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Feeding *Artemia* larvae with yeast heat shock proteins 82 (HSPs82) to enhance the resistance against abiotic stresses (hyperosmotic and high temperatures)

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Abstract

Feeding farmed *Artemia* with yeast heat shock proteins is a novel way to protect them from stress conditions during the culture. In this study, the effect of feeding with stressed new identified *Saccharomyces cerevisiae strain YG3-1* yeasts (containing induced heat shock proteins) on the survival of *Artemia* in stress conditions, was evaluated. For this purpose, heat shock proteins 82 (Hsps 82) of mentioned yeasts were induced by applying the high thermal (30, 35 and 40 ˚C) and high salinity (60, 120, 180 and 240 $g.l^{-1}$) stresses. After that, two different species of *Artemia* (*Artemia urmiana* and *A. franciscana*) were fed with treated yeasts during the culture. Then, to investigate the effects of *S. cerevisiae strain YG3-1* Hsps82 on *Artemia* survival, after the end of feeding, adult individuals of both species were exposed to authorized high salinity (230 $g\ L^{-1}$ and 280 $g\ L^{-1}$) and authorized high temperature (35 ˚C and 37 ˚C) as permitted stress for 48 h (hours). Finally, this administration resulted in the resistance of both species against the high salinity and high temperature ($p<0.05$). This result was confirmed by analysing total protein of *Artemia* using SDS-PAGE, and suggests that this administration can be used for enhancing the survival of *Artemia* in stress conditions.

Keywords: *Artemia*, Heat shock proteins, *Saccharomyces cerevisiae*, Stress, Survival

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Introduction

Artemia is one of the most important live foods in aquaculture (Garcia *et al.*, 2008), and is favored as a model organism for use in aquaculture biotechnology (Gavanda *et al.*, 2007). Heat shock proteins (Hsps) are a large class of proteins (Tkáčová and Angelovičová, 2012), and play a key role in the process of protein metabolism under normal and stress conditions, including the refolding of denatured protein, maintenance of structure integrity and other regulatory processes (Qin *et al.*, 2016). An increased accumulation of Hsps is essential for the survival of cells exposed to various stresses (Li *et al.*, 2006). There are multiple stressors in a changing world (Gunderson *et al.*, 2016). For example, the effects of global warming include rising mean annual temperatures and dramatic increase in the frequency and amplitude of severe temperature events (Xu *et al.*, 2016). So, farmed aquatic organisms experience many different environmental stresses including temperature fluctuation and salinity shift during culture (Aleng *et al.*, 2015). The production of heat shock proteins is one of the classical cellular responses of all organisms to environmental insult (Clark *et al.*, 2008). The enhanced expression of heat shock proteins in aquatic organisms can be detected in response to many kinds of the stressor (Shi *et al.*, 2015). Heat shock proteins are a kind of resistance mechanisms against environmental stresses such as hyperosmotic and high temperatures in *Artemia* and other aquatic crustaceans (Sankian *et al.*, 2011). Enhancement of Hsps synthesis promotes resistance of aquatic organisms against stress conditions (Sung *et al.*, 2012). Heat shock proteins can be produced in these organisms by exposure to stress (Sung *et al.*, 2008; Givskov Sorensen, 2010), and also through feeding with single cell organisms (Probiotics) containing induced Hsps (Sung *et al.*, 2009a). Examples include observations on Hsp70-induced thermotolerance generated in common carp (*Cyprinus carpio* L.,) against lethal ammonia toxicity and in coho salmon (*Oncorhynchus kisutch*) as result of a sublethal heat shock (Sung *et al.*, 2012; Arkush *et al.*, 2008). In another study, feeding with bacterial heat shock proteins protected *Artemia franciscana* larvae from *Vibrio campbellii* infection (Sung *et al.*, 2009a). Feeding of bacterially encapsulated heat shock proteins to invertebrates is a novel way to limit *Vibrio* infection. As an example, ingestion of *Escherichia coli* overproducing prokaryotic Hsps significantly improves survival of gnotobiotically cultured *Artemia* larvae upon challenge with pathogenic *V. campbellii* (Sung *et al.*, 2009b). Considering that in recent years researchers have investigated that the heat shock proteins play many important roles in aquatic organisms including *Artemia* and other aquatic crustaceans (Chaurasia *et al.*, 2015), potential applications for Hsps in the commercial production of fish, crustaceans and other aquatic organisms are indicated (Sung *et al.*, 2011), In the present study the effect of feeding larvae and adult individuals of *Artemia* with stressed new identified *Saccharomyces cerevisiae strain YG3-1* yeasts containing induced heat shock proteins 82 (Hsps82) as a novel way for enhancing the survival of *Artemia* in stress conditions was evaluated in larvae and adult individuals of *Artemia urmiana* and *Artemia franciscana* as test organisms. However, effects of live yeasts (*S. cerevisiae* strains) supplementation on the performance of aquatic organisms have been studied previously (Perrone *et al.*, 2013), but the effect of new identified *S. cerevisiae strain YG3-1* yeasts containing induced Hsps82 on the survival of *Artemia* and other aquatic organisms has not been studied until now. Hsp82 is a member of Hsp90 family in yeast. Members of the Hsp90 family stabilize misfolded proteins and interact with regulatory signaling proteins in yeasts (Seppä, 2005) and play important roles in multiple cellular stress responses of aquatic organisms (Wang *et al.*, 2016). Based on the findings mentioned previously, the present study was performed to investigate the effect of the function of Hsps82 (belonging to the *S. cerevisiae strain YG3-1*) on the survival of *Artemia* in stress conditions.

Materials and methods

Preparation of S. cerevisiae YG3-1 yeasts

All of the yeasts used in the present study were isolated from the intestine of endemic farmed rainbow trout (*Oncorhynchus mykiss*) in the West Azerbaijan province of Iran according to the procedure as described previously (Andlid *et al.*, 1995), and then identified by molecular methods as a new strain of *S. cerevisiae* yeasts.

Induction of Hsp82 proteins in yeasts

For this purpose, yeasts were cultured and grown using the yeast extract-peptone-glycerol (YPG) medium. In the stationary growth phase (after 3 days) yeast cells were harvested by centrifugation (5000 rpm for 10 m (minutes)) (Aoki *et al.*, 2002). Then, harvested yeasts were divided into three groups and stored at -20 ˚C. After that, production of Hsps82 was stimulated in the two groups of stored yeasts. In this way, heat shocks (30, 35 and 40 ˚C) were applied on yeasts in one group of them. At the same time, yeasts in the other group were exposed to high salinity (60, 120, 180 and 240 g L^{-1}) as stress. Both stresses were performed for 4 h (hours) (Sathiyaa *et al.*, 2001). Reverse transcription PCR (RT- PCR) was used to study the expression of *hsps* genes in the yeasts. Total RNA was isolated from each yeast sample and single-strand cDNA (complementary DNA) was synthesized from mRNA (messenger RNA that extracted from total RNA) using by 2-steps RT-PCR-Kit. Before synthesis, RNA concentration and quality had been verified using Biophotometer (Eppendorf Biophotometer plus, Germany). Then, the cDNA fragments were amplified (using Cinnagen Co PCR-Kit) using specific *hsp* primers combination as a forward primer ($Hsp82_{forward}$ 5’-AGT-TGC-CGA-CAG-AGT-TCA-GGT-TA-3’) and a reverse primer ($Hsp82_{reverse}$ 5'-AGA-ACC-ACC-AGC-GTT-GGA-TT-3'). These primers previously had been designed using theOligo software and produced by Cinnagene Co. PCR thermal cycling parameters for these specific primers were somewhat modified as: 35 cycles of 95 ˚C for 30 s (seconds), 45 ˚C for 40 s, 72 ˚C for 40 s with final extension of 72 °C for 5 min for amplification of *hsp*82. This program was applied with the Master cycler gradient Eppendorf thermal cycler. After amplification, all products were run on the 1.6% agarose gel electrophoresis, stained with ethidium bromide and visualized in a UV- transilluminator contained CCD camera (Sankian *et al.,* 2011).

Culture and feeding Artemia with yeast Hsps

Cyst samples (cysts of *Artemia urmiana* and *Artemia franciscana*) were obtained from the cyst bank of Urmia Lake Research Institute at Urmia University, Urmia, Iran. For optimal hatching, 1.5 g cysts of each population was incubated in artificial 0.45 µm filtered medium at a salinity of 35 g L^{-1}. After hatching, 500 individuals of instar-I nauplii were transferred directly into the 1 cylindroconical vials at an initial density of 2 nauplii ml^{-1} of 80 g L^{-1} culture medium (Sankian *et al.,* 2011). Finally, according to the standard protocol culture was performed in two treatments and four replicates for each treatment. Air during culturing was passed through a 0.22-µm filter (Van Stappen *et al.,* 1996). During the culture, both species of *Artemia* (*A. urmiana* and *A. franciscana*) were fed with two forms of yeast *S. cerevisiae YG3-1*: 1) Yeasts without any treatment (as Control treatment), 2) Yeasts with induced Hsp82 (combination of yeasts that were exposed to thermal and osmotic stresses as Hsp treatment), plus live *Dunaliella tertiolecta* algae. Feeding was performed in accordance with a feeding table which was adopted from a previously established procedure (Coutteau *et al.,* 1990).

Stress of Artemia; Determination of induced tolerance to stress in Artemia and statistical analysis

After the end of feeding, adult individuals of both species were exposed to high salinity (230 g L^{-1} and 280 g L^{-1}) and high temperature (35 ˚C and 37 ˚C) as permitted stress for 48 h (hours). They were fed with live *D.tertiolecta* algae during the stress (Sung *et al.,* 2008; Sankian *et al.,* 2011). Stresses were applied based on the guidelines (standard protocols adopted from previous literature) and in accordance with animals experimentations ethics and were approved by the Animal Experimentation Ethics Committee of the Urmia University, Urmia, Iran. To evaluate the effects of feeding with induced Hsps82 of *S. cerevisiae strain YG3-1* yeasts on the survival and stress resistance of *Artemia*, numbers of swimming larvae were determined and the survival percentage was calculated. Mortality and survival were compared by means of one-way ANOVA. The results of survival were expressed as percentage. One-way ANOVA and Duncan's test of SPSS16 software were used to identify differences among means, and significances were accepted at $p<0.05$ (Baxevanis *et al.*, 2004).

Protein extraction and sample preparation for SDS–PAGE

The whole body of adult *Artemia* individuals which survived from stresses was pulverized in 1.5 ml micro tube by appropriate tips. Then, 100 mg of the resulting powder, consisting of 10-15 adult *Artemia* individuals of both species, was homogenized in 500 µl of protein extraction buffer, containing buffer K (5 mM MgCl2, 5 mM NaH2PO4, 40 mM Hepes, 70 mM Potassium gluconate, 150 mM Sorbitol, pH 6.5) and protease inhibitor cocktail (Invitogene™ Mini from Roche Diagnostics GmbH). Protein concentration was determined by the Bio photometer (Eppendorf Biophotometer plus, Germany). Samples were heated at 95 ˚C for 5 min and subsequently cooled. After that, insoluble fragments were removed by low speed centrifugation (1600 rpm for 5 min) and then supernatants were electrophoresed (Clegg *et al.*, 2000; Sankian *et al.*, 2011).

SDS polyacrylamide gel electrophoresis (SDS–PAGE)

Flatbed SDS-PAGE was performed with a vertical system (BioRad System, USA). 10 % total acrylamide gel with 100×70×0.5 mm dimensions was prepared. 30 µl of samples were loaded on each gel track (Clegg *et al.*, 2000; Sankian *et al.*, 2011). The running buffer was prepared according to the described procedure previously (Diezel *et al.*, 1972). The buffer system in the strips formed a discontinuous buffer system together with the gel buffer. High molecular weight (250 KD) Ladder (Marker) was used for the detection of protein bonds on the gel. Electrophoresis was performed at a constant current of 50 mA (milli-amper) in BioRad electrophoretic apparatus with power supply, set at 150V for 1 h. The gel was stained with Coomassie blue G250 (Clegg *et al.*, 2000; Sankian *et al.*, 2011).

Results

RT-PCR results

Investigation of gene expression in stressed yeasts has shown that under different temperature and salinity, the Hsps gene expression of these yeasts has changed. So, the stress enhances the mRNA transcripts of Hsps in stressed yeast cells. RT-PCR gel electrophoresis of *hsps* genes expression indicated that 4h exposure to all of the stresses resulted in expression of *hsps*82 genes in *S. cerevisiae YG3-1*. Intensity of cDNA bands has shown that the temperature 35 ˚C and salinity 60 g L^{-1} are the best conditions for expression of Hsps genes in *S. cerevisiae YG3-1* (Fig. 1).

Figure 1: Semi quantitative RT-PCR gel electrophoresis of HSP 82 expression in various samples of *Saccharomyces cerevisiae YG3-1*. M: Marker (1 Kb ladder), lane 1: expressed HSP at 30 °C, lane 2: 35 °C, lane 3: 40 °C, lane 4: expressed HSP at salinity 60 g L^{-1}, lane 5: 120 g L^{-1}, lane 6: 180 g L^{-1}, lane 7: 240 g L^{-1}, lane 8: negative sample, lane 9: control. Semi quantitative RT-PCR revealed that the stress enhances the mRNA transcript of Hsp in stressed yeast cells. Intensity of bands shows the temperature 35 °C and salinity 60 g L^{-1} are the best conditions for expression of Hsps genes.

Enhanced survival of Artemia in stress conditions

Survival of *Artemia* individuals that were fed with Hsps in stress conditions significantly was improved and enhanced as compared to control *Artemia* individuals fed with non-stressed yeasts (p<0.05). So among the stressed *Artemia* adult individuals, the minimum percentage of survival in individuals of *A.urmiana* wasn't less than 60 % and Hsp-fed individuals in *A.franciscana* samples exhibited the highest survival (Table 1).

Table 1: Survival of *Artemia urmiana* and *A. franciscana.*

Treatment	Day 7	Day 11	Day 15
Control/ *A. urmiana*	89.50±17.90[a]	80.35±16.05[a]	77.75±15.55[a]
HSP/*A. urmiana*	89.99±17.99[a]	83.35±16.69[a]	80.95±17.55[a]
Control/*A. franciscana*	89.75±17.55[a]	82.50±16.40[a]	78.45±15.69[b]
HSP/*A. franciscana*	91.75±14.94[b]	89.75±17.55[b]	81.80±16.16[a]

Same characters in each column indicate insignificant differentiation. Hsp-fed individuals in *A. franciscana* samples exhibited the highest survival. The results are presented as the average of three determinations with standard deviations (mean±SD).

Synthesis of Hsps in Artemia

Staining of SDS polyacrylamide gels (SDS-PAGE of the survived adult *Artemia* in high salinity and high temperature) clearly demonstrated increased amounts of almost 80-kDa polypeptides in *Artemia* samples fed with yeast Hsps. While, *Artemia* individuals that fed with control yeast cells (non- stressed yeast cells with no Hsp) do not produce Hsp (Fig. 2).

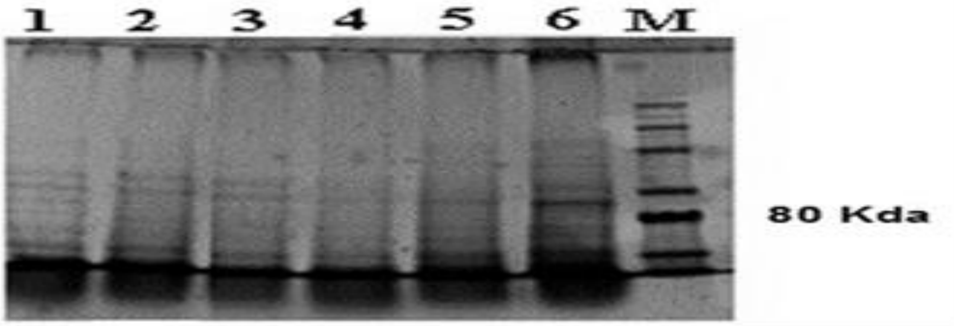

Figure 2: SDS-PAGE of total protein of the survived adult *Artemia* in different feeding treatments: 80 kDa protein bands were resolved in 10% SDS polyacrylamide gel. Lane 1: *A. franciscana* fed with Control treatment, lane 2: *A. franciscana* fed with HSP treatment, lane 3: *A. franciscana* fed with HSP treatment, lane 4: *A. urmiana* fed with Control treatment, lane 5: *A.urmiana* fed with HSP treatment, lane 6: *A. urmiana* fed with HSP treatment, M: Marker (250 KD ladder). As revealed by SDS-PAGE, administration of yeast Hsps enhances Hsp production in whole *Artemia* fed with Hsp. *Artemia* individuals that fed with control yeast cells (non- stressed yeast cells with no Hsp) do not produce 80-kDa polypeptides.

Discussion

The results of this study showed that feeding *Artemia* with induced Hsps of *S. cerevisiae* resulted in the induction of Hsps production in *Artemia* and confirmed that this feeding resulted in resistance of *Artemia* against stress. Maybe following the dietary administration with stimulated Hsps82 of *S. cerevisiae* strain *YG3-1* yeasts, chaperone activities of Hsps82 in *Artemia* were induced and these activities have resulted in stress tolerance in *Artemia*. Before this, ingestion of *E. coli* over-producing *Artemia* Hsp70 shelters brine shrimp against *V. campbellii*, possibly by triggering the innate immune response to produce anti-inflammatory substances and suppress infection. DnaK and *Artemia* Hsp70 exhibit 59.6% similarity in the peptide-binding domain and the protective capacity of these proteins, termed the innate immunity-activation portion, may reside within this molecular domain, a conclusion similar to that made for Hsp70 from dendritic cells (Sung *et al.*, 2009b; Sung and Macrae, 2011). In the present study, maybe there was a similarity in the domain of yeast Hsp82 and *Artemia* Hsps (that following the feeding with Hsp-enriched yeasts made from hemocytes of *Artemia*). Possibly, this similarity, has resulted in the induced-production of Hsps in *Artemia*. In addition, Hsps appear to stimulate the innate immune response of aquatic organisms and reinforce the function of the immune system (Sung and Macrae, 2011). Reinforcement of immune system function helps resistance against stressors and therefore enhances the survival in stress conditions (Aleng *et al.*, 2015). In the present study, yeast Hsps82 may reinforce the immune system function of *Artemia* individuals and so enhance their survival against stress conditions (hyperthermal and hyperosmotic stresses). Families of heat shock proteins, otherwise known as stress proteins or molecular chaperones, consist of conserved molecules found in all organisms. The expression of genes encoding Hsps is either constitutive or induced by stress and their products are essential for cell survival. Under normal conditions Hsps mediate nascent protein folding and assembly, translocate proteins through membranes into organelles such as mitochondria, and assist in the degradation of structurally aberrant proteins. Hsps, often when functioning cooperatively with one another, prevent the irreversible denaturation of proteins exposed to physiological stressors such as heat, toxins and disease, thereby facilitating protein refolding and protecting cells from damage (Sung and Macrae, 2011). Heat shock proteins were reported to inducte and enhance the resistance of aquatic invertebrates including crustaceans such as *Artemia* and shrimp against environmental stress. Also, previous studies showed that Hsps can be induced by external stresses. For example, expression of 90 KDa heat shock proteins in the brine shrimp *Artemia* Leach 1819, in response to high salinity stress protected *Artemia* against the high salinity stress (Sankian *et al.*, 2011). In addition, exposure of gnotobiotic *A. franciscana* larvae to abiotic stress (hypothermic and hyperthermic shocks) promoted heat shock protein 70 synthesis and enhanced resistance to pathogenic *V. campbellii* and showed a causal link between Hsp70 accumulation induced by abiotic stress and enhanced resistance to infection by *V. campbellii*, perhaps via stimulation of the *Artemia* immune system (Sung *et al.*, 2008). In many studies heat shock proteins have been used to enhance the stress tolerance in aquatic crustaceans such as *Artemia* and shrimp. Heat shock proteins can not only be produced by stress in all living animals, but can also be absorbed externally. Feeding of bacterially encapsulated heat shock proteins to invertebrates is a novel way to limit *Vibrio* infection. As an example, ingestion of *E. coli* overproducing prokaryotic Hsps significantly improves survival of gnotobiotically cultured *Artemia* larvae upon challenge with pathogenic *V. campbellii* (Sung *et al.*, 2009b). Moreover, feeding with non-pathogenic strains of *E.coli* including YS1, YS2 (containing 70-kDa bacterial Hsp, DnaK) and A_{native} (containing *Artemia* Hsp70 cDNA) containing induced Hsp70 proteins conferred protection to *A. franciscana* individuals against *V. campbellii* infection and enhanced their length (Baruah *et al.*, 2010). In a similar study, feeding with bacterial heat shock protein protected *A. franciscana* (Kellogg) larvae from *V. campbellii* infection (Sung *et al.*, 2009a). The described experiments showed HSPs can elevate survival performance of aquatic crustaceans in stressor conditions. Also, considering these results, feeding with yeast heat shock proteins can be considered as a novel way to make *Artemia* and other aquatic crustaceans resistant against stress conditions. As mentioned previously, effect of feeding with *S. cerevisiae strain YG3-1* yeasts containing induced heat shock proteins 82 on the survival of *Artemia* in the present study was evaluated and resulted in resistance of *Artemia urmiana* and *Artemia franciscana* (as a model organisms) against abiotic stress. The results of this study suggest that described yeasts can be used to protect the other species of *Artemia* from various abiotic stresses during culture. Previously, effects of β-glucan polysaccharides of 2-mercaptoethanol (2ME)- treated *S.cerevisiae* strain *YG3-1* on the growth of *Artemia* were investigated (Shekarchi *et al.*, 2016), but the effect of Hsps82 of *S.cerevisiae* strain *YG3-1* on the survival of *Artemia* in stress conditions has not been studied until now. So, considering the adverse side effects of antibiotics on the public health of aquatic organisms in the long term (Nikkhoo *et al.*, 2010), this administration can be used as a novel safe way to enhance the performance (improving growth and survival) of farmed *Artemia* during the culture.

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