysozyme production was evaluated.

The highest yield (75.06 U/mg) was at pH 5.8, 20°C, with 1.5% methanol without any a_w modification. This maximum yield increased approximately by 3.6 times compared with those cells cultured under the control conditions. In contrast, the lowest yield (25.1 U/mg) was obtained at pH 5.8, 20°C with 0.5% methanol addition in the modified a_w treatment.

Normality of the distribution of the population was tested using the Kolmogorov–Smirnov test. The null hypothesis (H_0) was that the data followed a normal distribution and the alternative hypothesis (H_a) was that data were non-normally distributed. For the responses variable, the H_0 was rejected in favor of the H_a. This was done with 95% confidence level **(Figure 4).** Moreover, the inequality of variance was revealed from the Levene's test.

One approach to address non-normal distributed datasets is to normalize the dataset using transformation prior to analyses. Johnson transformations were used because more normalized data sets were generated (P>0.05, Kolmogorov–Smirnov test). Clearly, the Johnson transformation method gave the new transformed data set which was closer to a normal distribution than the original one (see Figure 4).The original data sets of specific activity of lysozyme were thus transformed according to the following equation:

$$Y^* = -0.137633 + 1.05657 \times Asinh\left(\frac{(x - 44.3134)}{7.70277}\right) \quad \text{Equation 3}$$

In the equation, Y^* represents the transformed specific activity of lysozyme and x represents the original data.

The back-transformed data were calculated from the equation below:

$$y = 44.3134 + \left(3.851385\, e^{\left(\frac{(Y^*+0.137633)}{1.05657}\right)}\right) - \left(45.4094\, e^{\left(-\frac{(Y^*+0.137633)}{1.05657}\right)}\right) \quad \text{Equation 4}$$

where y represents back-transformed data, Y^* represents the transformed specific activity of lysozyme.

An ANOVA analysis was carried out on the transformed data in Equation 3. Both qualitative variables of this experiment including a_w modification showed no significant changes in specific activity of lysozyme (P>0.05). Among the studied variables, temperature had a significant effect on specific activity (P<0.05). The remaining variables, i.e., methanol concentration and pH, were non-significant within the range used in this study.

After the collected data were transformed and fitted, a linear first-order model in terms of the actual factors for specific activity was developed as shown below:

$$\mathbf{Y^* = 5.94182 - 0.42972\ pH - 0.13372\ temperature + 0.041146\ methanol\ concentration} \quad \text{Equation 5}$$

In the equation, Y^* represents the transformed specific activity of lysozyme.

The main effects plot is useful in the practical interpretation of the results. In term of the screening experiment, these plots can be used to compare the changes in the mean level to examine the tendency of the response on the tested level of factors providing the information for the subsequent experiment. **Figure 5** represents the main effects plots of pH, temperature and methanol concentration in terms of back-transformed data. Although the pH and methanol concentration factors were not significant (P>0.05), there was some useful information from these results. Better yield could be achieved by choosing a relative more acidic pH level and setting the methanol concentration at a much higher level.

ISSN: 0975-8585

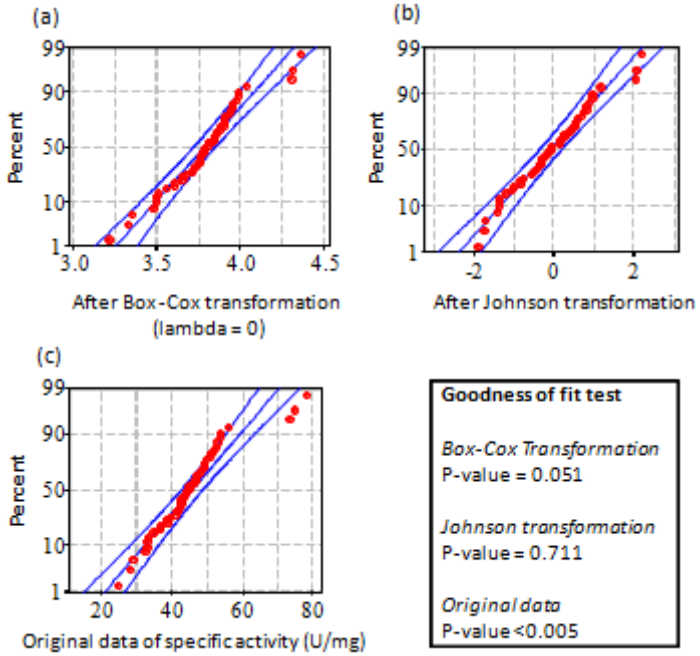

Figure 4: Normal probability plots of specific activity of original lysozyme data, transformed data with Box-Cox and Johnson transformations.

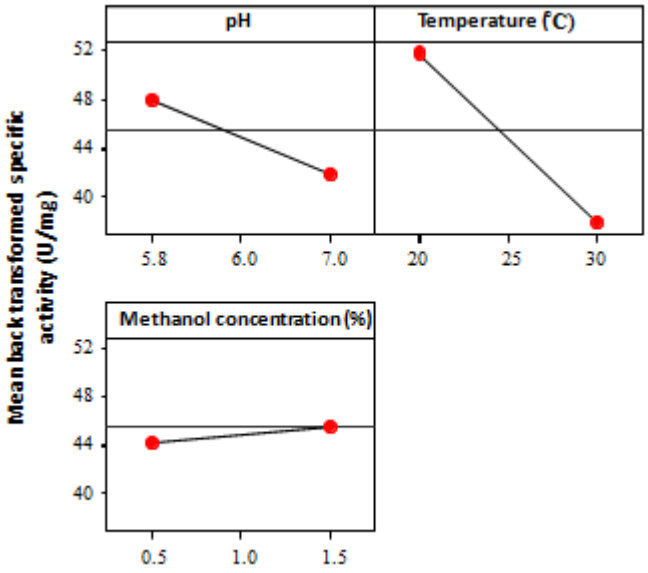

Figure 5: Main effects plot for the mean of back-transformed specific activity of lysozyme (U/mg).

Influence of different buffer systems on lysozyme production by *P. pastoris*

For the expression of recombinant protein in *P. pastoris*, a low pH value of 5.0-6.0 has been commonly used. A potassium phosphate buffer (0.1M) is recommended when preparing buffered media to maintain a stable pH condition. In order to examine the effects of different buffer systems on lysozyme production, liquid culture experiments with three different buffer systems, i.e., MES, potassium phosphate and phosphate citrate buffer were compared and interactions with temperature, aw and methanol concentration compared **(Figures 6 and 7).** The highest enzyme production (53.8 U/mg) was obtained at pH 6.8 + 25°C, with a methanol concentration of 0.6%, and 0.97 a_w using a MES buffer system (see Figure 6). When the potassium

ISSN: 0975-8585

phosphate buffered medium was used, the highest enzyme production (44.3 U/mg) was observed at pH 6.2, 20°C, with methanol concentration of 1.1% and 0.98 a_w. For phosphate citrate buffered medium, the maximum enzyme activity (28.9 U/mg) was observed at pH 6.8, 25°C, 0.6% methanol concentration and 0.99 a_w.

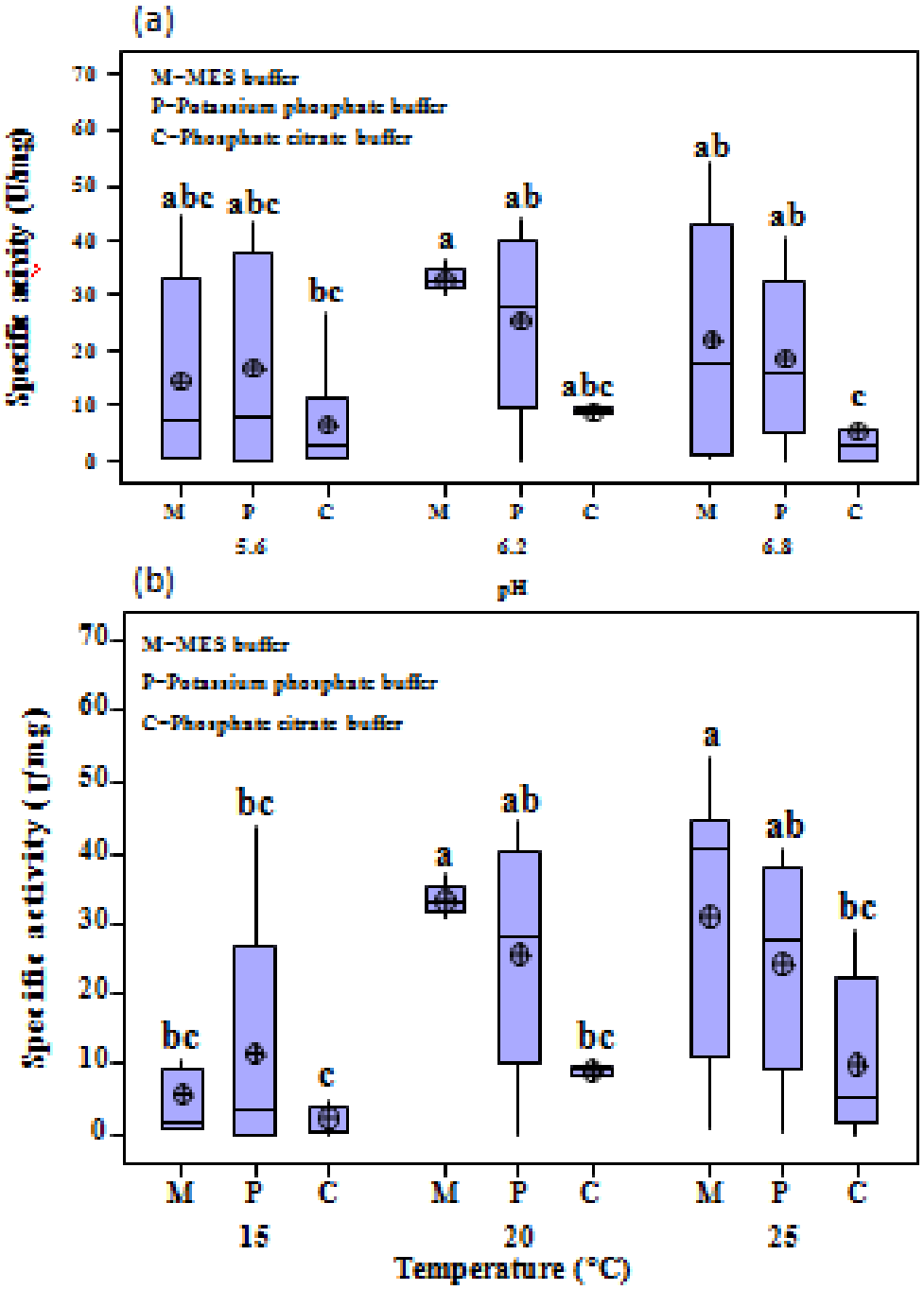

Figure 6: The box plot illustrates specific activity of lysozyme (U/mg) in the response to (a) pH and buffer systems and (b) temperature and buffer systems. Boxes contain the median (horizontal line within the box and the minimum and maximum values (whiskers). The circle inside the box is the mean. The non-parametric Dunn's multiple comparisons test was used with Bonferroni individual alpha for multiple comparisons is 0.028. The median of groups that share a letter are not significantly different.

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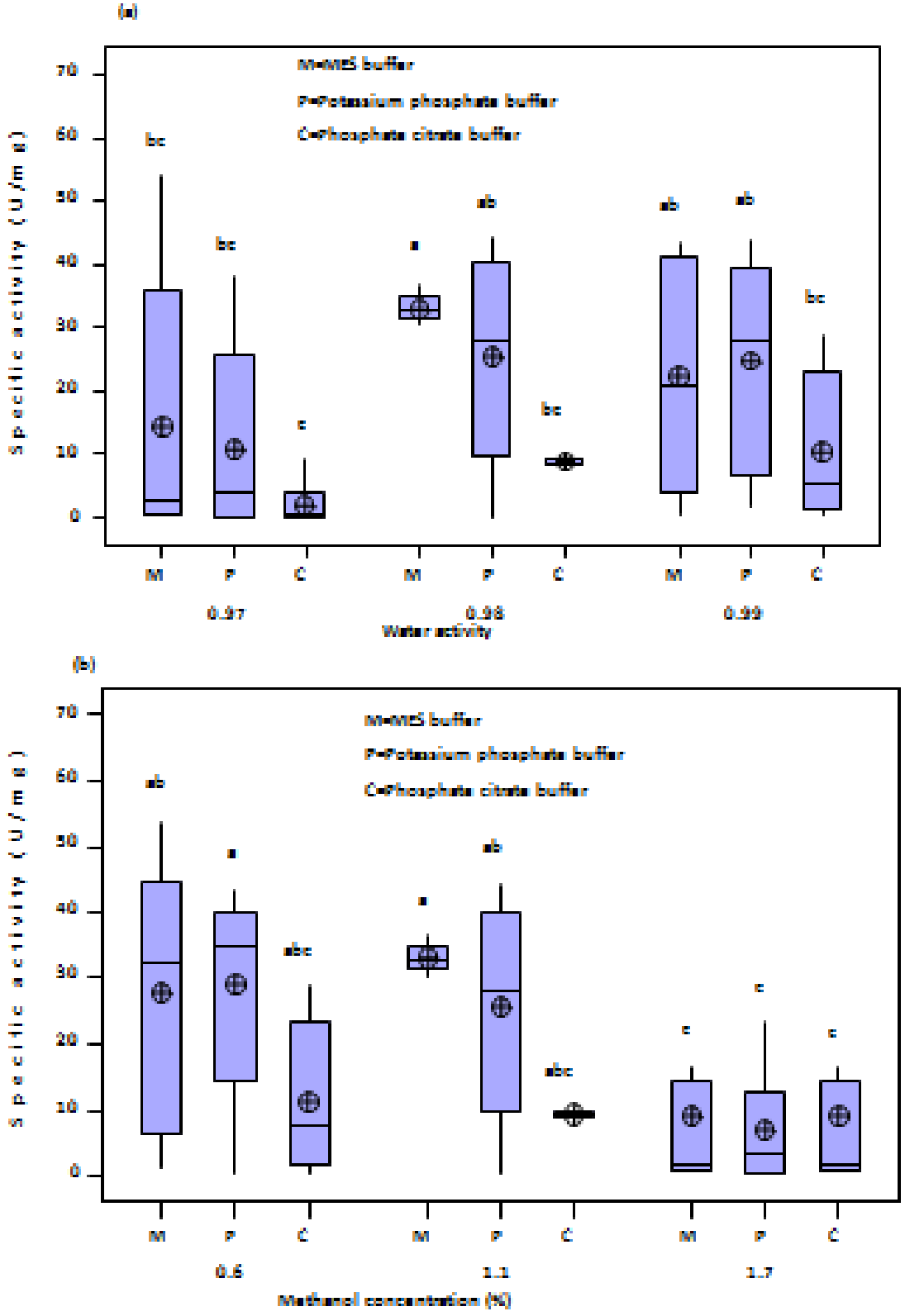

Figure 7: The box plot illustrates specific activity of lysozyme (U/mg) in the response to (a) water activity (a_w) and (b) methanol and buffer systems. Boxes contain the median (horizontal line within the box) and the minimum and maximum values (whiskers). The circle inside the box is the mean. The non-parametric Dunn's multiple comparisons test was used with Bonferroni individual alpha for multiple comparisons is 0.028. The median of groups that share a letter are not significantly different.

To illustrate the difference of the responses between groups, the non-parametric Dunn's multiple comparisons test was performed **(Table 3).** This showed that the highest median yield was in the MES buffered medium at pH 6.2 (P<0.028), whereas the maximum yield was obtained at pH 6.8. Using the phosphate citrate-buffered medium at pH 6.8, the production of lysozyme was significantly lower when compared to other pH levels and buffer systems used (P<0.028). The difference in the median lysozyme production between this condition and under the conditions favouring production (the highest median value) was approx. 30 times. Buffering the medium at pH 6.2, the higher median lysozyme production values were observed when comparisons were made within the same buffer system used.

Table 3: Analysis of variance for transformed data for lysozyme production

source	df	Mean square	F value	P value
pH	1	2.44	3.59	0.0649
temperature	1	14.31	21.05	<0.0001
methanol concentration	1	0.014	0.02	0.8884
a_w modification	1	4.923E-003	7.243E-003	0.9326
expression method	1	0.33	0.049	0.8264

Within the MES buffered pH media used, there was a significant difference between the decline in the median values (21.6–26.6 fold decline) occurred when cultured at lower temperatures (15°C) (P<0.028). The buffering conditions with phosphate citrate buffer system showed lower median values and also lower amounts of enzyme production regardless of the temperature used.

Using the buffered MES medium at 0.98 a_w gave the best yields of lysozyme. However, the maximum value of lysozyme production was obtained in the potassium phosphate buffer at the same a_w level. Phosphate citrate buffered media again supported the lowest median value and amount of enzyme production when cultured at 15°C.

The influence of methanol concentration in the different buffer systems on lysozyme activity showed that with 0.6% the highest median production was obtained (see Figure 7). However, this was similar to that observed for the MES buffered medium using 1.1% methanol (P>0.028). At methanol concentration >1.1 %, there was a dramatic decline in the median lysozyme values in all the buffered medium treatments used. The sharpest decline in production levels was observed at the highest methanol concentration (1.7%) with the MES buffer system. There was a 26.2 fold decline when compared with the highest median production (potassium phosphate buffered medium, 0.6 % methanol concentration).

DISCUSSION

This study has shown that *P. pastoris* was able to grow in the range 0.995 to 0.95 a_w. The solute type affected the total cell numbers produced. A higher average cell number was observed when using glycerol as the a_w depressor than when using the ionic solute NaCl at lowered pH values. The present study has also identified the ecological conditions of aw x temperature x pH range which are optimal and marginal for growth and lysozyme production. The better growth in the glycerol amended media may be due to the ability to transport glycerol across the cell membrane [14]. Transported glycerol serves not only as a carbon source for cell multiplication, but also as a compatible solute accumulating inside the cells directly, compensating for leakage. In contrast, growth at high NaCl concentration can be toxic and induce the response to unfolded proteins which leads to cell lysis.

Shake-flask culture is widely used as the first stage in the investigation of optimizing conditions. However, the levels of protein products are usually lower than when performed in a fermenter. The highest specific activity at 100 hrs (75.1 U/mg) obtained in the present study, was lower than some published reports. For example, Masuda *et al*. [15] reported a high specific activity of hen egg lysozyme of 1,500 U/mg when using a 3 L fermenter. Limited aeration is a critical factor for Mut^+ strains in shake-flasks, since there is no constant supply of air and methanol [10, 16, 17]. Thus, the potentially toxic molecule formaldehyde from methanol metabolism can be accumulated in the cells with a detrimental effect when the cells are exposed to

oxygen limitation [18, 19, 20]. The initial screening study was designed as a first step of an optimization process. However, the statistical test revealed that the optimal conditions were not located within the experimental parameters used and required a further more detail experimental design.

It should be noted that the recommended cultivation temperature (28–30°C) by Invitrogen had a negative effect on lysozyme production in the present study. This was shown by the higher average yields obtained at lower temperatures when compared with the higher recommended temperatures. A reduction in temperature also resulted in a positive effect on growth, and lysozyme productivity could be significantly linked to the proteolytic activity. It has been suggested that this activity is decreased at lower temperatures. In addition, the stability of cell membranes can be improved and the rate of protease release into the culture supernatant reduced [21, 22, 23]. Yu et al. [17] used a growth temperature of 30°C and an induction temperature for lysozyme of 23.5°C for optimum production. Interestingly, Huang and Demirci [4] found higher human lysozyme production by *Kluyveromyces lactis* was achieved under oxygen limitation and in acidic environments at both 250 ml and bioreactor scale at 25°C.

To express the recombinant protein from *P. pastoris* under standard conditions, a pH of 5-6 has been recommended [24]. However, the present study has shown that the highest average lysozyme production was achieved at lowered pH values. This could be due to the reduction of proteolytic activity [25]. To minimize proteolytic activity, lowering pH to 3.0 in the induction phase has been previously suggested [21, 26]. However, other studies have indicated that expression levels were increased at pH 7.0 –8.0 [27]. The lowest levels of protease activity were also detected at higher pH levels (pH 6.0, 8.0). Thus cultivation pH is probably product dependent.

The present study has shown that the best methanol concentration to use is between >1 to 1.5% for optimising lysozyme expression. However, a slight decrease in lysozyme production was observed when methanol concentration was added at 0.5% or >1.6%. This was probably due to the toxic effect of methanol. While *P. pastoris* is able to assimilate methanol, excess methanol is toxic to the yeast cells due to the accumulation of intracellular toxic compounds. Formaldehyde and hydrogen peroxide are the oxidized products from methanol which involve alcohol oxidase (AOX) and catalase in peroxisomes using oxygen molecules as an electron acceptor [20]. For *P. pastoris* which has a strongly inducible AOX1 promoter for protein expression, better control of the methanol concentration with sufficient dissolved oxygen in culture media may be necessary for optimising production. Increasing the concentration of methanol from 0.15% to 1.0% was optimal for yeast growth and increased the expression level [17]. Moreover, the toxicity of methanol is one of the main reasons for cell lysis and consequently host cell protein leakage into the culture supernatant; especially protease, which leads to lower yields and an increase in protein product impurities [21, 25].

As the minimum inhibitory a_w for growth of *P. pastoris* is 0.95–0.90 [27], it was possible to induce the host cells under osmotic stress and determine the effects of such stress on expression levels. Overall, in the present study a modified a_w of 0.97 gave lower expression levels than in the unmodified medium (0.99 a_w). Increasing osmolarity can have a significant impact on some cellular processes: particularly, protein folding, ribosome biogenesis and cell wall organization [28].

The use of different buffering pH compounds was to examine whether the effect was not just due to the high concentration of H^+ ions at low pH. Those tested have different buffering capacities. This had effects on lysozyme production, with a lower enzyme production when phosphate citrate buffered medium was used at identical pH values. This could be due to a strong chelating property of citric acid. The citric acid in a buffered medium may bind to the metal ions in the medium and sequester them in a complex form reducing the availability of trace elements for yeast cells [27]. The same effect occurred when examining the effect of pH on *P. pastoris* growth on YPD agar using this buffer system. Delayed growth and reduced populations of yeast populations were observed. This correlated with the low lysozyme production observed. Yu et al. [17] used a growth pH of 5.5 and then induction pH values of 4.5, 5.0 and 5.5 in a fed-batch study and found little difference in production of human lysozyme with optimum production after around 80 hrs by *P. pastoris*. They suggested that induction time, temperature and culture volume significantly affected production of lysozyme.

In conclusion, this study has provided information on the effect of key interacting environmental conditions on human lysozyme production by *P. pastoris*. The temperature, pH and aw, and their interactions

influenced growth and indeed induction of lysozyme by this yeast. The use of buffered pH values with specific compounds significantly influenced lysozyme production. The induction phase and optimum temperature for enzyme yield was different from that which was previously suggested. Overall, lower temperatures (20°C), lowered pH (5.8) and >1% methanol was optimum for enzyme production. Water stress affected the relative growth rate of *P. pastoris* but also reduced the lysozyme production by the yeast.

ACKNOWLEDGEMENTS

S.S. is grateful to the Royal Thai Government for financial support to carry out this research project.

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