Cross-Stress Resistance in Saccharomyces cerevisiae Cells

How does the exposure of combinations of distinct stress factors lead in *Saccharomyces cerevisiae* cells to develop cross-stress resistance in terms of concentration change and rate of fermentation?

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Contents

1. **Context** 3

2. **Background Information** 4

2.1. Yeast 4

2.1.1. Cellular Structure 4

2.1.2. *Saccharomyces cerevisiae* 5

2.2. Types of Stress Factors 5

2.2.1. pH Stress 6

2.2.2. Temperature Stress 6

2.2.3. Heavy Metal Stress 7

2.3. Coping Mechanisms 7

2.3.1. Common Stress Response 7

2.3.2. Specific Stress Response 8

2.3.3. Cross-Stress Resistance 8

3. **Preliminary Experiment and the Scope of Final Investigation** 9

4. **Hypothesis** 9

5. **Aim of the Investigation** 10

6. **Investigation** 10

6.1. Variables 10

6.2. Materials 11

6.3. Procedure 11

6.3.1. Procedure of First Stage of the Experiment 11

6.3.2. Procedure of the Second Stage of the Experiment 13

6.3.3. Aseptic Technique 14

6.3.4. Ethical Considerations 14

6.4. Methods 14

6.4.1. Determination of the Rate of Fermentation 15

6.4.2. Determination of the Yeast Cell Concentration 15

7. Results 16

7.1. Qualitative Observations 16

7.2. Raw Data 17

7.3. Calculations 18

7.3.1. Sample Calculations 18

7.4. Processed Data 19

7.5. Statistical Test 21

8. Discussion 23

9. Evaluation of Procedure 25

10. Evaluation of the Sources Used 26

11. Conclusion 26

12. References 28

Context

In order to fulfill the increasing demand (Easterbrook-Smith,2021) for *Saccharomyces cerevisiae*, efficiency in the yeast production should be maximized. However, many factors that drastically affect the viability of the yeast cells. One of the main elements that hinder efficient yeast production is the stress factors, such as temperature, pH, heavy metal, oxidative, and so on. In the past years, several reviews have been published centered around establishing the optimum conditions for the cell growth of Baker's Yeast (Almudhaffar,1978 and Salari et al, 2017). However, there is a lack of research focusing on the impact of a combination of stress factors on developing cross-stress resistance as a cellular response.

With the emerging global problems such as global warming, disruption of the ionic balance in the atmosphere and increasing heavy metal concentration in soil, organisms are exposed to a variety of different stress agents all at once. Considering the current environmental changes, investigation the effects of cross-stress agents are more important than ever.

With the research question **"How does the exposure of different combinations of distinct stress factors lead *S. cerevisiae* cells to develop cross-stress resistance, as measured by the recording of fermentation rate in accordance with the concentration change?",** this study focuses on the possible combinations that result in cross-stress resistance in *S. cerevisiae* cells, aiming to investigate the development an optimum strategy within the cell for minimizing the damaging effects of stress agents.

Background Information

Climate change is the change in the environmental conditions of the earth. Such changes have detrimental effects to the environment, such as the rising ocean levels and amount of CO_2 in the atmosphere. This leads to the development of new stress agents or the increase in the number of previous ones. Temperature has emerged as a new stress agent for living organisms, due to global warming. Ionic compounds that alter the pH and heavy metals are more prevalent in the soil (Oyewo, Opeyemi A. et al, 2020), which disrupts the balance in the ecosystem. Many organisms suffer from such negative effects, including yeast. Since this organism plays a big role in baking industry, an increase in such stress factors damage the efficiency in its production (Kahraman, 2004 and Parapouli et al, 2020).

Yeast

The physiology of yeasts does not differ from the physiology of other microorganisms. All microorganisms can live and multiply, and they can benefit from the nutrients they can find in their environment. Yeast cells vary significantly in size, shape, and color of the cell. Even yeast lineages within the same species show heterogeneity, which is due to physical and chemical environmental changes such as heat, water, pH, and nutrients (Kahraman, 2004).

Cellular Structure

Structural elements in the yeast cell are cell wall, cytosol, nucleus, mitochondria, secretory vesicles, vacuole, peroxisome, and plasma membrane (Parapouli et al, 2020). As seen in Figure 1, many of yeast cells have high-eukaryotic structural and functional features with the same structure, but in contrast to mammalian cells, fungal cells are surrounded by a rigid cell wall and cell division followed by bud sticks.

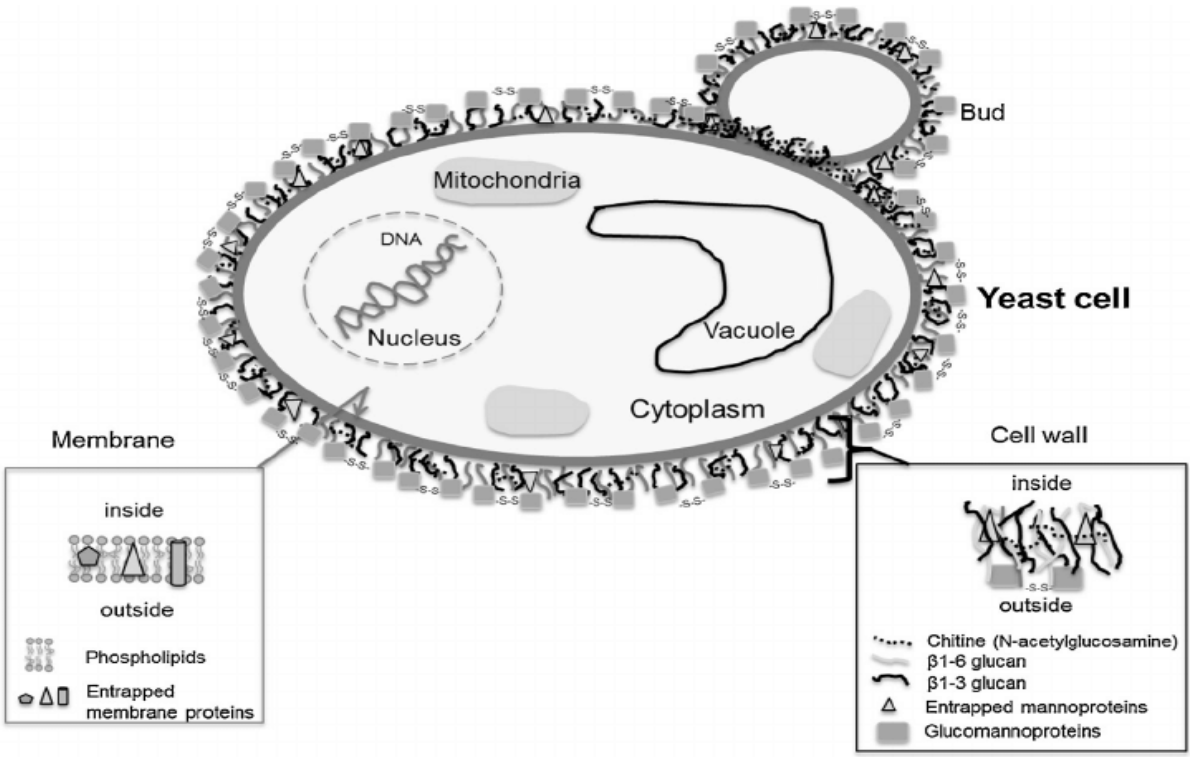


Figure 1 Schematic Structure of Yeast Cell (Chantal et al. 2014)

Saccharomyces cerevisiae

When it comes to yeasts, it has become synonymous with *Saccharomyces cerevisiae* in daily life and is also defined as a domesticated organism. Thus, they have always been close to humans, although they were used unknowingly until the last century as a source of food in the microbial world. *S. cerevisiae* is the specific type of fungus that is also described as a "sugar-eating fungus". This species has a reasonably vigorous fermentation and baking power.

Types of Stress Factors

There are constant changes in the natural habitat of an organism, such as but not limited to nutrient availability, average temperature, or presence of toxic materials. Organisms often elicit an adequate response to these changes, called stress factors. The organism may perceive stress factors as a threat, challenge, or physical and psychological barrier. *Stress resistance* is a

survival trait that makes an organism respond to the surrounding stress factors. The method of expressing stress resistance varies in accordance with the type of stress factor present in the environment. Studies of Eigenfield et al. 2021 has revealed that *S. cerevisiae* cells are exposed to a wide range of stressors which drastically affect its viability and vitality due to disrupted homeostasis during industrial processes. Three main stress types will be investigated in this study.

pH Stress

Although *S. cerevisiae* cells proved to be stress-tolerant between 3.0-11.0 pH levels (Rogowska et al., 2018), reproductive and fermentative ability as well as the cell size of the yeast are dependent on the pH of the growth medium. Hence, an imbalance between the internal and external pH would influence the viability of *S. cerevisiae* cells.

Heat Stress

When cells are exposed to extreme temperatures, the critical components of cells, such as proteins and plasma membrane, are dramatically affected (Beney and Gervais, 2001). Furthermore, most eukaryotic (Magerand Moradas Ferreira, 1993) and prokaryotic (Morozovet al.,1997) microorganisms can develop a degree of thermotolerance under particular conditions in order to protect their components and maintain homeostasis. Exposure to a moderate 24-hour incubation heat treatment leads yeast to develop thermotolerance, which is linked to heat shock factor and stress response element pathways (Mager and Moradas Ferreira, 1993; Morano et al., 1998; Parsell et al., 1994)

Heavy Metal Stress

In the natural environment, metals and metalloids are abundant. Metals enter organisms through natural sources or, more recently, anthropogenic sources such as the use of metals and metal compounds as fungicides and disinfectants (Waldron et al. 2009). Metals remain in cells and interfere with cellular homeostatic circuits as they cannot be destroyed or changed like toxic organic substances (Ballatori, 2002). Metal toxicity results from a variety of effects on the cellular and organismal levels, involving oxidative stress (Valko et al. 2005), changes in enzyme and protein function (Porwol et al. 1998), and DNA damage (Beyermen et al. 2008).

Coping Mechanisms

The stress response's biological purpose is to protect cell components not only against the potentially detrimental effects of present stressors but also to prepare them for potentially damaging elements of the same or other types of stressors. Among the variety of organisms that are conducted stress resistance research on, *Saccharomyces cerevisiae* has a distinctive position in terms of its complex stress response mechanisms.

Common Stress Response

In response to a stress factor, the metabolic activity of the cell alters owing to the suppression of most proteins generated in the cell under normal physiological conditions and the production of a unique set of proteins known as stress proteins. The division cycle is temporarily slowed or inhibited due to these modifications. This process is known as the common stress response, as it is the first reaction of an organism in the presence of a stressor.

Specific Stress Resistance

The activation of unique pathways for certain types of stress results in a specific stress response. It entails the activation of specific defense and repair systems, the result of which is resistance to stress factors. Many responses by *S. cerevisiae* cells are specific to eliminating a particular stress factor (Causton et al. 2001).

Cross-Stress Response

Cross-stress response is activated by a combination of different stress factors. The resistance developed as a response to cross-stress factors is detected not just in the stress reaction but also in cells that develop slowly or have a cell cycle arrested (Sheltzer et al., 2012). Davies et al. demonstrated that exposing yeast to conditions that disrupt the equilibrium between the production and neutralization of reactive oxygen species triggers an adaptation response that leads to a transitory resistance to higher levels of the same conditions. Tolerance to otherwise deadly levels of chemical and physical variables in a fermentation environment has been linked to increased protein synthesis during pre-exposure treatment, priming the cells to adapt and respond more efficiently as the environment exposes more stress to the organism (Aguilar-Uscanga and François,2003). Such studies indicate that yeast has the ability to improve its fermentation resilience if the relevant environmental and internal triggers are activated. Cross-stress resistance develops in cells when two stress factors with different intensity levels are exposed to cells simultaneously (Swiecito, 2016).

Preliminary Experiment and the Scope of Final Investigation

Conducting a set of preliminary experiments is beneficial in terms of narrowing down the research question and determining the scope of the actual experiment. During the experiments, previously optimized growth conditions (Dejean et al., 2000 and Salari et al., 2017) are modified to serve this investigation's needs best. This process achieved the optimum yeast concentration with a solution of 2.5g yeast and 2.5g glucose in 250 ml distilled water, resulting in the best fermentation rate after an hour of incubation time. The most desirable incubation time was determined to be between 30-60 minutes for the first round of measurements (without the stress factors) and further 60 minutes for the second round of measurements (after the stress factors are included). This makes a total of 90 to 120 minutes (assuming that the fermentation measurements for all samples are done within the 30 minutes after incubation) which corresponds to the doubling time of *S. cerevisiae* according to the research done by Salari et al. Reviews from Leon (2021) and Kireççi (2016) led to the use of Cu as the main component of heavy metal stress. Spectrophotometric measurements were taken at 412, as suggested by Elman (1959), and 600 nm, as it was is traditionally used for determining the optical density, which is proportional to the number of cells in the cuvette. It was found that measurements done at 600 nm gave a clear relation with the cell number and amount of stress factor applied.

Hypothesis

After a detailed literature review and a set of preliminary experiments, the hypothesis of this investigation is formed as follows: The exposure to complex stress factors leads *S. cerevisiae* cells to develop cross-stress resistance, which decreases the negative effects of a particular stress factor or overall negative effects of them.

Aim of the Investigation

The aim of the investigation is to determine whether cross-stress resistance due to combined stress agents decrease the detrimental effects of single stress factors exposed on *S. cerevisiae* cells.

Investigation

Table 1: A table summarising the variables and their role in the investigation

Variables		**Impact upon the investigation**	**How the variable will be changed/ measured/controlled**
Independent variable	Temperature	Changing temperature may cause denaturation of cellular proteins	Chosen values: -18, +4, +25 Each kept constant with heating/cooling surroundings
	pH	Changing pH would affect the ion balance inside and outside of the cell	Chosen values: 2, 5, 11 (pH) Each kept constant manually depending on the readings from pH Meter.
	Metal Concentration	Changing metal concentration would disrupt the metabolic pathways of the cell and thus, affect the viability	Chosen values: 0.5 , 1, 2 mg of $CuSO_4$ Each kept constant manually using sterile pipettes.
Dependent variable	Cell Concentration	Cell concentration is directly related with the yeast budding, thus a good measure to answer the research question	Readings are taken from the UV spectrophotometer at 600 nm.
	Rate of fermentation	Rate of fermentation is directly related with the viability of the yeast cells	Calculations for determining reaction rate is required.
Control variables	Incubation time	Both of these variables might impact the data collected by increasing/decreasing the budding rate	Incubation time will be kept constant by conducting the experiment simultaneously
	Volume of yeast suspension		Volume of yeast suspension will be kept constant by using sensitive scales.

Materials

Table 2: A table showing the materials used in the experiment

Measurement Equipment	Apparatus	Other Materials
(9) 0.5 cm^3 graduated pipettes (± 0.005 cm^3)	Incubator (+25°C)	Cotton (for incubation)
(1) electronic weighing scale (±0.0001g)	Refrigerator (+4°C)	Aluminum foil of size 10cm x10cm (for incubation)
(1) electronic weighing scale (±0.01g)	Freezer (-18°C)	Clean soft towel
(1) 1000 cm^3 graduated cylinder ± 5cm^3	Laminar air flow	Distilled Water
25cm^3 volumetric pipette ±0.03 cm^3	Shimadzu UV-2700i Spectrophotometer	70% Ethanol (for sterilization)
(24) 500 cm^3 beaker ± 3 cm^3	Vernier pH Meter	
(3) 250 ml erlenmeyer	LabQuest O_2 Meter	

Procedure

The experiment consists of two consecutive stages. First stage includes the separate application of different stress factors, being temperature, pH and heavy metal stress. Second stage consists of combined stress stimuli for to determine whether cross-stress resistance would develop in the *S. cerevisiae* cells.

Procedure of First Stage of the Experiment

250 mL distilled water and 2.5 g glucose containing medium was prepared for the growth and proliferation of *S. cerevisiae* used in the experiment. The number of repetitions for each group was (n) = 5. After the medium was prepared, a cotton plug is used to close the flask.

It incubated for 30 minutes. Fermentation is expected during this time, hence if bubbles were observed at the end of the incubation, the solution was divided into the following groups;

Control Group: For *S. cerevisiae* cells in this group, medium containing 2.5 g of yeast extract and 2.5 g of glucose in 250 mL of distilled water was prepared. No stress is exposed to the cells in this group.

Temperature Stress Groups: For *S. cerevisiae* cells in this group, after the medium containing the growth medium was prepared, each group is placed to the following temperatures: -18C, +4 C, +25 C. The refrigerator is set to +4 degrees, the freezer to -18 degrees. Cells are then left for 1 hour.

pH Stress Groups: For *S. cerevisiae* cells in this group, after the medium containing the growth medium was prepared, 15 mL of 2%, 5%, 10% NaCl concentrations are added to the 25 mL yeast solution. A control group is prepared with 15 mL of distilled water and 25 mL of yeast solution. Cells are then left for 1 hour at 25°C.

Heavy Metal Stress Groups: For *S. cerevisiae* cells in this group, after the medium containing the growth medium was prepared, 15 mL of 0.5%, 1% and 2% mg of $CuSO_4$ solution was added to the culture mediums as 25 mL. After inoculation, cultures were incubated at 25°C for 1 hour.

At the end of the incubation period, after measuring the cell densities at 600 nm using spectrophotometer and the rate of fermentation was measured under laboratory conditions as explained in the “Methods” section.



Figure 2 Experiment groups (white cylinder with cotton attached, pH stress agents (middle line) and heavy metal stress agents in different concentrations (volumetric flasks with blue solutions)

Procedure of the Second Stage of the Experiment

The second stage of the experiment, which includes cross-stress groups, is designed based on the aforementioned results obtained[1]. The most desired results from each category was recorded as being 25°C, 2 pH and 0.5 molar Cu stress. This group is named as the group A.

The exact opposite is true for the least desired results (-18°C, 10pH and 2 molar Cu solution) meaning that yeast cell viability and thus, fermentation rate decreased drastically in these stress groups, which named as the group B.

Chosen groups are used to create the second set of variables, which are presented in the Table3.

[1] See Processed Data section.

Table 3: A table showing the experiment groups for the cross-stress exposure

Name of the Group	Temperature Stress Factor	pH Stress Factor	Heavy Metal Stress Factor
A1	25°C	2pH	-
A2	25°C	-	0.5M $CuSO_4$
A3	25°C	2pH	0.5M $CuSO_4$
B1	-18°C	10pH	-
B2	-18°C	-	2M $CuSO_4$
B3	-18°C	10pH	2M $CuSO_4$
B4	25°C	10pH	2M $CuSO_4$

Aseptic Technique

All the materials were sterilized with distilled water and followed by 70% ethanol before used. During the experiment, gloves were also worn and all containers were closed while not in use. (Working with Yeast, n.d.). Experiments conducted in a biological safety cabinet (Thermo-Fisher Laminar Air Flow) to avoid any contamination.

Ethical Considerations

Since there are no specified ethic rules for working with yeast cells (Levy, N., 2012), this study fulfills the ethical clearance requirements.

Methods

Two different test methods have been determined in order to test the functions of yeast cells in each of the nine groups to adapt to the environment with a stress response or to test their viability:

Determination of the Rate of Fermentation

After the incubation, 20 ml of the yeast solution and H_2O_2 solution was mixed in a 200 ml Erlenmeyer. After calibration, an O_2 sensor was placed at the top and covered to prevent gas escape. Maximum O_2 levels and time taken for that value were recorded.



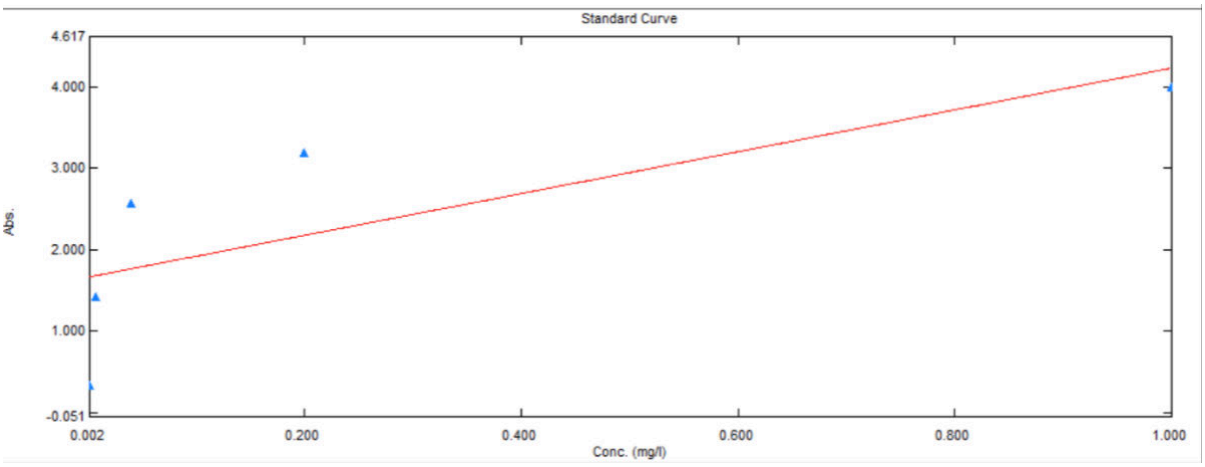
Figure 3 Readings from O_2 meter

Determination of the Yeast Cell Concentration

Once the incubation time was over, the solutions were added in cuvettes with sterile pipettes and then placed in a calibrated Shimadzu UV-2700i spectrophotometer. As seen in the Graph 1 below, a "Standard Curve" was prepared with solutions in different concentrations, each were % 50 diluted. All readings were taken consecutively to prevent further budding. Before each measurement, the solution was shaken slightly for homogeneity.

The whole procedure was then repeated five times.

Graph 1: Standard Curve used in the spectrophotometer



Results

Qualitative Observations

- After the yeast solution is prepared and left for the first incubation period, it was observed that the yeasts settled at the bottom. In order to prevent a possible uneven distribution within the solution, the samples are shaken before each measurement.
- Solutions that are incubated in room temperature was condense and more bubbles are observed at the surface than solutions incubated in the refrigerator, whereas solutions at -18°C was frozen and no bubbles were present.
- During the spectrophotometric readings, it was observed bubbles are produced on the surface of the cuvette. This suggests that yeast solution continued fermentation during the measurement process.
- Groups that $CuSO_4$ solution is added turned a brown color when mixed with H_2O_2 solution during the measurements, suggesting that for these experiment groups there might be other reactions affecting the rate of O_2 production, as well as yeast fermentation.

Raw Data

Table 4: Raw data obtained from the first and the second stages of the experiment

1st Stage		Concentration (mol/L)					Absorbance (WL 600,0)					Amount of O_2	
		1	2	3	4	5	1	2	3	4	5	Max %	Time (s)
Control		0,312	0,302	0,310	0,308	0,395	2,464	2,437	2,459	2,455	2,678	15,58	175
Temperature	-18	0,389	0,394	0,395	0,398	0,398	2,663	2,675	2,676	2,684	2,685	24,35	738
	4	0,415	0,416	0,410	0,434	0,420	2,729	2,732	2,715	2,778	2,759	19,84	290
	25	0,397	0,536	0,438	0,108[2]	0,442	2,682	3,039	2,788	1,940	2,812	15,39	155
pH	Control	0,398	0,571	0,493	0,502	0,497	2,686	3,130	2,929	2,953	2,932	15,85	191
	2	0,340	0,317	0,334	0,380	0,353	2,536	2,476	2,520	2,638	2,585	13,39	254
	5	0,219	-0,083	0,208	0,224	0,220	2,225	1,451	2,196	2,238	2,231	10,88	286
	10	0,152	0,191	0,159	0,163	0,172	2,053	2,152	2,072	2,081	2,181	10,40	235
Heavy Metal	Control	0,222	0,291	0,225	0,263	0,302	2,234	2,897	2,239	2,337	2,437	18,97	239
	0.5	0,141	0,117	0,109	0,157	0,134	2,026	1,963	1,944	2,067	1,985	28,75	1030
	1	0,446	0,435	0,421	0,466	0,421	2,808	2,781	2,744	2,859	2,744	31,92	1418
	2	0,387	0,384	0,372	0,368	0,352	2,656	2,649	2,617	2,607	2,470	26,48	1406

2nd Stage	Concentration (mol/L)					Absorbance (WL 600,0)					Amount of O_2	
	1	2	3	4	5	1	2	3	4	5	Max %	Time (s)
Control	0,312	0,302	0,310	0,308	0,275	2,464	2,437	2,459	2,455	2,368	18,93	234
A1	0,414	0,415	0,416	0,415	0,403	2,726	2,727	2,730	2,729	2,697	13,26	238
A2	0,325	0,238	0,268	0,268	0,376	2,497	2,273	2,350	2,351	2,628	19,54	619
A3	0,431	0,421	0,420	-0,187	0,420	2,769	2,745	2,742	1,183	2,742	26,75	963
B1	0,525	0,522	0,523	0,522	0,523	3,022	3,003	3,005	3,002	3,005	11,59	375
B2	0,399	0,398	0,396	0,397	0,398	2,688	2,685	2,680	2,682	2,684	19,24	427
B3	0,291	0,286	0,285	0,279	0,291	2,410	2,397	2,395	2,380	2,411	23,43	953
B4	0,365	0,366	0,367	0,366	0,368	2,600	2,604	2,604	2,604	2,607	25,21	647

[2] The highlighted values are anomalies as they are far from the other values and are not included in the calculations.

Calculations

The formula for calculating the average concentration:

$$Avr.Cont. = \frac{1st\ trial + 2nd\ trial + 3rd\ trial + 4th\ trial + 5th\ trial}{5}$$

The formula for calculating the rate of fermantation:

$$Rate\ of\ ferm.(\%) = \frac{Max\ ferm\ x\ 60}{time\ taken\ (s)}$$

The formula derived for determining the stress-response rate of cross-stress factors in *S. cerevisiae* cells (for both concentration and rate of fermentation):

$$Stress\text{-}response\ rate\ (\%) = \frac{Value\ of\ Experiment\ Group\ x\ 100}{Value\ of\ Control\ Group}$$

Sample Calculations

→ Calculating the average concentration for the control group

$$Avr.Cont. = \frac{0{,}510 + 0{,}498 + 0{,}492 + 0{,}488 + 0{,}495}{5} = 0{,}497$$

→ Calculating the rate of fermantation for the control group

$$Rate\ of\ ferm = \frac{15{,}58\ x\ 60}{175} = 5{,}685\ \%$$

→ Calculating the stress-response rate in -18°C experiment group

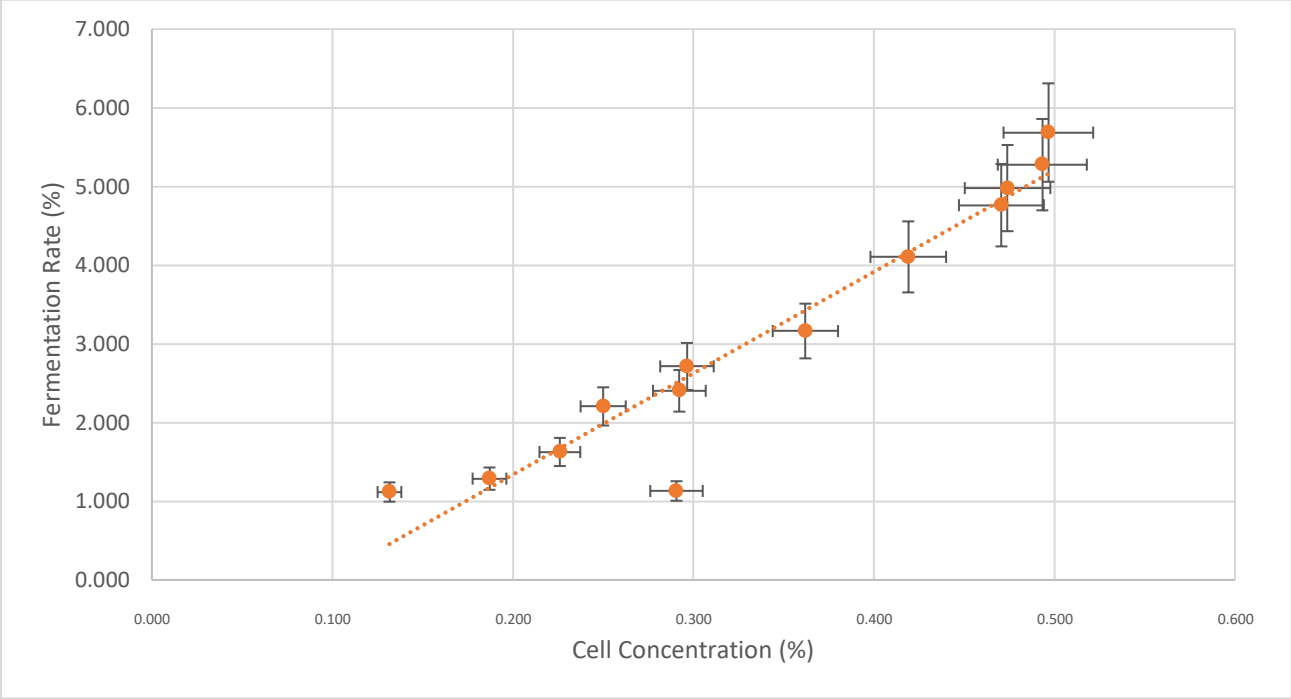
$$Stress\text{-}response\ rate = \frac{0{,}292\ x\ 100}{0{,}497} = 59\%$$

Processed Data

***Table 5**: A table showing the average cell concentration at 600nm in % of 5 trials, standard deviation, the rate of fermentation of the yeast solutions at different levels of stress exposure and the rate of stress - response based on them*

Experiment Groups		Average Concentration (mol/L)	Standard Dev.	Stress-response rate for cont. (%)	Fermentation Rate	Stress- response rate for ferm. (%)
Not incubated yeast		0,296	0,008	-	2,713	-
Control		0,497	0,008	-	5,685	-
Temperature (°C)	-18	0,292	0,005	59%	2,403	42%
	4	0,419	0,009	84%	4,105	72%
	25	0,493	0,176	99%	5,277	92%
pH	Control	0,474	0,008	-	4,979	-
	2	0,362	0,011	76%	3,163	63%
	5	0,250	0,152	52%	2,205	44%
	10	0,187	0,011	39%	1,287	25%
Heavy Metal (mol/g)	Control	0,471	0,028	-	4,762	-
	0.5	0,132	0,019	27%	1,117	34%
	1	0,226	0,006	47%	1,626	26%
	2	0,263	0,010	61%	1,130	13%

Graph 2: A graph showing the correlation between rate of fermentation and cell concentration



Trends Noted from the Graph[3]

- There is a strong correlation between the cell concentration and the rate of fermentation, meaning that the more yeast cells present in the solution, the faster fermentation occurs.
- However, it also seems that for some values fermentation rate decreases even there is a high concentration of yeast cells.
- There are two anomalies which are belong to the 0.5 and 2 molar $CuSO_4$ stress groups respectively.

Second experiment groups, which consist of different combinations of distinct stress factors, are designed in accordance with the results obtained in the first stage. Same methods are used to obtain the measurements.

Table 6 A table showing the average cell concentration at 600nm in % of 5 trials, standard deviation and rate of fermentation at different levels of combined stress exposure

Experiment Group	Average Concentration (mol/L)	Standard Dev.	Stress- Response Rate (Cont.)	Fermentation Rate	Stress- Response Rate (Ferm.)
Control	0,300	0,015	-	5,854	-
A1	0,423	0,273	83%	3,343	32%
A2	0,412	0,005	59%	1,894	28%
A3	0,302	0,055	85%	1,667	57%
B1	0,288	0,005	105%	1,854	46%
B2	0,523	0,001	80%	2,704	39%
B3	0,367	0,001	57%	0,475	5%
B4	0,398	0,001	73%	2,338	8%

[3] Further explained under the Discussion section.

Observations Noted from the Table 3

- Combinations of different stress agents altered the response of the yeast cells. Most apparent change occurred in the experiment groups with heavy metal stress factor.
- Temperature seems to have a defining role on the increase in cell concentration. Yeast solutions that are exposed to low temperature stress observed to have lower concentration (signaling that cells failed to bud) and slower fermentation rate irrespective of other stress agents included.
- Mild temperature stress seemed to decrease the effects of pH stress. Yeast cells are observed to have a better viability (in terms of their fermentation ability) when exposed to both pH stress and temperature than pH stress only.
- Cells in B3 group responded the combined stress factors aggressively, which results in a clear decline in the fermentation rate.
- No overall increase in the viability is observed in the yeast cells that combined stress factors are applied. Instead, a decrease in the destructive effects of individual stress agents is detected.

Statistical Test

Since there is a positive correlation was expected, Pearson Correlation Test was used to ensure the results were statistically significant.

- H_0 (Null Hypothesis): No cross-stress resistance is developed in *S. cerevisiae* cells since there is no linear relationship between the cell concentration and the fermentation rate.
- H_1 (Alternate Hypothesis): There is a pattern of cross-stress resistance in *S. cerevisiae* cells due to the linear relationship between the cell concentration and the fermentation rate.

Table 7: Calculation of Pearson's Correlation Test

Experiment Groups (n)			Conct. (x)	Ferm. (y)	xy	X^2	Y^2
1	Not incubated yeast		0,296	2,713	0,804	0,088	7,360
2	Control (incubated 30min)		0,497	5,685	2,823	0,247	32,319
3	Temperature	-18	0,292	2,403	0,702	0,085	5,774
4		4	0,419	4,105	1,720	0,176	16,851
5		25	0,493	5,277	2,603	0,243	27,847
6	pH	Control	0,474	4,979	2,360	0,225	24,790
7		2	0,362	3,163	1,145	0,131	10,005
8		5	0,250	2,205	0,551	0,063	4,862
9		10	0,187	1,287	0,241	0,035	1,656
10	Heavy Metal	Control	0,301	5,854	1,762	0,091	34,269
11		0.5	0,132	1,117	0,147	0,017	1,248
12		1	0,226	1,626	0,367	0,051	2,644
13		2	0,291	1,130	0,328	0,084	1,277
Sum (Σ)			4,220	41,544	15,554	1,535	170,903

To find the Pearson's correlation test, the below-mentioned equation is used,

(Pearson's Test Correlation Coefficient- Excel guide, 2017)

$$r = \frac{n(\Sigma xy) - (\Sigma x)(\Sigma y)}{\sqrt{[\, n\Sigma x^2 - (\Sigma x)^2 \,][\, n\Sigma y^2 - (\Sigma y)^2 \,]}}$$

$$r = \frac{(13\ x\ 15{,}554)\ - (4{,}220\ x\ 41{,}544)}{\sqrt{(13\ x\ 1{,}535 - 17{,}808) x\ (170{,}903 - 1725{,}904)}}$$

$r = 0{,}823$ (to 3 decimal place)

$Degrees\ of\ freedom = 13 - 2 = 11$

Using the critical values for Pearson's correlation test from an academic website[4], the Pearson's correlation coefficient value of 0.823 is smaller than but close to the critical value of 1 for the degree of freedom of 11, such that the null hypothesis is rejected and the results of this experiment are statistically significant.

Discussion

During analysis of literature regarding stress response of *S. cerevisiae*, it was observed that none were experimenting with cross-stress. Rogowska et al. (2018) investigated different levels of heat and pH stress factors, and Leon (2021) emphasizes that Cu is the main heavy metal stress that arrests the cell cycle of *S. cerevisiae* cells. Such studies fail to address a combined stress exposure as it usually occurs in real life. Thus, this study is important in terms of investigating cross-stress resistance.

The result of the experiment shows that cross-stress resistance due to combined stress agents decrease the detrimental effects of single stress factors exposed on *S. cerevisiae* cells. The validity of the results is supported by the low standard deviations (ranging between 0,005 to 0,273), suggesting the consistency of the cell viability in different trials. The statistical test also provides evidence that the results of this test are statistically significant, confirming that a combination of the stress factors decreases the overall damage of individual stress factors. However, more trials will be needed in order to identify anomalies that occurred due to random errors. The anomalies in this investigation were also avoided in calculations to obtain reliable results.

The relationship between the yeast cell concentration and fermentation rate in different experiment groups with various stress stimuli are demonstrated in the Graph 1, which suggests that the higher the concentration, the faster the fermentation rate. Therefore, as the gradient line

[4] Media3.bournemouth.ac.uk, n.d.

implies, these two variables are correlated. This indicates there is a cross-stress resistance have developed. A discussion prompt would be whether the dead cells present in the solutions affect the concentration. During the incubation time, yeast cells increase as the doubling time is provided to the cells. Thus, the concentration of the solution increases. However, since the stress factors can seriously harm the cells, it is expected to be dead yeast cells within the solution. However, the fermentation rates confirm the extent to which dead cells increase the concentration. If a solution is rich in terms of viable cells, the fermentation rate would be faster since the dead cell do not interfere with the fermentation process.

Two values belonging to the 0.5 and 2 molar $CuSO_4$ stress groups are outside the best fit line. This indicates that in some cases, concentration or fermentation rate might be affected by external factors. For instance, The $CuSO_4$ particles might increase the concentration irrespective of the number of yeast cells, as well as the reactions occur during O_2 measurements since $CuSO_4$ and H_2O_2 molecules tend to undergo a reaction (C. Zhang et al., 2016) that may cause further O_2 production, which is, therefore, a limitation of the experiment.

For the second stage of the experiment, no clear correlation is detected between variables. Since the experiment groups are created with combinations of different stress factors, thus a random distribution has occurred. However, some patterns signal cross-stress resistance developed in the yeast cells. Comparing Tables 3- 4 and 1- 2 reveals that mild temperature stress reduces the effects of pH stress. When exposed to both pH stress and temperature simultaneously, yeast cells show higher viability in terms of their fermentation potential than when exposed to only pH stress. Besides, the destructive effects of heavy metal stress factors seem to be reduced with cross-stress exposures. The only exception is the B3 group with a 5% stress-response rate, signaling that yeast cells aggressively respond to the combined stress factors. This suggest that three severe stress stimuli excess the response capacity of the *S. cerevisiae* cells and create no room for the development of cross-stress resistance.

The strengths of these results are limited as the results also suggest that some combinations of different stress agents can damage the yeast cells more than an individual stress factor exposed. Further investigation is needed to determine the specific stress combinations in which cross-stress resistance develops in the yeast cells as a response. Despite this, the results are promising, meaning that if the correct strategy is used, cross-stress resistance can be developed during the industrial processes that include Baker's yeast and the detrimental effects of the combined stress agents due to climate change can be reduced significantly.

Evaluation of Procedure

Table 8: A table demonstrating the limitations of the investigation and possible improvements

Limitations	Improvements
Need a longer incubation and exposure time to understand the effects of continuous stress factor exposure.	Carry out the experiment with 120 minutes incubation time and 180-240 minutes of stress exposure time. Thus, the doubling time for yeast cells would be there times longer.
The O_2 measurement was not taken at the same time for the different samples. This means that the yeast may bud more depending on the measurement delay.	A number of the same instrument (for this experiment, O_2 meter) can be used to record the rate of fermentation simultaneously.
It is possible that the experiment has started before the cells were fully active.	Check for bubbles with a sterile gas tube submerged in distill water to be sure fermentation has started before the experiment.

The yeasts may not have been distributed evenly throughout the yeast solution.	Use a magnetic stirrer to keep the yeast solution at maximum homogeneity.
Despite the use of sterile procedures, the sample may still be contaminated.	Carry out the whole experiment, including taking measurements, in a laminar air flow. Wear sterile gloves and face masks throughout the experiment.
There are some anomalies in the data, getting a more accurate result is needed.	Carry out the experiment with 10 repeats

Evaluation of the Sources Used

Secondary sources included in this research appear to be credible because they were collected from published journal papers (the majority of which were published within the previous 15 years) or online databases. However, since science advances so quickly, the older the secondary source, the less credible the information. As a result, relying on resources released in the recent 5 years may have been an improvement.

Conclusion

In conclusion, this study provides an answer to the research question. The results, support the hypothesis that cross-stress resistance does develop when certain combinations of different stress stimuli are exposed to *S. cerevisiae* cells despite some anomalies.

When multiple stress stimuli are given to yeast cells, there seems to be no overall increase in viability. Rather, a reduction in the damaging effects of specific stress agents is observed, which is the aim of cross-stress resistance In some cases, the combined stress was detrimental to the yeast cells, which eventually caused apoptosis and death of the cell. This

may, in turn, be used in the yeast industry to increase the efficiency and produce more resistant *S. cerevisiae* cells so they can adapt to the environmental changes caused by climate change.

However, these results are only relevant for these specific experiment groups. Further investigation is needed to determine the type and amount of the stress agent that will be exposed to the *S. cerevisiae* cells for the development of cross-stress resistance response.

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