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EFFECT OF PIGMENTS ISOLATED FROM BACTERIA OF ARSENIC CONTAMINATED AREAS ON HSP-70 EXPRESSIONS IN TRANSGENIC *DROSOPHILA MELANOGASTER* (BG^9)

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ABSTRACT
The present study demonstrates the toxic potential of some carotenoid pigments (ADSYR4, ADSYR5 and ADSYR1) isolated from bacterial population of arsenic prone areas of West Bengal. *Drosophila melanogaster* (transgenic to hsp70-lacZ) was used for toxicological assessment of the pigments. 25 % concentration of ADSYR4 triggered higher HSP70 expression (Score 19) than when treated with 50% pigment concentration (Score 11). Conversely, other two pigments (ADSYR5 and ADSYR1) showed comparatively greater HSP70 expression at higher pigment concentration (50%). The scores for ADSYR5 and ADSYR1 at 25% pigment concentration were recorded as 7 and 8 whereas the same at 50% pigment concentration were found as 30 and 11 respectively. This finding claims that, the carotenoid pigments were pro-oxidative in nature and the efficacy of the pro-oxidative potential depends on treatment concentrations. Furthermore, use of reporter gene assay from transgenic *Drosophila melanogaster,* in this study yet again validates the use of alternative model organisms for toxicological studies.

INTRODUCTION

Arsenic is the 20th most abundant element in the Earth's crust, 14th in the seawater, 12th in the human body (Woolson, 1975) and is also widely distributed throughout the nature in soil, rock water and air (Singh and Choudhary, 2013; Santra and Samal, 2013). The easy availability of this element is a result of weathering, dissolution, fire, volcanic activity and anthropogenic input (Cullen and Reimer, 1989). Interestingly the metal or metalloid contaminated soil and water can harbor various microorganisms (Zettler *et al.,* 2002; Baker and Banfield, 2003). Microorganisms like bacteria that survive in these contaminated environments adapt very well to the changes in the milieu. Generally, they acclimatize with the environmental adversities through detoxification mechanisms, metal homeostasis, precipitation, redox transformations or by metabolic exploitation (Bruneel *et al.,* 2006; Hetzer *et al.,* 2006; Guiné *et al.,* 2007). They are the common chemical decomposers in the nature (Shyamala and Belagali, 2012). In addition, they often synthesize certain kind of pigments which help them in mitigating the toxicity induced by the foreign chemicals (Carepo *et al.,* 2004). Pigments are mostly antioxidant in nature but occasionally they are also found to be pro-oxidative, *i.e.,* they may induce stress response in the organisms that produce them (Young and Lowe, 2001).

Carotenoids, the most common bacterial pigments are well known antioxidants that can protect against various types of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radicals and superoxide anions (Manimala and Murugesan, 2014). These compounds are among the most diverse natural products which are synthesized by a variety of organisms, including animals, plants, and microorganisms. Carotenoids absorb light in the range of 400 to 550 nm, which gives them their characteristic yellow-orange color.

Besides their antioxidant activity, Carotenoids are also seen to manifest some pro-oxidative results depending upon several determining factors such as oxygen tension, carotenoid concentration as well as interaction with other antioxidant molecules (Palozza, 1998), but the number of investigations dealing with pro-oxidative behavior of these pigments is rare. Hence the present work was aimed to assess stress inducing potentials of some carotenoid pigments in *Drosophila melanogaster,* Bg9 transgenic for hsp70-lacZ.

Organisms facing any kind of chemical or physical stress try to survive by expressing some specific genes (Lee et al., 2006; Das *et al.,* 2011), majority of which encode a group of proteins referred to as the heat shock protein (HSPs) or stress proteins (Lakhotia and Prasanth, 2002). HSPs, particularly HSP70 provide first line of defense and shows increased expression during heat and chemical insults. Since elevated levels of these proteins are considered as an indication of stress, the present study was focused on localization of HSP70 expression which helped authors to indentify stressed parts of the organism under pigment insult.

Drosophila melanogaster (Bg9) was used as a model organism for the present study due to their short life cycle, easy culture

procedures as well as ease in genetic manipulation. Various toxicological studies have been successfully conducted using *Drosophila* as a non-target organism (Rajak *et al.,* 2013; Rajak *et al.,* 2014; Podder and Roy, 2014; Rajak *et al.,* 2015). Moreover, National Research Council Committee on Toxicology recommended use of *Drosophila*, as model organism in risk assessment of several toxicants (Rand, 2010).

Thus, in the present work, Carotenoid pigments isolated from bacterial strains of arsenic prone areas were tested for their toxic potential in transgenic *Drosophila melanogaster* model.

MATERIALS AND METHODS

Experimental organism

3rd instar larvae of transgenic *Drosophila melanogaster* (hsp 70-lacZ) strain which express bacterial β-galactosidase as a response to stress was used during this study. Larvae were cultured on standard *Drosophila* food medium (SDM) containing Agar agar, corn meal, sugar and yeast extract powder at 24 ± 1ºC inside environmental chamber. Nepagin and propionic acid were added to SDM as antifungal agents.

Treatment schedule

3rd instar larvae of transgenic *D. melanogaster* (Bg9) were exposed to 25% and 50% concentrations of isolated carotenoid pigments (such as ADSYR4, ADSYR5 and ADSYR1 dissolved in 7% glucose solution) for a period of 24 hours. After completion of the exposure time, larvae were processed and dissected for the β-galactosidase staining assay. Control sets were simultaneously maintained on 7% glucose solution without the test pigments.

X-Gal staining assay (β-galactosidase staining assay)

X-Gal staining assay was performed following the method of Sarkar *et al.,* 2015 with little modifications. Briefly after completion of the exposure time, larvae were dissected in Poel's salt solution (PSS) to expose the gut and different parts of the body which were then fixed in 2.5% glutaraldehyde for 10 mins. Later, tissues were washed with 50mM sodium phosphate buffer and stained with X-gal staining solution for 20-30 mins at 37ºC in dark condition.

Scoring

Pattern and intensity of blue color appeared in different parts of dissected larvae were examined minutely and were scored following the method of Krebs and Feder, (1997). Scoring pattern was as follows: dark blue: 4, large blue batches: 3, Small blue patches: 2, Light blue: 1, No blue color: 0.

RESULTS

Larvae treated with ADSYR4 (25%) pigment appeared to express HSP70 on both anterior as well as posterior parts of the larval body. But anterior part (covering mouth parts, proventriculous and salivary glands) showed more blue colored patches, an indication of higher HSP70 expression there, than the posterior parts (Fig.1a) of the body. Cuticles were also seen to have blue coloration. Larvae treated with ADSYR4 (50%) pigment manifested a little bit different result. Here, posterior regions including the cuticle showed darker colour than the anterior regions (Fig1b). Therefore, toxic effect of the pigment at 50% concentration is more on the structures of posterior part than the anterior regions. Overall 25% concentration of the ADSYR4 showed higher toxic response in the larvae than the 50% concentration of the pigment.

The larvae treated with 25% pigment of ADSYR5 sample has imparted very less colour. Small blue patches were visible only in some regions of mid gut and mouth parts which is an indication of lesser HSP70 production (Fig 1c). Larvae treated with 50% pigment of ADSYR5 sample showed significant increase in blue color. Mouth parts, salivary glands, gastric caeca, malpighian tubules and entire hind gut were seen to take dark blue colour (Fig 1d). Cuticles were also marked with blue coloured patches. Therefore, larvae treated with 50% concentration of ADSYR5 showed greater degree of HSP70 expression and therefore under higher degree of toxic stress than the 25% solution.

The larvae treated with 25% pigment of ADSYR1 sample has imparted blue colour in the mid gut region only (Fig 1e). Cuticles and mouth parts were found to be unaffected. But cuticles of larvae treated with 50% pigment of ADSYR1 appeared to produce HSP70 which is an indication of stress. Other structures such as salivary glands, proventiculus, brain, foregut, Malpighian tubules and hindgut were also marked with prominent blue colour (Fig1f). Therefore, 50% concentration of ADSYR1 sample is found to be more toxic than the 25% solution. Table 1 provides scores for HSP70 expression in case of different pigments.

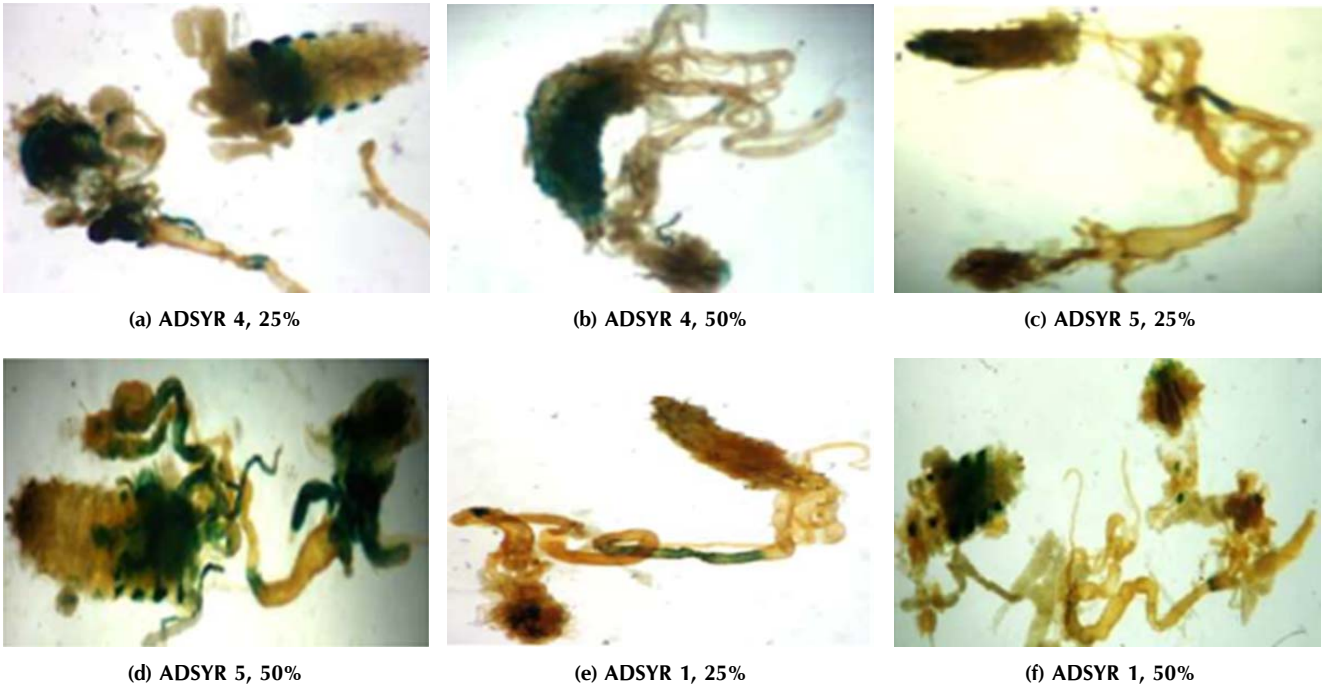
DISCUSSION

Present finding successfully demonstrated pro-oxidative

Table 1: Summary of β-galactosidase staining in the tissues/body parts of third instar larvae of transgenic *Drosophila melanogaster* (hsp70-lacZ) Bg⁹ exposed to different concentration of bacterial pigments (ADSYR1, ADSYR4, ADSYR5). The experiments were carried out in triplicate sets and each set consisted of 20 larvae. Along with treated larvae, control (without any shock, Figure 2) have been maintained for clear demarcation from the ones expressing pigment-induced stress (scoring of the controls not shown in the table). Score considered are as follows: Dark blue:4, Large blue patches: 3, Small blue patches: 2, Light blue: 1, No blue color: 0

Sl.No.	Pigment concentrations for treatment	MP	SG	PV	HC	FG	MG	HG	MT	C	Scores
1.	(ADSYR 4), 25%	4	1	4	3	1	2	1	0	3	19
2.	(ADSYR 4), 50%	2	2	0	0	0	2	1	1	4	11
3.	(ADSYR 5), 25%	1	0	0	0	0	1	3	0	2	7
4.	(ADSYR 5), 50%	4	4	0	4	4	2	4	4	4	30
5.	(ADSYR 1), 25%	2	0	0	0	0	1	4	0	1	8
6.	(ADSYR 1), 50%	3	0	1	1	0	1	1	0	4	11

Abbreviations used in the table stand for MP: mouth parts, SG: salivary glands, PV: proventriculous, HC: hepatic caeca, FG: foregut, MG: mid gut, HG: hind gut, MT: malpighian tubules, C: cuticle. In the present study scoring pattern followed was similar to that of Sarkar et al. 2015



(a) ADSYR 4, 25% (b) ADSYR 4, 50% (c) ADSYR 5, 25%

(d) ADSYR 5, 50% (e) ADSYR 1, 25% (f) ADSYR 1, 50%

Figure 1: β-Galactosidase staining pattern in different tissues of third instar larvae of transgenic *D. melanogaster* (hsp70-lacZ) after treatment with 25% and 50% concentrations of different bacterial pigments (ADSYR4, ADSYR5 and ADSYR1) for a period of 24 hours. Differential appearance of blue colour indicates differential expression of HSP70 in the various parts of the body

behavior of bacterial pigments in *Drosophila melanogaster*. The differential expression of HSP70 in different treatment categories can be visualized in Figure 3. Larvae treated with ADSYR4 (25%) manifested more HSP70 expression than treatment with higher concentration (50%). From Table: 1, the cumulative score of pigmentation is seen to be 19 and 11 respectively which suggests that the pro-oxidant potential is higher at low treatment concentration. This observation was in line with the finding of Kumar *et al*. (2011) where a chemical methyl methane sulfonate induced more HSP 70 expression at lower doses than the higher ones. The reduced cellular viability at higher chemical concentration might be responsible for apparently less HSP70 production/ appearance in the body tissues. In case of ADSYR4 treatment, the lower concentration manifests greater HSP70 activity in the anterior part of the body which might be due to the fact that, larvae consume the pigments along with glucose while feeding. Hence the mouth parts and associated areas go through maximum exposure and therefore express the coloration manifesting the stress. But with higher treatment concentration the expression of HSP70 is diminished. This might be due to avoid of feeding by larvae due to the presence of greater pigment concentration. This observation gets support from the finding of Podder and Roy (2014) where greater chemical concentration restrained the larvae from food intake.

Contrary to above finding, the 25% concentration of ADSYR5 resulted in lesser HSP70 production than its higher counterpart. The scores were 7 and 30 respectively for lower and higher treatments as can be seen in Table 1. This finding suggests that lower concentration of ADSYR5 is less toxic to the larvae and hence lesser HSP70 is expressed. But higher concentration might have generated an intense stress on larvae which elicited higher stress protein production and hence deep blue color was observed. Or otherwise the result can be explained with the findings of Krebs and Feder, (1997) where they suggested that HSP70 being an active chaperon can help in protection of several other vital proteins during stressed condition. Hence a surge in HSP70 expression is quite natural with higher toxic insult.

Figure 2: β-Galactosidase staining pattern in control, with third instar larvae of transgenic *D. melanogaster* (hsp70–lacz) maintained at optimum temperature without any stress. No particular colour development is observed

Similar trend of protein expression was seen in case of ADSYR1 where treatment with 25% produced less color than treatment with 50% pigment concentration. The score in Table 1 appeared to be 8 and 11 respectively. Thus, the bacterial

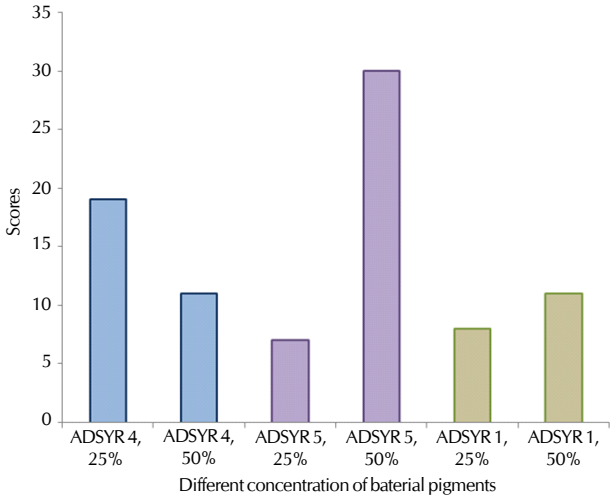


Figure 3: The column graph represents the variation in the degree of expression of HSP70 in the different categories of treatment. The scores in each category represent the cumulative score of coloration from all body parts. Score considered are as follows: Dark blue:4, Large blue patches: 3, Small blue patches: 2, Light blue: 1, No blue color: 0 Abbreviations used in the table stand for MP: mouth parts, SG: salivary glands, PV: proventriculus, HC: hepatic caeca, FG: foregut, MG: mid gut, HG: hind gut, MT: malpighian tubules, C: cuticle. In the present study scoring pattern followed was similar to that of Sarkar *et al.,* 2015

pigments show a definite shift from the expected antioxidant property towards an effective pro-oxidant nature or otherwise, it might be suggested following the idea of Krebs and Feder, (1997), that the treatment with bacterial pigments in *Drosophila melanogaster* has conferred the ability in the flies for greater production of chaperon (HSP 70), thereby making the flies better adapted to fight stress.

Thus, the study very well clarifies the fact that bacterial pigments isolated from arsenic contaminated medium might have critical roles in eliciting stress response in *Drosophila melanogaster* depending on the particular type of the pigment as well as their concentrations of exposure.

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