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Evaluation of antagonistic property and osmotic stress tolerance of yeast based biocontrol formulation

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Abstract
The application of yeast based biocontrol formulation is safe and economic alternative for reducing post-harvest fungal decay of fruits. Development of formulation is an important step in the application of a biocontrol agent. When the yeast is in an environment with high osmolarity, water flows from the cell into the extracellular medium. This results in a decrease in the cellular volume and turgor pressure and may result in cell death. Tolerance to osmotic stress is a prerequisite for development of biocontrol yeast formulation. The current study was aimed to evaluate the antagonistic property of yeast isolate and its osmotic stress tolerance capacity. The antagonistic property of Y-27 (*Meyerozyma caribbica*) was evaluated against *Colletotrichum musae* by dual culture method in vitro. The viability of antagonistic yeast in molasses urea based liquid formulation was evaluated after exposing them to osmotic stress with molasses urea media containing salt at different concentration and also with changing the concentration of molasses. The number of viable cells (Colony forming units per millilitre) was counted with modified serial dilution method. *Meyerozyma caribbica* showed an antagonistic property against the test pathogen. The cells were grown in the control formulation showed a significant change in the cell size and shape compared to the formulations which induced osmotic stress. It was generally observed that as salt concentration increases, the viability of cells increases but the cell multiplication rate decreased proportionally. The formulation prepared with yeast isolate was found highest viability in 1% NaCl and 15% molasses added separately up to 120 and 60 days of storage respectively.

Keywords: Biocontrol yeast, osmotic stress, NaCl, molasses urea, *Colletotrichum musae*

Introduction
Postharvest decay of fruits by fungal pathogens is often controlled with synthetic chemical fungicides. However, consumer concerns about the safety of synthetic fungicides, as well as their environmental effects, prompted the adoption of safer alternatives (Droby *et al*. 2009) [6]. Yeasts are naturally occurring unicellular microbial organisms that has been found to control a variety of postharvest pathogens (Droby *et al*. 1993; Wisniewski *et al*. 2007; Piano *et al*., 1997; Zhimo *et al*. 2017) [5, 11, 16, 17]. In the development of a biocontrol product, formulation is critical. The formulation determines how well biocontrol agents are delivered, as well as their shelf life, stability, and effectiveness in commercial conditions. Antagonistic yeasts are exposed to a wide range of adverse stresses during pre- and post-harvest application, as well as during the production process, including competition, predation, temperature changes, osmotic stress, variable pH, oxidative stress, and the availability of nutrients and water, all of which may affect their viability and efficacy. Cells must physiologically be able to respond to such osmotic stress in water shortage environments, either due to the presence of a high concentration of salts or in intermediate moisture agricultural products, to expand and reproduce (Magan, 2007) [10]. The mechanism of osmolarity has been demonstrated by Tamas and Hohmann, 2003 [14]. As a result of increment in the osmolarity of extracellular environment, the surrounding water becomes less available for the cell. Water appears to flow from a compartment with low osmolarity to one with higher osmolarity, so if yeast is in an atmosphere with high osmolarity, water flows from the cell into the extracellular medium, resulting in a water efflux and cell death. Based on the above background the present research work was conducted to evaluate the antagonistic property of yeast isolate and its osmotic stress tolerance capacity in the formulation.

Material and Methods
Biocontrol yeast and test pathogen
The yeast isolate *Meyerozyma caribbica* (Y-27; NCBI accession no-MN873568) was obtained

from Departmental Culture collection of Department of Plant Pathology, Bidhan Chandra Krishi Viswavidyalaya, Mohanpur, Nadia, West Bengal. *Colletotrichum musae* causing anthracnose of banana was used for studying the antagonistic potential of the yeast isolate.

Evaluation of antagonistic potential of the yeast isolates against the test pathogen

Antagonistic potential of the yeast against *Colletotrichum musae* was confirmed by following dual culture technique. 6 mm diameter disc of 5 days old grown test pathogen were inoculated on 90 mm Petri plates, containing Potato Dextrose Agar medium supplemented with yeast extract (1.0 g L^{-1}), at a distance of 1 cm from the periphery. A streak from a yeast cell suspension (1.2-1.4 × 10^8 CFU ml^{-1}) was made 1 cm from the periphery of opposite end. The plates were incubated at 28 ± 1^0C till 7 days. A similar plate streaked with sterile distilled water was a kept as control. The radial growth of the fungi was measured and the percentage mycelial growth inhibition of the pathogen by the yeast isolate was calculated using the formula: (radial of pathogen on control plate- radial growth of pathogen on treatment)/radial growth of pathogen on control plate) × 100 (Skidmore and Dickinson, 1976) [12].

Evaluation of osmotic stress tolerance of the yeast formulations

Molasses Urea media (Cane Molasses, 50 g/l; Urea, 1.2 g/l) were used for multiplication and formulating the yeast cells. CFU/ml was counted using haemocytometer. The tolerance of osmotic stress of yeast Y-27 *Meyerozyma caribbica* was evaluated in Molasses urea media (cane molasses, 50gL^{-1}; urea, 1.2gL^{-1}) at pH 6.5 at storage temperature of 28 ± 1^0C. Sugar stress tolerance was assessed using the following concentrations of cane molasses in the molasses urea media: 5%, 15%, 20%, 25%, 30%, 35, 40%, and 45 percent. The salt tolerance was evaluated by adding different concentrations of Sodium chloride at the rate of 1%, 1.5%, 2%, and 2.5% in the formulation. Fresh yeast cells were grown as a starter culture in molasses urea media for 72 hours in a 250ml Erlenmeyer flask, and after the yeast cells reached exponential phase, 1 ml aliquots of this suspension were aseptically added to each flask containing different concentrations of molasses and salt. Yeast cells in aliquots with a concentration of 1-1.4x10^8 CFU ml^{-1} were added to replicated flasks at 28 ± 1^0C. Taking 10µl (composite sample of replicated flasks) aliquots from each formulation and plating on YPDA plates after 4, 10, 20, 30, 60 and 120 DAS determined the number of viable cells (CFU ml^{-1}) using modified serial dilution method (Thomas *et al*., 2015) [15].

Modified serial dilution method

The steps followed during modified serial dilution method as described by Thomas *et al.,* 2015 [15]. Media was prepared and poured in the plates (15-20 ml per 9 cm diameter pre-sterilized plates). The media was allowed to set in the laminar airflow (LAF) cabinet. Serial dilutions in sterilized distilled water up to 10^{-6} were prepared. The micro-tips were flushed 4-5 times and changed after each dilution. Reverse side of the petriplates were marked. 10µl aliquots from the dilutions were applied as micro-drops in marked areas. Plating was started from the last dilution and using the same tip. The plates were allowed to dry in the LAF cabinet for 4-6 minutes. Plates were sealed and incubated at 28±1°C for 24 hours. Colonies were counted in each sector and the CFU ml^{-1} of the sample was worked out by applying the formula: n x 10^{d+2} where, n = no. of colonies in 10µl sample; d = dilution level yielding countable colonies.

Study on relation between cell morphology in different formulations with osmotic stress

The morphology of the yeast cells in the formulation which showed viability (for 1 month) and non-viability under sugar and salt concentrations was observed by preparing slides and staining with cotton blue. The cells were examined under the phase contrast optical microscope (Carl Zeiss AxioScope, Germany) at 100X magnification. The length and breadth of the cells were measured and recorded along with shape. The measurements were taken using AxioVision Rel. 4.8 software.

Data analysis

The lab experiments were arranged in Completely Randomized Design (CRD). Data analysis was done using OPSTAT and Microsoft Excel software.

Results and Discussion

***in-vitro* test for antagonism of the yeast isolate**

The antagonistic potential of yeast isolate against *Colletotrichum musae* was evaluated by dual culture method. The yeast isolate was showed about 22.2% mycelial growth inhibition against pathogen (Fig 1). The control plates observed with profuse mycelial growth which has crossed the streak with sterilized distilled water. The inhibition of the pathogenic fungal growth is mainly due to the completion for the nutrients in the medium. Antibiosis, competition for nutrients and space, parasitism or direct contact with the pathogen, and induction of resistance in the host tissue have all been proposed as potential biocontrol mechanisms against post-harvest rots on fruit (Droby *et al*., 1994) [4]. There are many reports from the present lab which confirms the antagonistic effect of yeast isolates against *C. musae* (Zhimo, 2017) [17].

Evaluation of osmotic stress tolerance on yeast in the formulation

The cell number of Y-27 in molasses urea media containing salt (NaCl) at different concentration *viz* 1%, 1.5%, 2%, 2.5% was improved by varying degrees in all the concentrations compared to the population in molasses urea formulation without NaCl which kept as control at 28±1 °C (Table 1). It was generally observed that cell multiplication rate decreased proportionally with increased salt concentration.

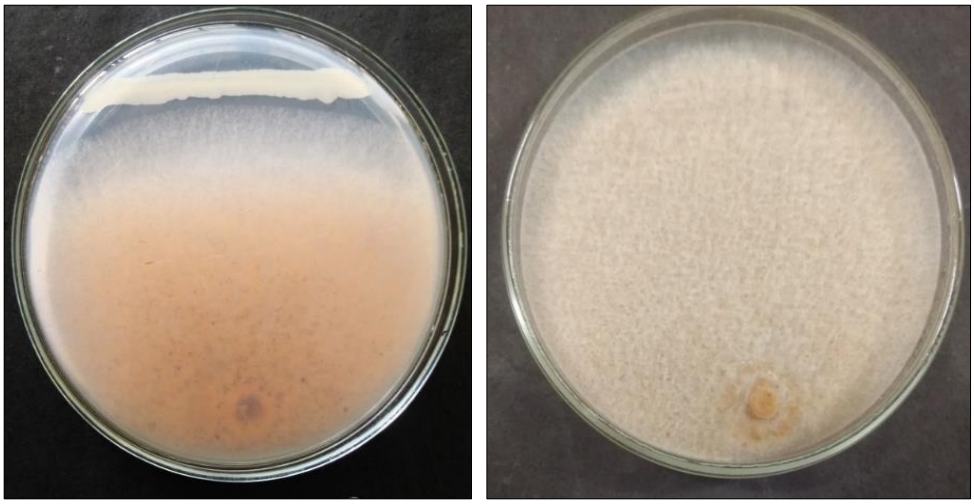

Fig 1: Mycelial growth inhibition of *Colletotrichum musae* by Y-27 yeast isolate *in vitro*

Table 1: Population of Y-27 yeast isolate in molasses urea medium based liquid formulation with different concentration of NaCl as osmotic stress agent at different days after storage (DAS) at 28 ±1 °C

Salt (NaCl) concentration (%)	Population of yeast cells (Log_{10}CFU/ml) at days after inoculation						
	0	4	10	20	30	60	120
1.0	6.25*	7.87	7.89	7.73	7.76	7.57	7.54
1.5	6.25	7.82	7.76	7.67	7.71	7.54	7.49
2.0	6.25	7.77	7.64	7.62	7.68	7.42	7.37
2.5	6.25	7.68	7.60	7.57	7.67	7.36	7.29
CONTROL	6.25	8.10	8.15	7.76	7.79	7.78	2.82
SEM±		0.009	0.008	0.007	0.013	0.014	0.092
C.D (5%)		0.027	0.025	0.022	0.040	0.043	0.280

*Values are means of four replications. Log_{10}CFU/ml are presented in the table.

The highest cell number was obtained with an initial count of 1.8 x 10^7 CFU ml^{-1} in the formulation containing 1% NaCl (3.3 x 10^7 CFU ml^{-1}) as additive and the number of the cells in control formulation decreased drastically to 6.6 x 10^2 CFU ml^{-1} after 4 months of storage 28 °C. In control formulation, the number of cells increased up to 10 days of storage and then decreased. This may be due to the depletion of nutrients in the formulation. Even though the formulations with salt observed with low growth rate, they showed high cell population after 4 months of storage. It indicates that under stress conditions, the cells preserve the nutrients and accumulates some compatible solutes as osmo-protectant. However, it was observed that during experiment, the cell number decreased with increased concentration of salt.

Stelios *et al*. (2007) [13] evaluated the growth and viability of *S. cerevisiae* (VIN 13) in the presence of 0% 4%, 6%, 10% w/v NaCl in glucose-based defined medium and observed more viability at 10% concentrations of salt compared to control. Under stress conditions yeast cells enhance intracellular accumulation of osmolytes, and polyols in particular glycerol and compatible ions such as, amino acids and fatty acids in cell membranes (Butinar *et al*., 2005) [3]. Compatible solutes are intracellular organic compounds that halophilic and halotolerant bacteria accumulate to protect themselves from osmotic stress caused by high NaCl concentrations (Galinski and Truper, 1994).

The cell number of Y-27 in the formulation containing molasses up to 45% were observed up to 60 days of storage at 28±1 °C (Table 2). The formulation containing up to 25% molasses maintained the cell population even after 2 months of storage. The formulation with 45% concentration of molasses, the number of yeast cells reduced to zero after 4 days storage at 28^0C. The number of the cells reduced to zero in the formulations containing >30% molasses after 20 days of storage. The highest cell number was obtained with initial count of 1.9 x 10^6 CFU ml^{-1} in the formulation containing 15% molasses It has been observed that number of cells were highest in 15% concentration (5.63 x 10^7 CFU ml^{-1}) compared to the control (5.28 x 10^7 CFU ml^{-1}) which was formulated with 5% molasses even after 60 days of storage at 28^0 C. At 25% molasses concentration, the cell number decreased after 20 days of storage. But it was increased after 30 days of storage and the maintained the viability even after 60 days of storage at 28°C. This might be due to the multiplication of yeast cells which has been adapted to higher osmotic stress. The findings of the present experiment is comparable with the studies conducted by Abadias *et al*. (2001) [1] have shown that viable counts of cells grown at reduced water activity (0.98 aw) media were equal to or higher than cells grown on unmodified media, with freely available water (0.996 aw). Water stress resistance increased by endogenous contents of glycerol and erythritol. Glycerol and arabitol were the main solutes accumulated by *C. sake* cells grown in the molasses medium in response to lowered water activity. Upon a shift to high osmolarity, yeast cells rapidly stimulate a mitogen-activated protein (MAP) kinase cascade, the high-osmolarity glycerol (HOG) pathway, which coordinate part of the transcriptional response (Hohmann, 2002) [8].

Table 2: Population of Y-27 yeast isolate in molasses urea medium based liquid formulation with different concentration of molasses as osmotic stress agent at different days after storage (DAS) at 28 ±1 °C

Sugar concentration (%)	Population of yeast cells (Log_{10}Cfu/ml) at days after inoculation					
	0	4	10	20	30	60
15	6.27*	8.15	8.11	7.93	7.94	7.82
20	6.27	8.13	7.75	7.78	7.78	7.67
25	6.27	8.09	6.85	6.75	7.82	7.68
30	6.27	7.82	5.18	0	0	0
35	6.27	6.71	0	0	0	0
40	6.27	3.04	0	0	0	0
45	6.27	0	0	0	0	0
5 (control)	6.27	8.07	8.05	7.81	7.78	7.72
SEM±		0.022	0.020	0.023	0.022	0.013
C.D (5%)		0.065	0.061	0.070	0.065	0.041

*Values are means of three replications. Log_{10}CFU/ml are presented in the table.

Effect of osmotic stress in the cell morphology

The effect of osmotic stress on the morphology of yeast cells were observed under 100X magnification and evaluated the changes in cell size and morphology. The cells were grown in the control formulation showed a significant change in the cell size and shape compared to treatments. The cells in control formulation were observed with almost circular in shape about 5.26- 5.42 µm in diameter and smooth cell wall (Figure 2). In general, the salt and sugar treated cells has shown difference in cell size and shape. In stressed condition it has been found that the shape of cells changed to somewhat oval. At high sugar stress condition (formulation containing 45% molasses) were observed with wrinkles and cell size of 5.20- 5.47 x 4.39- 4.90 µm (Figure 3). In contrast to sugar stress, the cells under salt stress were observed round to oval in shape (5.21-5.41 x 4.81-5.36 µm without significant difference in cell size compared to control (Figure 4).

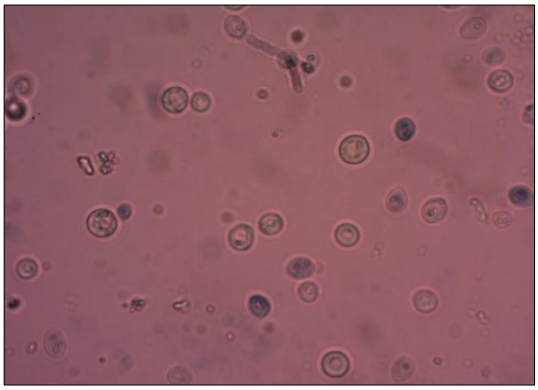

Fig 2: Population of Y-27 in the control formulation under 100X magnification

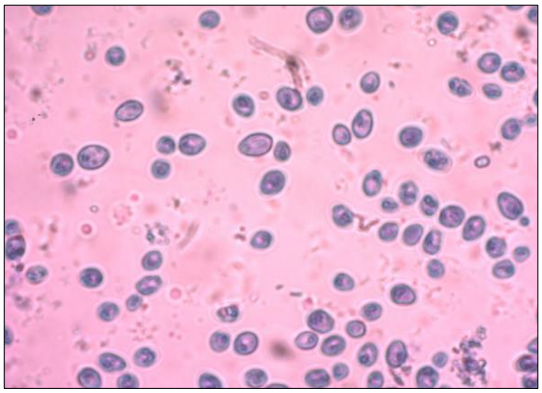

Fig 3: Population of Y-27 in the formulation containing 45% molasses under 100X magnification

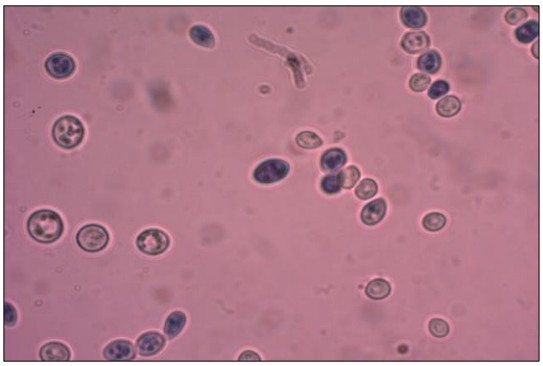

Fig 4: Population of Y-27 in the formulation containing 2.5% salt under 100X magnification

The results are comparable with the studies conducted by Avila-Reyes, *et al*. (2016) [2] in yeast *S. boulardi.* In the yeasts with salt stress treatment, cells were observed with wrinkles and with diverse degrees of plasmolysis. The changes occur at osmotic stress are aimed at establishing a balance by which the force pushing water across the osmotic gradient into the yeast cell is neutralized by turgor pressure against the plasma membrane and cell wall (Levin, 2011) [9].

Conclusion

The major obstacle in the commercialization of biocontrol products is the development of a shelf-stable formulated product that retains biocontrol activity at a level similar to that of fresh cells. The development of cheap yeast based bio control formulation with high stress tolerance capacity and longer shelf life for post-harvest disease management at commercial scale is very important aspect. The cells were grown in the control formulation showed a significant change in the cell size and shape compared to the formulations which induced osmotic stress. The formulation prepared with antagonistic yeast isolate Y-27 (*Meyerozyma caribbica*) had highest viability in 1% NaCl and 15% molasses after 4 and 2 months of storage respectively. It indicates that under stress conditions, the cells preserve the nutrients and accumulates some compatible solutes as osmo-protectant.

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