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RESEARCH ARTICLE

ANTIFUNGAL ACTIVITY OF VARIOUS WEAK ORGANIC ACIDS AND THEIR SYNERGISTIC EFFECT AGAINST *S. CEREVISIAE*.

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Abstract

Antifungal activity of four weak organic acids against *S. cerevisiae* cells were examined. Antifungal effects of hexanoic (C6), octanoic (C8), decanoic (C10) and benzoic acids were determined through Minimum Inhibitory Concentration (MIC), and inhibition zone measurements. The most effective weak acid was decanoic acid (MIC: 0.2-0.3 mM) according to MIC results. In order to have some insight in the inhibition mechanism of these weak acids, their efficiency was compared with that of hydrochloric acid (HCl) giving the same amount of drop in extracellular pH. Results demonstrated that the inhibition of yeast cells by weak acids is not simply due to acidity, but toxic effect of the anion and the insertion of the weak acids inside the cellular membrane may play a role. Moreover, synergistic effects of weak acids were examined and it has been shown that combinations of weak acids are more effective than using weak acids alone. Thus, this research not only opens new perspectives on antifungal activity mechanisms of weak acids, but also help their usage in combination widely.

Introduction:-

Weak acids have been extensively used as preservatives in food and beverage industries against yeast including *Saccharomyces cerevisiae* (Lambert and Stratford, 1999; Mollapour et al., 2006; Papadimitriou et al., 2007; Ullah et al., 2012). However, yeast strains may sometimes become resistant to these preservatives, therefore their more effective usage and understanding the essence of their inhibitory mechanism is crucial.

Fatty acids (hexanoic/caproic, octanoic/caprylic, decanoic/capric) are carboxylic acids characterized by the presence of a carboxyl group (-COOH) at one end and a methyl group ($-CH_3$) at the other end. Octanoic and decanoic acids are well known as fermentation inhibitors produced by yeasts during alcoholic fermentation (Legras et al., 2010).

Antifungal effect of these fatty acids against *S. cerevisiae* has been reported (Bergsson et al., 2001; Cabral et al., 2001; Kumar et al., 2011). The toxicity of short to medium chain fatty acids is correlated with their lipophilic properties (Tenreiro et al., 2002) and their key effect was proposed to be the cell membrane damage (Desbois and Smith, 2010; Ricke, 2003). These lipophilic weak acids can cross the plasma membrane by passive diffusion and dissociate their liposoluble form in the neutral cytosol at higher internal pH, leading to decrease of the intracellular pH and accumulation of toxic anion (Cabral et al., 2001; Legras et al., 2010; Viegas and Sá-Correia, 1997).

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Benzoic acid as a weak organic acid is significant for preventing microbial spoilage (Hazan et al., 2004; Kresnowati et al., 2008) and there are number of theories that explain the antifungal properties of weak acid, but the exact mechanism is still unknown (Ullah et al., 2012). Several studies have supported the notion that weak organic acids function through disruption of membrane organization through acidity. (Hatzixanthis et al., 2003; Holyoak et al., 1999). However, another study indicated that this acidification does not correlate with growth inhibition (Bracey et al., 1998), and therefore, acidification does not alone represent antifungal mechanism of benzoic acid.

In this study, we carried out an investigation of yeast response to hexanoic, octanoic, decanoic and benzoic acid and their combinations. Due to our continuing interest on the mode of action of various chemicals on yeast membranes, we set out to unearth the possible membrane dependent action and to show that the inhibition of yeast cells is not only due to the acidity of weak acids.

Materials and methods:-

Minimum inhibitory concentration (MIC) measurement:-

S. cerevisiae YPH499 strain was cultured overnight at 25˚C in YPD broth. Test strain was suspended in YPD to give a final density of 1×10^6 CFU/mL (Kumar Tyagi et al., 2014). Weak acids dissolved in DMSO were diluted to the mentioned concentrations in 24-well microtiter plate and then *S. cerevisiae* cells were added to each well. Plates were incubated for 48 h at 25°C with shaking. After incubation, MIC values were determined as the lowest concentration of acids by detecting the appearance of any visible growth of yeasts (Kumar Tyagi et al., 2014; Shimazaki et al., 2016).

Inhibition zone measurement:-

The antifungal activities of weak acids against YPH499 strain was determined by employing the standard disks diffusion method (Bauer et al., 1966; Rattanachaikunsopon and Phumkhachorn, 2010). In this method, 500 µL of fresh culture of YPH499 was spread on the YPD agar media plates. 5 mm diameter wells were opened. Weak acids were diluted in DMSO at 10% concentration and 55 µL of acids were filled into each well. Plates were incubated at 25˚C for 48 hours. After incubation, the diameters of the inhibition zones were measured in centimeter.

Extracellular pH measurement :-

YPH499 strain was cultured overnight at 25˚C in 20 mL of YPD broth. After incubation, the yeast cells were centrifuged at 3200 rpm for 5 min and pellet was washed twice with sterilized dH_2O. The pellet was then resuspended in sterilized dH_2O. About 50 mg wet weight of yeast cells were used for each experiment (Gášková et al., 2013; Wang et al., 2015). The weak acids dissolved in DMSO were added to the above resuspended solution. %2 glucose (zero point) and weak acids (18th min) were manually injected to the mentioned final concentrations. Extracellular pH was recorded at the indicated times with an HI 98127 water proof pH meter (HANNA, USA).

Statistical Analyses:-

MIC, inhibition zone assay, extracellular pH and extracellular conductivity measurements were repeated several times. Averages of all experimental groups were calculated and standard deviations were determined using Microsoft Excel.

Results and Discussion:-

S. cerevisiae is one of the common yeasts associated with food spoilage and acquired resistance to weak acids by increasing expression of the ABC (ATP-binding cassette) transporter, Pdr12p, which promotes the active leakage of these compounds (Hatzixanthis et al., 2003; Holyoak et al., 1999; Mollapour et al., 2006). Control of fungal spoilage and growth is a critical problem for food industry, medical field and other areas.

After a thorough search of the literature we uncovered that the information on the antifungal activity against *S. cerevisiae* using MIC and inhibition zone assay was limited. More importantly experimental data reported in the literature are based on studies with different experimental conditions preventing a simple comparison of the data with each other. Thus, at the beginning of our studies we set out to determine the MIC and inhibition zone values as a measurement of antifungal activity of weak acids (Table 1).

Table 1:- Antifungal activity of weak acids presented as zones of inhibition and MIC values. Weak acids were mixed with DMSO to increase solubility.

#	Weak acids	Inhibition zones (cm)*	MIC (mM)
1	hexanoic acid	3.3±0.3	4.0 – 8.0
2	octanoic acid	2.7±0.2	1.5 – 2.5
3	decanoic acid	2.9±0.3	0.2 – 0.3
4	benzoic acid	2.0±0.2	4.0 – 8.0

* 10% of weak acid was used.

According to MIC results, decanoic acid (0.2–0.3 mM) is more toxic than octanoic and other weak acids. On the contrary, hexanoic acid (3.3±0.3) is most effective weak acid in inhibition zone results. Our results supported the findings of previous studies (Alexandre et al., 1996; Viegas et al., 1989), where decanoic acid was reported to be more toxic than octanoic acid since it is more liposoluble causing an increase in cell permeability (Alexandre et al., 1996; Bergsson et al., 2001; Kumar et al., 2011).

On the other hand, addition of weak organic acids, caused a sudden decrease of extracellular pH (Fig 1). When yeast cells were treated with HCl in the as much necessary amount to provide the same pH drop (i.e. up to 10^{-3}M) (Fig 2), they continued to survive (Table 2). Thus inhibitory effect of weak acids does not occur solely through acidity, toxic effects of the anion and the increased permeability of the cell membrane due to the lipophilicity of the weak acid must be the underlying phenomenon in antifungal activity.

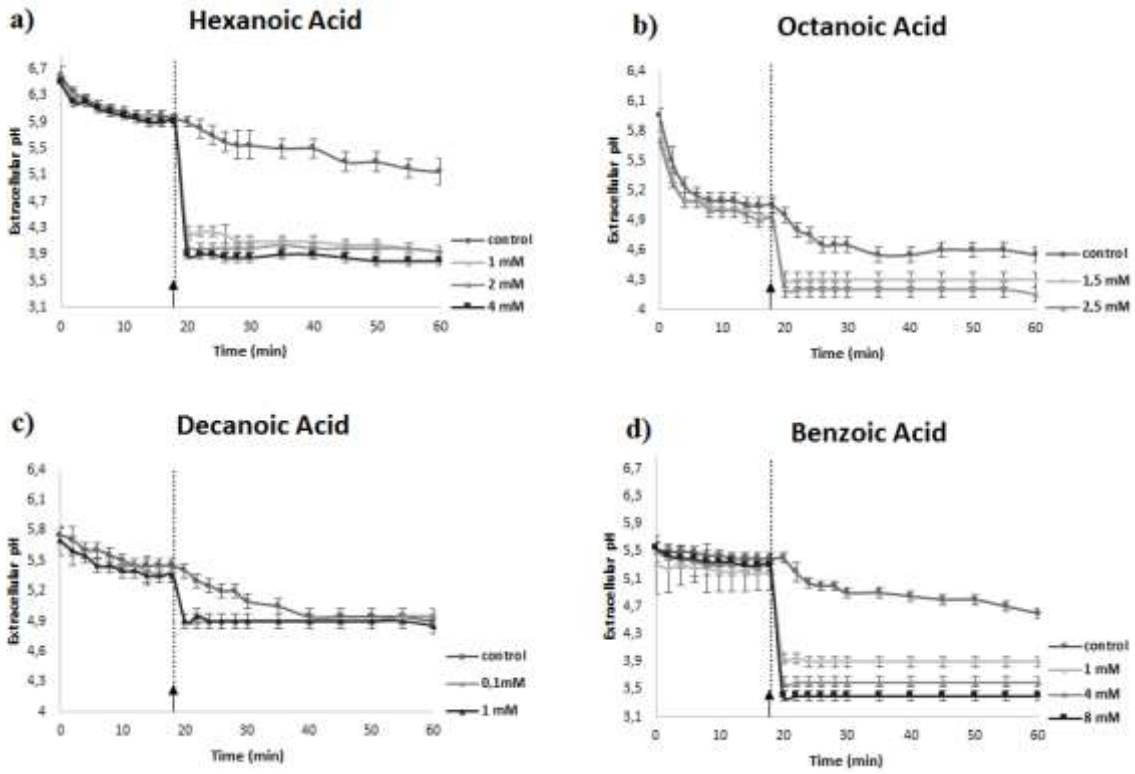

Fig 1:- Effects of weak acids on extracellular pH of *S cerevisiae* in glucose-induced medium. The arrows indicate the time of addition of **a)** hexanoic acid, **b)** octanoic acid, **c)** decanoic acid and **d)** benzoic acid. Horizontal axis – time (minute); vertical axis – extracellular pH.

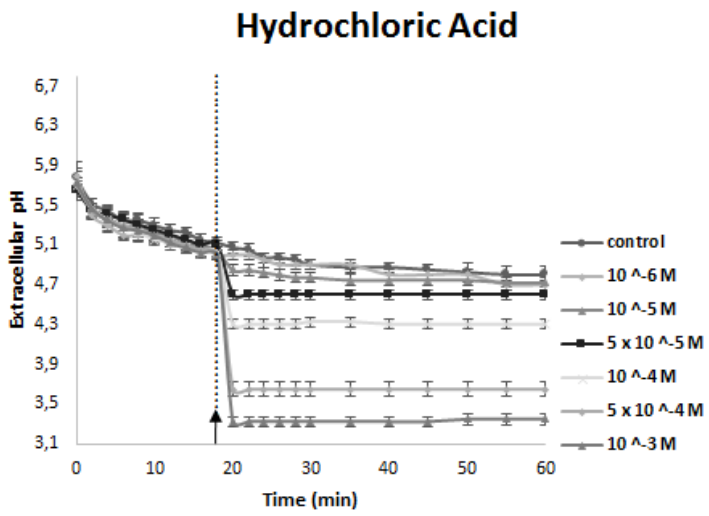

Fig 2:- Effects of HCl in various concentrations which provide the same pH drop as weak acids on extracellular pH of *S cerevisiae* in glucose-induced medium. The arrows indicate the time of addition of HCl.

Table 2:- Viability of *S. cerevisiae* cells upon exposure to HCl, *ND*: not determined.

#	HCl concentration (M)	Cell viability	Final extracellular pH
1	10^{-4}	+	4.30
2	10^{-3}	+	3.35
3	10^{-2}	+	ND
4	10^{-1}	-	ND

These results suggested us that the combination of these weak acids may enhance their antifungal activity and we set out to investigate the synergistic effect of their combinations. Although the inhibition effects of weak acids have been investigated before (Alexandre et al., 1996; Hazan et al., 2004; Jarboe et al., 2011; Legras et al., 2010), the antifungal activity of their combinations has not been reported against *S. cerevisiae*. Thus, various combinations of acids were prepared and viability of yeast cells in the presence of these mixtures were determined (Table 3).

Table 3:- Synergistic effects of weak acids against *S. cerevisiae* cells. Weak acids were mixed with DMSO to increase solubility.

a		Hexanoic Acid						d	Benzoic Acid					
	mM	0	1	2	4	8			**mM**	0	1	2	4	8
Octanoic Acid	0	+	+	+	+	-		**Hexanoic Acid**	0	+	+	+	+	-
	0.5	+	+	+	-	-			1	+	+	+	+	-
	1	+	+	-	-	-			2	+	+	+	-	-
	2,5	-	-	-	-	-			4	+	+	-	-	-
									8	-	-	-	-	-
b		Octanoic Acid						e	Benzoic Acid					
	mM	0	0.5	1	1.5	2.5			**mM**	0	1	2	4	8
Decanoic Acid	0	+	+	+	+	-		**Octanoic Acid**	0	+	+	+	+	-
	0.1	+	+	+	-	-			0.5	+	+	+	+	-
	0.2	+	-	-	-	-			1	+	+	-	-	-
	0.3	-	-	-	-	-			2.5	-	-	-	-	-
c		Hexanoic Acid						f	Benzoic Acid					
	mM	0	1	2	4	8			**mM**	0	1	2	4	8
Decanoic Acid	0	+	+	+	+	-		**Decanoic Acid**	0	+	+	+	+	-
	0.1	+	+	+	+	-			0.1	+	+	+	+	-
	0.2	+	+	+	-	-			0.2	+	+	-	-	-
	0.3	-	-	-	-	-			0.3	-	-	-	-	-

Our results demonstrate that it is possible to achieve same inhibitory effect using less amounts of combinations of these weak acids. In general combinations of fatty acids within themselves are more effective than combinations with benzoic acid (Compare for example Table 3b with 3e and 3f). In particular octanoic acid-decanoic acid combination is the most powerful one.

Conclusion:-

In conclusion, we have shown that inhibition of yeast cells by weak organic acids is not associated with their acidity. More importantly, using weak acids in combinations increase their potency as antifungal agents, enabling their minimal amount of usage as preservatives. These results enrich our knowledge about the mechanism of action of weak acids against *S. cerevisiae* cells and help us widen their usage in food, cosmetic and pharmaceutical industries.

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