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Response of *Streptococcus zooepidemicus* to Oxidative Stress in Hyaluronic Acid Fermentation

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ABSTRACT

Streptococcus zooepidemicus (*SZ*) is an aerotolerant bacteria and its ability to survive under reactive oxidant raises the question of the existence of a defense system against oxidative stress. As a characteristic of lactic acid bacteria, *Streptococcus* lacks an ordinary anti-oxidative stress enzyme, catalases and an electron transport chain. Whether this bacterium resists oxidative stress prior to an exposure to a higher level of an oxidizing agent H_2O_2 in hyaluronic acid fermentation is not known. This paper describes that *Streptococcus* cells, once treated with lower concentrations of H_2O_2 (i.e. 0.25, 0.50 and 1.0 mM) at least, were prepared for a subsequent higher concentrations of H_2O_2 such as 20.5 and 100 mM. At low concentrations (i.e. 0.25, 0.50 and 1.0 mM), H_2O_2 was found to act as a stimulant for HA synthesis, but it became toxic if presented at a very high level (100 mM H_2O_2). The highest HA yield to glucose consumed ($Y_{HAtotal/glu}$) was 0.017 gg^{-1} for the cells pre-treated with 0 mM of H_2O_2, and then exposed to 20.5 mM H_2O_2. Thus, this implied that this bacteria might possess a defense mechanism against oxidative stress and that this system was inducible.

Keywords: Hyaluronic acid, hydrogen peroxide, oxidative stress, protective response, *Streptococcus zooepidemicus*

ABBREVIATIONS

DNA	deoxy-ribonucleic acid
HA	hyaluronic acid
H_2O_2	hydrogen peroxide
OD	optical density
ROS	reactive oxygen species
SBA	sheep blood agar
*s*HA	soluble hyaluronic acid
*t*HA	total hyaluronic acid

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$Y_{HAtotal/glu}$	hyaluronic acid yield to glucose consumed
$Y_{H2O2/glu}$	hydrogen peroxide yield to glucose consumed
$Y_{X/glu}$	biomass yield to glucose consumed

INTRODUCTION

The ability to persist and thrive under oxidative stress is necessary for the growth of bacteria, under aerobic or reactive oxidant challenges. In bacteria, the association of enzymatic and non-enzymatic systems, with reactive oxygen species (ROS), provides a mechanism for a cellular response against deleterious effects of the molecules. Of the various reactive molecules (O_2^-, H_2O_2, • OH), hydrogen peroxide (H_2O_2) is particularly derived from non-radical oxygen. Though H_2O_2 is chemically less reactive, it is still a threat to the structure and function of cells (Halliwell and Gutteridge, 1989). Likewise, it can readily diffuse across the cellular membranes and oxidatively damage a number of vital cellular components, including membrane lipids, enzymes and DNA (Miller and Britigan, 1997; Porter, 1984).

An adaptation to hyperoxidative environment provides organisms with a wider selection of ecological niches for survival. Like many other lactic acid bacteria, most *Streptococcus* species have manganese form of superoxide dismutase (Mn-SOD) for detoxification of ROS (Jakubovics, Smith and Jenkinson, 2002). However, this might not be the only mechanism in bacteria. The binding of the metal ions onto superoxide dismutase (SOD) or catalases in forms, which was unable to accelerate radical (O_2-, H_2O_2, • OH) or non-radical reactions (Halliwell and Gutteridge, 1999), was thought to form the basis for an alternative chemically detoxification of H_2O_2. These involved reactions are described as (Bannister, Bannister and Rotilio, 1987): $2O_{2-} + 2H^+ \rightarrow H_2O_2 + O_2$ (Superoxide dismutase), $2(H_2O_2) \rightarrow 2H_2O_2 + O_2$ (Catalase) and $H_2O_2 + RH_2 \rightarrow 2H_2O + R$ (Non-specific peroxidase). Such a system might be necessary in reducing the levels of H_2O_2 and damaging their production. However, as is the characteristic of lactic acid bacteria, *Streptococcus* species does not synthesize heme and lacks catalase (Condon, 1987). The specific mechanisms by which they adapt to peroxide stress are still unknown.

Traditionally, hyaluronic acid (HA), which is an industrially important biopolymer, was extracted from rooster comb. However, an increasing trend to streptococcal fermentations is rapidly expending in view of its emerging applications in the medical and cosmetic industries (Lerner, 1996). Remarkly, *Streptococcus zooepidemicus* (*SZ*) were also greatly used as the risk of cross species viral infection which can be avoided (Huang *et al.*, 2006). Nevertheless, there were some drawbacks in relation to *Streptococcus* HA production routes. According to Goh (1998), under aerated conditions, HA production was found to be reduced as a result of the growth inhibition by hydrogen peroxide (H_2O_2), since this compound is inherently toxic and reactive, as well as *Streptococcus zooepidemicus* being catalase negative (Hardie and Whiley, 1995). Mashitah *et al.* (2005) reported that $H_2O_{2,}$ produced by *Streptococcus zooepidemicus* cells, did not affect the growth of cell, but influenced the production of HA. Accordingly, the production of H_2O_2 took place during the growth phase, and this was only started after the growth had reached its late exponential phase, that is, when H_2O_2 in the culture media had depleted. Whether such cells are able to resist oxidative stress prior to exposure to higher level of H_2O_2, this has not been clearly defined. In this study, the researchers examined the response of *Streptococcus zooepidemicus* cells to oxidative stress, prior and during the HA fermentation. For this purpose, H_2O_2 was used as an oxidizing agent.

MATERIALS AND METHODS

Strain

Streptococcus equi sub-species *zooepidemicus* ATCC 39920 was obtained from the American Type Culture Collection (Rockville, MD., USA). It was maintained on sheep blood agar (SBA) slants and kept at 4^{o}C.

Culture medium

The composition of the medium used in all the experiments comprised of (gl^{-1}) glucose 30, yeast extract 10, KH_2PO_4 0.5, $Na_2HPO_4.12H_2O$ 1.5, and $MgSO_4.7H_2O$ 0.5, respectively. The pH of the medium was adjusted to 7.0 with 5 M NaOH prior to autoclaving it at 121^{o}C for 20 min. This glucose solution was autoclaved separately and mixed aseptically with the other components on cooling.

Cell suspension

Cell suspension of the shake flask culture was prepared by inoculating aseptically a stock culture of *S. zooepidemicus* onto Sheep Blood Agar (SBA)-plates and incubated overnight at 37^{o}C. The formed colonies were punched by a sterile cork borer to obtain ten round disks of 0.85 cm in diameter. The disks were then put in a sampling bottle containing 50 ml of sterile distilled water. The sampling bottle was vortexed for 3 min so that the cells could evenly be distributed in the liquid.

The Effect of H_2O_2 Pre-treatment

20 SBA-disks full of *S. zooepidemicus* colonies, were pre-incubated with 100 ml of H_2O_2 solution (0, 0.25, 0.50 and 1.0 mM) in an orbital shaker at 37^{o}C, 150 rpm for 30 min. These cells (15 ml) were then re-treated with H_2O_2 (15 ml) at the indicated concentrations (0, 20.5 and 100 mM) into a flask containing 120 ml culture media. The cells were left to be in contact with H_2O_2 in an orbital shaker at 37^{o}C, 250 rpm for 24 h. After 24 h, the samples were analyzed for cell biomass, H_2O_2 and HA production, and glucose consumption. The cell biomass was taken as indices of the cells growth during the fermentation period.

Analytical Methods

The cell concentration was determined by measuring the optical density (*OD*) at 600 nm by Jenway Spectrophotometer and dry cell method. A correlation between the dry cell weight and OD_{600} was established. The concentration of H_2O_2 was analyzed using the spectrophotometric method as suggested by Emiliani and Riera (1968), with a slight modification. Hyaluronic acid (HA) and glucose concentrations were determined using the method described by Mashitah *et al.* (2002), and the hexokinase method (Sigma Diagnostic, Glucose HK, Procedure No 16-UV), respectively. The HA soluble (HA_{sol}) represented the hyaluronic acid (HA) which had been solubilised or released from *Streptococcus zooepidemicus* capsule during the fermentation. The HA total (HA_{total}) represented the combination of HA which was solubilised from the capsule during the fermentation and the HA which was released from the ruptured capsule into the solution, after being treated with sodium dodecyl sulphate and vortexed for 3 mins.

RESULTS AND DISCUSSION

The Effect of H_2O_2 Pre-treatment on Growth and Biomass Yield

The ability of *S. zooepidemicus* to respond to H_2O_2 pre-treatment, prior to HA fermentation, was investigated and the results are shown in *Fig. 1*. It was suggested that prior to treating the *Streptococcal* cells directly with a high level of H_2O_2 in the production medium, the cells were pre-treated in the absence or in the presence of 0.25, 0.50 and 1.0 mM H_2O_2 at 37°C, 150 rpm for 30 mins. This was done to allow the cells to acclimatize with the low levels of H_2O_2 before being introduced to a medium with higher H_2O_2 concentrations, since exposing to higher doses of H_2O_2 at the start of the culture would significantly decrease the growth and HA production (Mashitah *et al.*, 2005; Mashitah, 2006).

As shown in *Fig. 1(a)*, the *S. zooepidemicus* biomass was found to slightly increase when pre-treated with lower doses of H_2O_2. For a 0 mM added H_2O_2 with 1.0 mM H_2O_2 pre-treatment, it led to a slight increase in biomass as compared to the one without any pre-treatment. As for 20.5 mM added H_2O_2, with 0.5 mM H_2O_2 pre-treatment, a slightly lower level of cell biomass was detected. A similar trend was also observed for 0.25 mM pre-treated cells. However, it was also observed that with 100 mM H_2O_2 medium, not more than 1% of either 0, 0.25, 0.50 or 1.0 mM pre-treated population could survive as compared to the non-pretreated control. This showed that the treated cells when exposed to lower levels of H_2O_2, were better able to cope with subsequent toxic doses. This also meant that the growth was unaffected or even enhanced by pre-treating the cells to H_2O_2. The treatment with the highest H_2O_2 concentration (100 mM) led to a depletion of the biomass values. Thus, indicating that the organism might have been killed or destroyed at this level of added H_2O_2.

The Effect of H_2O_2 Pre-treatment on HA and Extracellular H_2O_2 Production and Glucose Consumption

As observed in *Fig. 1* (*a, b, c, d* and *e*), the biomass and HA production (HA total and HA soluble) were closely related. For the cells treated with 0 mM H_2O_2, lesser amounts of extracellular H_2O_2 and glucose consumption were detected (*Fig. 1(d)*). This could be due to the fact that during H_2O_2 exposure, *S. zooepidemicus* might consume H_2O_2 and glucose during growth. Therefore, when the maximum biomass was attained, H_2O_2 became limited, and hence the inhibition was reduced to increase the production of HA. This showed that some cells might have evolved highly efficient and often redundant repair mechanisms to remove lesion from DNA, proteins and membrane lipids. For example, all damages produced by free radical attack on DNA molecules were repaired by a universal DNA repair process known as the base-incision repair (Demple and Harrison, 1994; Thibessard *et al.*, 2001; Zhang, 2002), indicating that the appearance of ROS, H_2O_2 had led to the development of defense mechanisms which either kept the concentration of the oxygen derived radicals at acceptable levels or repaired oxidative damages.

The findings of the current study also showed that the main physiological benefit of adaptive response was clear to protect *Streptococcus* cells from higher doses of a toxic agent. Such a protective response also indicated that the cell, once exposed to the H_2O_2, expects, or at least is prepared for a subsequent lethal dose. Besides that, a protective mechanism could have occurred; the cocoid cells associated into strands by the HA capsule, where the reduced surface-to-volume ratio and the limited H_2O_2 diffusivity in the capsule shielded the cells from H_2O_2. In other words, the streptococcal cells synthesized HA excessively for a reduced rate of H_2O_2 uptake.

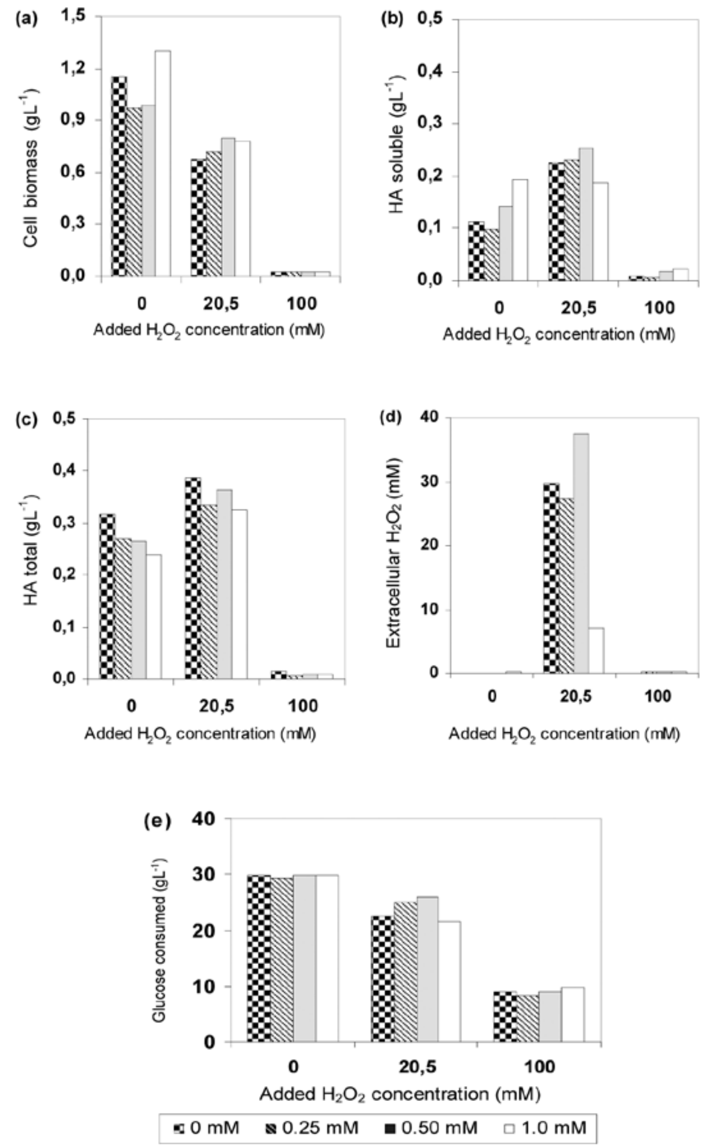

Fig. 1: The effect of hydrogen peroxide pretreatment on the growth and HA production, H_2O_2 released and glucose consumption by S. zooepidemicus *after 24 hr of fermentation period. (X-axis - concentration of treated H_2O_2; legend – concentration of the pre-treated H_2O_2)*

When pre-treated cells were treated with higher doses of H_2O_2 (100 mM), the growth and HA production was negligible (*Fig.1*). As a result, a lesser amount of glucose was utilized *(Fig. 1 (e))* and lower extracellular H_2O_2 was detected in the media. This also means that a significant damage, due to H_2O_2, indiscriminately reacted with various macromolecules in the cells had occurred, thus leading to a variety of biochemical and physiological lesions, and resulting in metabolic impairment and cell death (Totter, 1980; Harman, 1981; Ames, 1983).

From Table 1, it can be observed that the highest HA yield to glucose consumed ($Y_{HAtotal/glu}$) was 0.017 gg^{-1} for the cells pre-treated with 0 mM, and then exposed to 20.5 mM H_2O_2. As for the cells pre-treated with 0.50 mM and exposed to 20.5 mM H_2O_2 medium, the detected amount of extracellular H_2O_2 in the media was found to be the highest. The highest by-product yield ($Y_{H2O2/glu}$) to glucose consumed was 1.44 mM g^{-1}. According to Ryan and Kleinberg (1975), the concentration of H_2O_2 increased continuously during the growth due to the catabolic activity, and the categorization of this organism as H_2O_2 producer was therefore appropriate.

TABLE 1

Yield parameters of *S. zooepidemicus* at various pre-treated H_2O_2 concentrations, which were re-treated with 20.5 mM H_2O_2 medium (Condition: T = 37°C, agitation 250 rpm)

Pretreated H_2O_2 conc. (mM)	Biomass, X (gL^{-1})	Hyaluronic acid total, HA_{total} (gL^{-1})	Hydrogen peroxide, H_2O_2 (mM)	$Y_{x/glu}$ (gg^{-1})	$Y_{HA/glu}$ (gg^{-1})	$Y_{H2O2/glu}$ (mM g^{-1})
0	0.672	0.388	29.7	0.0299	0.017	1.32
0.25	0.716	0.334	27.2	0.0288	0.013	1.09
0.50	0.798	0.364	37.3	0.0308	0.014	1.44
1.0	0.781	0.326	6.99	0.0362	0.015	0.32

CONCLUSIONS

In the response of *S. zooepidemicus* cells to oxidative stress in hyaluronic acid fermentation, several important features were found. Streptococcal cells were aerotolerant bacteria; when treated to lower levels of H_2O_2 (0, 0.25, 0.5 and 1.0 mM) it was better able to cope with the subsequent higher toxic levels of the oxidizing agent (20.5 mM). H_2O_2, at low level, acts as a stimulant for the cells to synthesize HA. At higher level (100mM), the cells might be destroyed or killed. Furthermore, for the Streptococcal cells to survive against the oxidative stress, a defense system which is inducible must exist.

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