Increase in enzyme productivity by induced oxidative stress in *Bacillus subtilis* cultures and analysis of its mechanism using microarray data

Surabhi Mishra[a], S.B. Noronha[a,b], G.K. Suraishkumar[c,*]

[a]*School of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India*
[b]*Department of Chemical Engineering, Indian Institute of Technology Bombay, Powai, Mumbai 400076, India*
[c]*Biotechnology Department, Indian Institute of Technology, Madras, Chennai 600 036, India*

Abstract

Treatment of microbial cultures with chemical agents such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) induces production of reactive oxygen species (ROS) in culture. These ROS inducing (treatment) agents increased the growth rate as well as the maximum specific levels of extracellular enzymes such as α-amylase and protease in *Bacillus subtilis* cultures. Treatment with hypochlorous acid increased maximum specific α-amylase level by 2.2-fold and maximum specific protease level by 2.6-fold, respectively. Similarly, treatment with H_2O_2 increased specific α-amylase and specific protease level by 1.5- and 1.9-fold, respectively. Increases in specific enzyme levels were correlated with levels of specific intracellular ROS in cultures. The mechanism of increase in enzyme productivity under induced oxidative stress was also traced at the genetic level through analysis of available microarray data. The microarray data showed an induced level of signal peptidase gene (*sip*T), which is the most important secretory apparatus component, and suggested that increased efficiency of secretory apparatus as a result of treatments with ROS inducing agents also leads to increased productivity of α-amylase.

Keywords: Reactive oxygen species (ROS); α-Amylase; Protease; Hydrogen peroxide; Hypochlorous acid; Microarray; SipT

1. Introduction

Bacillus subtilis and other bacilli have a high capacity for the production of native extracellular enzymes [1] and are therefore used in industry for large-scale production of such enzymes [2]. Industrial production of enzymes frequently involves protein engineering techniques such as modification of proteolytic cleavage sites to protect the product from proteolysis by host proteases after secretion [3]. These approaches result in significant improvement in activity of enzymes under desired conditions, but the introduced modifications are not always beneficial with regard to production and secretion of enzymes [3]. This emphasizes the need for the development of alternative methods to enhance production without lowering the economics of large-scale production. Induced oxidative stress can increase enzyme production and growth rate in *Bacillus subtilis* cultures. The method for improving productivity through induced oxidative stress involves a mere treatment of the inoculum for a short period before inoculation into the seed tank.

Oxidative stress results when the rate of production of reactive oxygen species (ROS) exceeds the capacity of the cell for disposal [4]. Under physiological conditions, ROS are continuously generated in cells from metabolic processes such as respiration and fatty acid biosynthesis in aerobic organisms or environmental agents such as near UV or ionizing radiations [5]. Further, ROS can be induced in cultures by treatment with chemicals such as hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), menadione, paraquat, etc. [6].

The effects of ROS produced by hydrogen peroxide treatment have already been studied at the transcriptional level using a microarray technique in *B. subtilis* [7]. The microarray data provides information about expressions of genes under specified growth conditions. The microarray data under hydrogen peroxide induced oxidative stress showed changes in transcriptional levels of a large number of genes.

In the present work, we have studied the effect of ROS inducing (treatment) agents such as hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl) on cell growth and enzyme productivity in *B. subtilis* cultures. We report significant increases in the specific activities of extracellular α-amylase and protease, and consequent increases in total enzyme concentration with increase in the levels of intracellular ROS in the ROS range studied. We have further analyzed the microarray data of hydrogen peroxide mediated oxidative stress in *B. subtilis* to investigate the possible relationship between genes induced and increased production of α-amylase and protease.

2. Materials and methods

2.1. Bacterial strain and media

The microorganism used in the study, *B. subtilis* 748 (*trpC*2) was obtained from the Bacillus Genetic Stock Center, Ohio State University, OH, USA. The bacteria were grown in Luria Bertani (LB, Miller) medium (Hi-Media) (10 g l^{-1} casein enzymic hydrolysate, 5 g l^{-1} yeast extract and 10 g l^{-1} sodium chloride, supplemented with kanamycin, 10 μg m l^{-1}) at 37 °C under shaking conditions.

2.2. Treatment procedures

The bacterial cultures in mid-exponential phase, at a concentration of 1.8 g l^{-1} were exposed to freshly prepared 10 mM HOCl (prepared by dissolving an equal volume of sodium hypochlorite in distilled water), corresponding to 5.55 mmol HOCl (g cell)$^{-1}$. This exposure was followed by 10 min of incubation in the dark at a shaker speed of 180 rpm maintained at 37 °C. The residual free chlorine was quenched with an equal volume of 0.2 M sterile sodium thiosulphate (Na_2SO_3) solution. To remove the excess sodium thiosulphate, cells were centrifuged and re-suspended in fresh media. The treated cells were used for inoculation (10%, v/v) in fresh LB media.

In the case of H_2O_2 treatment, cells at a concentration of 0.9 g l^{-1} (early exponential phase) were exposed to 2.5 mM pulses of H_2O_2 at 30 min intervals. Since H_2O_2 is a weak inducer of ROS compared to HOCl, a continuous-mode exposure to H_2O_2 was chosen.

For HOCl toxicity studies, various concentrations of HOCl, i.e. 1, 2.5, 5, 10, 20, and 40 mM which corresponded to 0.56, 1.38, 2.78, 5.55, 11.11, and 22.22 mmol (g cell)$^{-1}$, respectively, were used, and the procedure mentioned above was followed. For H_2O_2 toxicity studies, one time exposures of mid-exponential phase cells to different H_2O_2 concentrations, i.e., 0.5, 1, 2.5, 5, 10 and 20 mM, which corresponded to 0.55, 1.11, 2.78, 5.55, 11.11 and 22.22 mmol (g cell)$^{-1}$, respectively, were used. The treatment doses of HOCl and H_2O_2 were chosen based on the results of the toxicity studies, from the number of colony forming units (CFU) under various concentrations of HOCl and H_2O_2, as detailed in Section 3.

2.3. Enzyme assays

Assays were performed for α-amylase (extracellular as well as intracellular) and the family of proteases (referred to as protease). Standard methods were used for enzyme assays: the azocasein degradation method for protease [8] and spectrophotometric measurement of blue coloured complex of leftover starch with iodine at 620 nm for α-amylase [9]. The specific enzyme levels were determined by normalizing the total enzyme concentration with the cell concentrations.

2.4. Intracellular enzyme assay

One milliliter of the fresh bacterial culture was centrifuged (7000 × *g*, 5 min and 4 °C) and cell pellets were washed with 0.2 M phosphate buffer saline (PBS) (pH 7.0) twice to remove all traces of media with extracellular secretions. The pellets were suspended in an equal amount of 0.2 M PBS containing 2.5 mg ml^{-1} lysozyme, 100 mM sucrose, 132 μM EDTA and 2.5 mM $MgCl_2$, and incubated at 37 °C for 15 min to generate protoplasts. The solution was centrifuged at 10,000 × *g* for 10 min at 4 °C to recover the protoplasts. The resulting protoplasts were washed with the same suspension buffer without lysozyme. The protoplasts were pelleted by centrifugation and then lysed by osmotic shock or addition of 1 ml of distilled water. This extract was used for intracellular enzyme assays using standard protocols as in the case of extracellular enzyme assays.

2.5. ESR spectrometry

The procedure used for ESR spectrometry was similar that one used in earlier studies [10]. One milliliter of the fresh bacterial culture was centrifuged (12,000 × *g*, 10 min and 4 °C) and cell pellets were washed with 0.9% saline three times to remove traces of media with extracellular secretions. The pellets were suspended in an equal amount of 100 mM Tris buffer (pH 8.0, saturated with nitrogen). Then 2.5 mg ml^{-1} lysozyme, 100 mM sucrose, 132 μM EDTA and 2.5 mM $MgCl_2$ were added to the cells which were incubated at 37 °C for 15 min. The solution was centrifuged at 12,000 × *g* for 10 min at 4 °C to recover protoplasts. The resulting protoplasts were washed properly with the same suspension buffer without lysozyme. To the

protoplasts, 80 mM dimethyl-1-pyrroline-N-oxide (DMPO, Sigma) as a spin trap was added. Subsequently, the volume was made up to 1 ml using distilled water saturated with nitrogen and mixed thoroughly to break open the protoplasts. The final two steps, which include addition of spin trap and lysis of protoplasts, were performed under red light. The derivative ESR spectra were obtained using a Varian E-112 EPR, spectrometer. The area under the absorption curve was obtained by double integration of the derivative ESR spectrum, and a standard curve was used to obtain the actual ROS concentrations. The free radical concentration is proportional to the area under the absorption curve [11]. The specific intracellular ROS level was obtained by normalizing the ROS concentrations with the corresponding cell concentrations. The ROS type was identified by comparison with standard spectra in the literature [12].

ESR spectrometer recording settings were as follows: field set 3380 G; microwave frequency, 950 MHz; microwave power, 5 mW; modulation frequency, 100 kHz; modulation amplitude, 2 G; and receiver gain $2 \times 2 \times 10^4$.

3. Results and discussion

3.1. Toxicity of treatment agents

The toxicity levels of the treatment agents (HOCl and H_2O_2) were determined by exposing the bacterial cultures in the exponential phase with different concentrations of treatment agents, as detailed in Section 2. The colony forming units obtained under various treatment conditions are presented in Table 1. It can be observed that at 20 mM HOCl (11.11 mmol (g cell)$^{-1}$) and 5 mM H_2O_2 (5.55 mmol (g cell)$^{-1}$) concentration, the number of colonies were half those in to the untreated culture. Such concentrations are referred to as LC_{50} values. The LC_{50} values for HOCl and H_2O_2 for *B. subtilis* were higher than that of another bacterium *Xanthomonas campestris*, which were 4.5 mmol (g cell)$^{-1}$ and 3 mmol (g cell)$^{-1}$ for HOCl and H_2O_2, respectively [13]. The highest colony counts and maximum cell concentrations were obtained with 10 mM HOCl (5.55 mmol (g cell)$^{-1}$) and 2.5 mM H_2O_2 (2.78 mmol (g cell)$^{-1}$), and therefore these concentrations were chosen for further study.

Table 1
Effect of various treatments on the number of colony forming units (CFU)

Treatments	Treatment doses (mmol/g cell)	Number of colony forming units (CFU)
Untreated	0	132 ± 7
HOCl treatment	0.56	126 ± 6
	1.11	62 ± 4
	2.78	212 ± 12
	5.5	246 ± 11
	11.11	66 ± 7
	22.22	0 ± 0
H_2O_2 treatment	0.56	122 ± 8
	1.11	104 ± 4
	2.78	183 ± 10
	5.55	67 ± 6
	11.11	0 ± 0
	22.22	0 ± 0

It is also clear from Table 1 that the toxicity of treatment agents to bacteria follows a bimodal pattern. The extent of cell death was higher with lower doses of treatment followed by a decline with increase in concentration of HOCl or H_2O_2, and later a sharp increase with further increase in concentrations. The bimodal pattern of cell toxicity to H_2O_2 has earlier been reported in HeLa cells [14] as well as in *Escherichia coli* [15], but there are no such reports in response to HOCl in the literature.

The effect of ROS inducing agents on survival rate of bacteria varies with phases of growth [16]. In one case, the survival rate after 10 mM H_2O_2 treatment has been reported to be 0.01–0.1% with treatment done in early exponential phase [16]. Therefore, the results of viable cell counts obtained after treatment with varying doses of ROS inducing agents are applicable only to that particular phase of growth.

3.2. Effect on growth

The growth profiles of *B. subtilis* cultures grown in the presence of ROS inducing agents in comparison to normal cells are presented in Fig. 1. The maximum cell concentration achieved in HOCl treated culture was 5.2 g l^{-1}, which was about 29% higher than untreated culture. Similarly, in the case of H_2O_2 treatment, maximum cell growth was 4.69 g l^{-1}, 16% higher than the untreated culture. The growth was simulated using a Riccati (logistic) equation:

$$\frac{dX}{dt} = kX\left(1 - \frac{X}{X_S}\right)$$

where k is the growth rate constant, and X_S the parameter that equals the maximum cell concentration, and the simulations are also shown in Fig. 1. Since the logistic equation does not

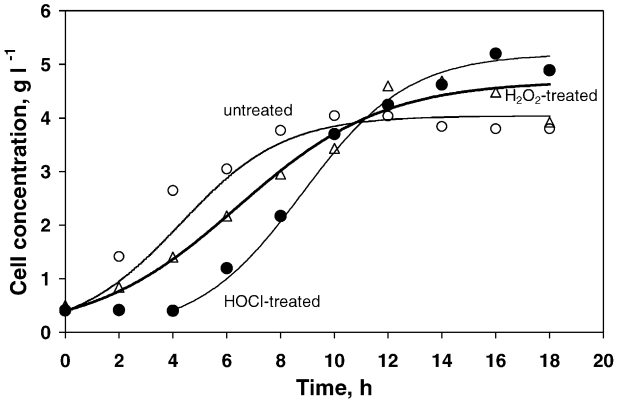

Fig. 1. Comparison of growth profiles from untreated (open circles), H_2O_2-treated (open triangles), and HOCl-treated (filled circles) *B. subtilis* cultures.

describe distinct lag-phases, the HOCl-treated culture growth was simulated after the lag-phase. The values of k (growth rate constant) obtained through nonlinear regression analysis were comparable for HOCl-treated, and untreated cultures (0.53 and 0.52 h^{-1}, respectively), whereas it was lower at (0.37 h^{-1}) for H_2O_2-treated cultures.

The immediate response to ROS inducing agents was a prominent lag phase, and it was more prominent in HOCl-treated cultures compared to the H_2O_2-treated cultures. The prominent lag phase may be due to time taken by bacteria to recover and adapt to new environment. Interestingly, the specific intracellular ROS levels (siROS) were also the highest during the lag phase, compared to other phases of growth (data not shown).

3.3. Effect on enzyme productivity

3.3.1. Increased production of α-amylase and protease

The time profiles of extracellular α-amylase concentrations obtained when untreated, H_2O_2-treated, and HOCl-treated cells were cultivated are given in Fig. 2. From the figure, it is clear that both H_2O_2 and HOCl treatments increased production of α-amylase. The maximum α-amylase concentration in the H_2O_2 treated case was 1.8-fold, and that in the HOCl treated case was about 2.6-fold of the value obtained in the cultivation with untreated cells.

Similarly, the H_2O_2 and HOCl treatments increased extracellular protease productivity; the maximum protease concentration in the H_2O_2 treated case was two-fold, and that in the HOCl treated case was about 3.5-fold of the value obtained in the cultivation with untreated cells as shown in Fig. 3.

Although the above results establish that ROS inducing treatments increased total enzyme concentration, it does not clearly depict the effect of the treatment at the cellular level. For example, the increase in total enzyme concentration could have been due to increases in both the specific enzyme level (per cell view) and the cell concentration. To investigate the effect of ROS inducing treatments at a cell level (per cell view), we compared the specific enzyme levels among the three cultivations were compared. The specific enzyme levels when the maximum total enzyme concentrations were observed are given in Table 2. The entries in this table shows that the specific α-amylase levels when the maximum α-amylase concentrations were reached, with H_2O_2 and HOCl treatments were 1.5-fold and 2.2-fold, respectively, of the value obtained with untreated cells. The specific protease levels when the maximum protease concentrations were reached, with H_2O_2 and HOCl treatments were 1.9- and 2.6-fold, respectively, of the value obtained with untreated cells. The specific enzyme levels clearly show that each cell produces higher enzyme levels as a result of the ROS-inducing treatments.

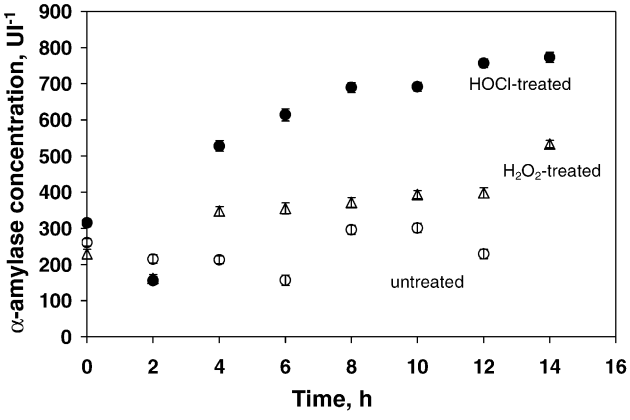

Fig. 2. Comparison of α-amylase productivities from untreated (open circles), H_2O_2-treated (open triangles), and HOCl-treated (filled circles) *B. subtilis* cultures.

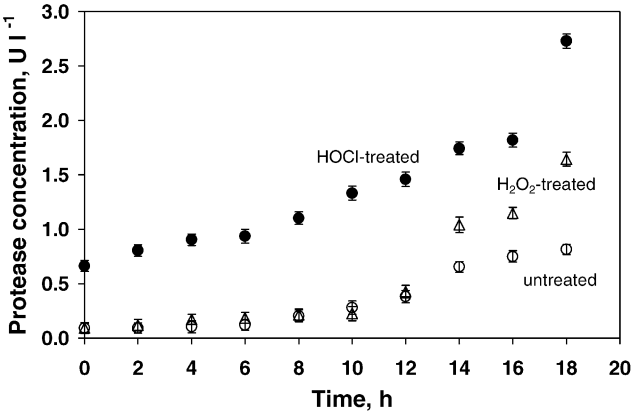

Fig. 3. Comparison of protease productivities from untreated (open circles), H_2O_2-treated (open triangles), and HOCl-treated (filled circles) *B. subtilis* cultures.

3.3.2. Increased siROS increases specific enzyme productivity

The relationship between the maximum specific level of enzymes and maximum siROS is presented in Fig. 4. The maximum siROS values were obtained in the 6 h of cultivation with both treated cultures. Also, as expected, the siROS was higher in the case of HOCl treatment as compared to H_2O_2, with HOCl known to be a better inducer of ROS [13].

Table 2
Maximum specific levels of extracellular enzymes produced under different treatment conditions

Treatment conditions	Enzymes	Specific enzyme level (U (g cell)$^{-1}$) at maximum total enzyme concentration
Untreated	Extracellular α-amylase	74.6
	Extracellular protease	0.22
H_2O_2 treatment	Extracellular α-amylase	113.7
	Extracellular protease	0.42
HOCl treatment	Extracellular α-amylase	167.2
	Extracellular protease	0.56

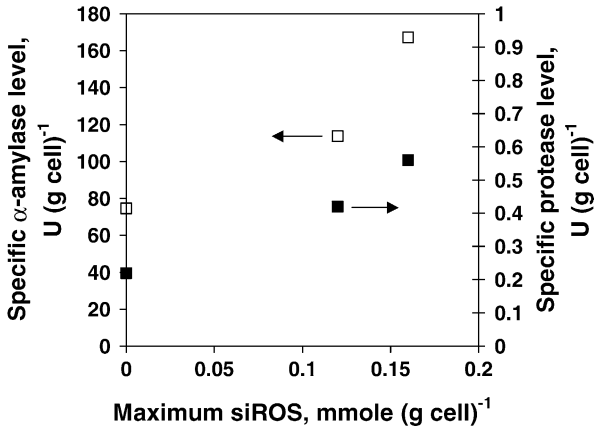

Fig. 4. Correlation between maximum specific extracellular enzyme levels (α-amylase: open squares; protease: filled squares) at maximum enzyme concentrations, and maximum siROS.

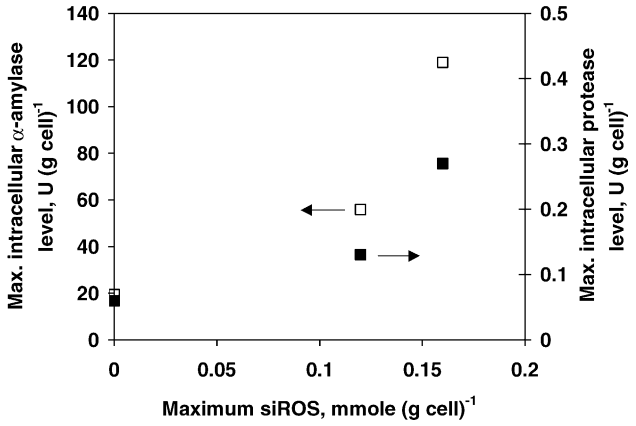

Fig. 5. Correlation between maximum specific intracellular enzyme levels (α-amylase: open squares; protease: filled squares), and maximum siROS.

The increased level of siROS poses a stress condition for the cells, and enhanced syntheses of molecular chaperones, foldases, extracellular degradative enzymes and proteases have been reported in bacteria under various kinds of stresses [17,18].

Therefore, increased specific enzyme levels were expected from the ROS inducing treatments. From Fig. 4, it can be seen that the maximum specific levels of both α-amylase and protease increase with siROS. Straight line approximations to the effect of maximum siROS on maximum specific enzyme levels yielded 520 U $(\text{mmol})^{-1}$ for α-amylase and 2.02 U $(\text{mmol})^{-1}$ for protease. Thus, the maximum siROS seems to improve α-amylase production better than protease production at a per cell level.

Interestingly, the specific enzyme levels of both enzymes obtained immediately after inoculation were higher compared to any other value subsequent in time to that value. This observation was made with both treated and untreated cultures, which rules out the possibility that the treatments cause the initial high value. The possibility of enzyme carryover in the inoculum did not explain the initial high specific level. Changes in environment osmolarity and consequent increases in amino acid pool and glycolysis rate are known to occur when the cells are inoculated in fresh media [19]. Changes in redox potential due to a change in environment as a result of inoculation in fresh medium can be a reason for a higher specific enzyme levels. Increased enzyme production on inoculation may be one such adaptive response to a change in the environments conditions. This aspect is discussed further in a later section.

3.4. Mechanistic aspects of increase in enzyme production

The discussion above established that specific extracellular enzyme level increased with ROS inducing treatments. Whether the increase resulted from higher rate of intracellular production or higher rate of secretion into the extracellular space, or a combination of both the processes was unclear. The contributions of intracellular production and secretion toward the observed increases in extracellular enzyme productivities were therefore investigated.

3.4.1. Intracellular enzyme production increased with siROS

The maximum intracellular α-amylase and protease levels are presented as a function of the maximum siROS in Fig. 5. It can be seen that the maximum intracellular enzyme production increased with siROS and thus the siROS levels are positively correlated with the increased production in extracellular enzymes.

3.4.2. Enzyme secretion can also increase with ROS

To investigate whether enzyme secretion is also responsible for the increased production of extracellular enzymes under oxidative stress (increased ROS), microarray data on hydrogen peroxide mediated stress on *B. subtilis*, which is available in the literature [7] was reviewed.

3.5. Analysis of microarray data

The microarray data available in literature includes overall transcriptional responses of *B. subtilis* cultures treated with micromolar concentrations of hydrogen peroxide as well as organic peroxides during mid-exponential phase of growth [7]. Analysis of the microarray data showed that approximately 75 genes were upregulated and other about 120 genes were downregulated [7] under conditions of oxidative stress (hydrogen peroxide exposure). Table 3 shows a compilation of the fold-inductions of some genes relevant for secretion in extracellular medium along with their respective functions, which were obtained using the SubtiList interface (http://genolist.pasteur.fr/SubtiList/). The induced genes were under the control of three different

Table 3
A list of *B. subtilis* genes that were significantly induced under peroxide stress, and their functions

Genes	Fold induction	Function
gbsab	7.62	Osmotic stress
sipt	4.25	Signal peptidase I
raph	3.21	Response regulator aspartate phosphatase

regulators, namely, PerR, OhrR and σ^B; PerR is the major response regulator of peroxide stress, OhrR controls a single gene [20] and σ^B is a general stress response regulator [7].

3.5.1. Increased efficiency of secretory apparatus causes increased extracellular α-amylase levels

The microarray data showed derepression of a gene belonging to the secretory apparatus of *B. subtilis, sipT* (signal peptidase T) when exposed to hydrogen peroxide [7]. The secretory apparatus in *B. subtilis* comprises of a group of enzymes, and precursor protein translocases [21], and secretion of α-amylase is a multi-step process. The various steps involved in that multi-step process are as follows:

1. Targeting of proteins across the cell membrane—a process that is assisted by 15–30 amino acids long signal peptides [22–25] and chaperones [2].
2. Translocation across the membrane by secretory translocase, SecA, Y, E, G, and F [26–28].
3. Pre-protein processing by signal peptidases (SipS, SipT, SipU, SipV, and SipW) [26,29,30].
4. Protein folding on the *trans* side of the membrane, and passage through the cell wall [3]—processes assisted by a lipoprotein, PrsA and metal ions such as Ca and Fe^{3+} [21].

Of all the above components, multiple signal peptidases (SPases) are major players in secretion of α-amylase in *B. subtilis*, and the rate limiting step in the secretion is the release of the signal peptide processed form of the enzyme [21]. SPases enable the cell to modulate the secretion machinery to adapt to changing environmental conditions. Studies with various Sip mutants in *B. amyloliquefaciens* also showed that *sipTis* needed for secretion of α-amylase (AmyQ) [2]. In another case, it has been shown that SipT and SipS play a major role in the processing of secretory preproteins and other chromosomally encoded SPases (SipU, SipV and SipW) are considered to be of less importance in *B. subtilis* [29].

The microarray data shows 4.25-fold induction for *sipT* under oxidative stress [7]. The increased levels of SipT suggest an increase in the processing of the α-amylase enzyme for secretion into the extracellular space. The increased activity of preprotein processing machinery can result in efficient enzyme release into the extracellular space. Therefore, an induced level of SipT is in correlation with the observed increase of α-amylase levels in culture. Thus, increased efficiency of the secretory apparatus can also be a reason for the increase in α-amylase productivity.

The microarray data also showed other interesting features such as induction of osmotic stress genes, *gbsAB*. These genes code for the production of osmo-protectant glycine betaine which is associated with osmotic stress. These genes are also induced in exponential phase of cultures [31]. The microarray data showed approximately 7.6-fold induction of *gbsAB* genes under peroxide mediated stress [7] and suggests a change in the osmolarity of the culture as a result of treatment with ROS inducing treatment agents. The observation that changes in the osmotic environment can cause changes in the levels of amino acids and glycolysis rate have been discussed previously. These changes probably lead to the observed increase in the specific enzyme productivities upon inoculation (discussed earlier) in culture under osmotic stress condition as an adaptive response. The microarray data also showed induction of the *rapH* gene, which codes for response regulator aspartate phosphatase. Rap phosphatase genes containing plasmids are used industrially for increased enzyme secretion in *B. subtilis* cultures [32]. Rap phosphatases act as cell-density signaling molecules in *B. subtilis* [33] and they increase extracellular enzyme production by inhibiting secretory proteases causing their proteolysis under low density cell culture or exponential phase growing cultures [33]. Immediately after inoculation, the cell density is low and *rapH* gene induction could also be a reason for the observed increase in specific α-amylase level upon inoculation.

3.5.2. Increased protease activity as an adaptive response to oxidative stress

The microarray data available in literature did not include any protease specific genes. Nevertheless, the present our experimental results clearly showed an increased protease level as a result of treatment with ROS inducing agents, which finds support from other available data on transcriptional profiling in literature. These transcriptional profiling data showed a large number of *spo* genes involved in stress management, some of which encode for proteases and peptidases [34,35]. There are also reports of a major class of ATP dependent proteases, Clp proteases, being induced in a wide range of environmental stresses [36–38].

4. Conclusions

B. subtilis cultures were treated with sublethal doses of ROS inducing agents (HOCl and H_2O_2). The treatment increased maximum cell growth concentration and maximum specific activities of enzymes such as α-amylase and protease. The increases in the enzyme activity and growth were higher in HOCl treated cultures as compared to H_2O_2-treated cultures. Further the increase correlated with levels

of ROS. Analysis of microarray data on transcriptional induction of genes under H_2O_2 mediated stress in *B. subtilis* showed that the increase in growth and enzyme activities were a result of adaptive responses induced under oxi dative stress.

Acknowledgements

We would like to thank Susmita Sahoo and Kapileswar Nayak, graduate students, Department of Chemical Engineering, Indian Institute of Technology, Bombay, India for their help in this work.

References

[1] Ferrari E, Jarnagin AS, Schmidt BF. Commercial production of extracellular enzymes. In: Sonenshein AL, Hoch JA, Losick R, editors. *Bacillus subtilis and other Gram-positive bacteria: biochemistry, physiology* and molecular genetics. Washington: American Society for Microbiology; 1993. p. 917–37.

[2] Bron S, Bolhuis A, Tjalsma H, Holsappel S, Venema G, van Dijl JM. Protein secretion and possible roles for multiple signal peptidases for precursor processing in bacilli. J Biotech 1998;64:3–13.

[3] Jensen CL, Stephenson K, Jorgensen ST, Harwood C. Cell-associated degradation affects the yield of secreted engineered and heterologous proteins in the *Bacillus subtilis* expression system. Microbiology 2000;146:2583–94.

[4] Sies H. Oxidative stress: oxidants and anti-oxidants.. New York: Academic Press; 1991.

[5] Georgiou G. How to flip the (redox) switch. Cell 2002;111:607–10.

[6] Storz G, Imlay A. Oxidative stress. Curr Opin Microbiol 1999;2:188–94.

[7] Helmann JD, Winston MF, Gaballa A, Kobel PA, Morshedi MM, Fawcett P, et al. The global transcriptional response of *Bacillus subtilis* to peroxide stress is coordinated by three transcription factors. J Bacteriol 2003;185:243–53.

[8] Sarath G, Motte RSDL, Wagner FW. Proteolytic Enzymes: A Practical Approach. Oxford: IRL Press; 1990.

[9] Sahoo S, Verma R, Suresh AK, Rao KK, Bellare J, Sureshkumar GK. Macro-level and genetic-level responses of *Bacillus subtilis* to shear stress. Biotech Prog 2003;19:1689–96.

[10] Sahoo S, Rao KK, Suraishkumar GK. Induced reactive oxygen species improve enzyme production from *Aspergillus niger* cultivation. Biotech Lett 2003;25:821–5.

[11] Borg DC. Applications of electron spin resonance in biology. In: Pryor WA, editor. Free Radicals in Biology, Vol. 1. New York: Academic Press; 1976. p. 69–147.

[12] Zweier JL. Measurement of superoxide-derived free radicals in the reperfused heart. J Biol Chem 1998;263:1353–7.

[13] Manjularao Y, Sureshkumar GK. Improvement in bioreactor productivities using free-radicals: HOCl-induced overproduction of Xanthan gum from *Xanthomonas campestris* cells in culture. Biotechnol Bioeng 2001;72:62–8.

[14] Nakamura J, Purvis RE, Swenberg JA. Micromolar concentrations of hydrogen peroxide induce oxidative DNA lesions more efficiently than millimolar concentrations in mammalian cells. Nucleic Acids Res 2003;31:1790–5.

[15] Luo Y, Han Z, Chin SM, Linn S. Three chemically distinct types of oxidants formed by iron-mediated Fenton reactions in the presence of DNA. Proc Natl Acad Sci USA 1994;91:12438–42.

[16] Dowds BCA. The oxidative stress response in *Bacillus subtilis*. FEMS Microbiol Lett 1994;124:255–64.

[17] Msadek T, Kunst F, Henner D, Klier A, Rapoport G, Dedonder R. Signal transduction pathway controlling synthesis of a class of degradative enzymes in *Bacillus subtilis*: expression of the regulatory genes and analysis of mutations in *degS and degU*. J Bacteriol 1990;172:824–34.

[18] Pummi T, Leskela S, Wahlstrom E, Gerth U, Tjalsma H, Hecker M, et al. ClpXP protease regulates the signal peptide cleavage of secretory preproteins in *Bacillus subtilis* with a mechanism distinct from that of the Esc ABC transporter. J Bacteriol 2002;184:1010–8.

[19] Wang H, Lee P, Liu L, Su J. Effect of sorbitol induced osmotic stress on the changes of carbohydrate and free amino acid pools in sweet potato cell suspension cultures. Bot Bull Acad Sin 1999;40:219–25.

[20] Fuangthong M, Atichartpongkul S, Mongkolsuk S, Helmann JD. OhrR is a repressor of *ohrA*, a key organic hydroperoxide resistance determinant in *Bacillus subtilis*. J Bacteriol 2001;183:4134–41.

[21] Leloup L, Haddaoui EA, Chambert R, Petit-Galtron MF. Characterization of the rate limiting step of the secretion of *Bacillus subtilis* a-amylase overproduced during the exponential phase of growth. Microbiology 1997;143:3295–303.

[22] Driessen AJM. How proteins cross the bacterial cytoplasmic membrane. J Membr Biol 1994;142:145–59.

[23] Pugsley T. The complete general secretory pathway in Gram-negative bacteria. Microbiol Rev 1993;57:50–108.

[24] Tjalsma H, Bolhuis A, van Roosmalen ML, Wiegert T, Schumann W, Broekhuizen CP, et al. Functional analysis of the secretory precursor processing machinery of *Bacillus subtilis*: identification of a eubacterial homolog of archeal and eukaryotic signal peptidases. Genes Dev 1998;12:2318–31.

[25] van Wely KHM, Swaving J, Klein M, Freudl R, Driessen JM. The carboxyl terminus of the *Bacillus subtilis* SecA is dispensible for protein secretion and viability. Microbiology 2000;146: 2573–81.

[26] Bolhuis A, Sorokin A, Azevedo V, Ehrlich SD, Braun PG, de Jong A, et al. *Bacillus subtilis* can modulate its capacity and specificity for protein secretion through temporally controlled expression of the *sipS* gene for signal peptidase I. Mol Microbiol 1996;22: 605–18.

[27] Overhoff B, Klein M, Spies M, Freudl R. Identification of a gene fragment which codes for the 364 amino-terminal amino acid residues of a SecA homologue from *Bacillus subtilis*: further evidence for the conservation of protein export apparatus in gram-positive and gram-negative bacteria. Mol Gen Genet 1991;228:417–23.

[28] von Heijne G. The signal peptide. J Membr Biol 1990;115:195–201.

[29] Tjalsma H, Noback MA, Bron S, Venema G, Yamane K, van Dijl JM. *Bacillus subtilis* contains four closely related type I signal peptidase with overlapping substrate specificities. J Biol Chem 1997;272:25983–92.

[30] van Dijl JM, de Jong A, Vehmaanpera J, Venema G, Bron S. Signal peptidase I in *Bacillus subtilis*: patterns of conserved amino acids in prokaryotic and eukaryotic type I signal peptidases. EMBO J 1992;11:2819–28.

[31] Caldas T, Caulet-Demont N, Ghazi A, Richarme G. Thermoprotection by glycine betaine and choline. Microbiology 1999;145:2543–8.

[32] Meijer WJJ, Wisman GBA, Terpstra P, Thorsted PB, Thomas CM, Holsappel S, et al. Rolling-circle plasmids from *Bacillus subtilis*: complete nucleotide sequences and analyses of genes of pTA1015, pTA1040, pTA1050 and pTA1060, and comparisons with related plasmids from Gram positive bacteria. FEMS Microbiol Rev 1998;21:337–68.

[33] Koetje EJ, Hajdo-Milasinovic A, Kiewiet R, Bron S, Tjalsma H. A plasmid borne Rap-Phr system of Bacillus subtilis can mediate cell density controlled production of extracellular proteases. Microbiology 2003;149:19–28.

[34] Sakamoto JJ, Sasaki M, Tsuchido T. Purification and characterization of a *Bacillus subtilis* 168 nuclease, YokF, involving in chromosomal DNA degradation and cell death caused by thermal shock treatments. J Biol Chem 2001;276:47046–51.

[35] Serrano M, Hövel S, Moran CP, Henriques AO, Völker U. Forspore-specific transcription of the *lonB* gene during sporulation in *Bacillus subtilis*. J Bacteriol 2001;183:2995–3003.

[36] Derre I, Rapoport G, Devine K, Rose M, Msadek T. ClpE a novel type of Hsp 100 ATPase, is part of the CtsR heat shock regulon of *Bacillus subtilis*. Mol Microbiol 1999;32:581–93.

[37] Gerth U, Kruger E, Derre I, Msadek T, Hecker M. Stress induction of the *Bacillus subtilis clp* gene encoding a homologue of the Clp protease and the involvement of ClpP and ClpX in stress tolerance. Mol Microbiol 1998;28:787–802.

[38] Michael RV, Landini P. Transcriptional responses to DNA damage. Curr Opin Microbiol 2001;178–85.