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Free radical aspects of *Xanthomonas campestris* cultivation with liquid phase oxygen supply strategy

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Abstract

Reactive oxygen (free radical) species such as hydroxyl, superoxide or peroxyl radicals significantly affect cell function and, therefore, determine bioreactor productivities. The specific intracellular reactive oxygen species (ROS) levels as well as the ROS type, during the log and stationary phases of several *Xanthomonas campestris* (both untreated and HOCl-treated cells) cultivations in the bioreactor, with and without antioxidants, were measured through electron spin resonance (ESR) spectroscopy. Depending on the cultivation conditions, intracellular hydroxyl or superoxide radicals at different levels were detected. The oxygen supply during the cultivations was through need-based, pulse additions of hydrogen peroxide (H_2O_2), which was subsequently decomposed by culture catalase to yield oxygen. Using data from all cultivations, it was found that the growth rate constant decreased with the log-phase intracellular ROS level; the specific productivity of xanthan gum was found to be optimal at an intracellular ROS level of 0.33 mmol per g cell in the stationary phase. The rheology of the xanthan gum produced in various cultivations is discussed. Further, a model, which can simulate and predict the specific total intracellular ROS level in the cultivations with untreated cells have been developed.

Keywords: ROS level; *Xanthomonas campestris*; H_2O_2; Antioxidants; Xanthan gum

1. Introduction

Oxidative stress is understood as the condition that results when an excess of reactive oxygen species (ROS) such as hydroxyl, superoxide, peroxyl free radicals or singlet oxygen are present in the organism [1–3]. Oxidative stress has been closely related to cancer, ageing [2], many other degenerative diseases [4] and damage to DNA, membrane and proteins [2,5,6]. However, sub-lethal levels (levels which do not cause a significant loss in cell viability, e.g. about 0.4 mmol per g cell for *Xanthomonas campestris*) of induced ROS can improve bioreactor productivity as well as the product profile [7,8].

In this study, specific intracellular ROS levels during various cultivations of the model system, *X. campestris* producing xanthan gum, were quantified using electron spin resonance (ESR) spectroscopy, to determine the relationship between ROS level and growth/productivity. The type of ROS was also identified. Quantification of ROS levels in bioreactors has not been reported so far, except briefly, in a related paper [7]. In that related paper, aeration was used as the oxygen source, whereas, H_2O_2 pulses have been used to supply oxygen in all cultivations described in this article.

Different specific intracellular ROS levels during cultivations were obtained using untreated or HOCl-treated cells and growing them in the presence/absence of antioxidants such as ascorbic acid (vitamin C), tocopherol (vitamin E) or a combination of the antioxidants. HOCl was used earlier to induce intracellular ROS [7,8], which resulted in higher productivity. The particular antioxidants were chosen because ascorbic acid is water soluble and hence can be effective against ROS in the hydrophilic regions of the cell such as the cytoplasm and in the medium; tocopherol is lipid-soluble and hence can be effective against ROS generated in the hydrophobic regions of the cell, especially, in

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the cell membrane. Several steps of the oxidative phosphorylation pathway, which lead to the formation of metabolic free radicals (ROS), take place in the membrane [9].

Further, a model to simulate and predict the total ROS level in the culture when untreated cells are grown under different conditions, has been developed. The rheology of the xanthan gum (0.1% w/w xanthan gum solutions in de-ionised water) produced in the above cultivations has been studied. In addition, correlations between growth rate parameters and the log phase specific ROS levels on the one hand, and between gum production parameters and stationary phase specific ROS levels on the other, have been explored.

2. Materials and methods

2.1. Culture

The microorganism used in this study was *X. campestris* (MTCC 2286, IMTECH, Chandigarh, India) producing xanthan gum. The stock cultures were maintained at 4 °C on agar slants containing 25 g l^{-1} glucose, 8 g l^{-1} yeast extract, 10 g l^{-1} $CaCO_3$ and 20 g l^{-1} of agar. The growth medium (standard medium) consisted of 40 g l^{-1} glucose, 3 g l^{-1} yeast extract, 5 g l^{-1} K_2HPO_4, 0.6 g l^{-1} of $MgSO_4 \cdot 7H_2O$ and 0.4 g l^{-1} of urea in tap water (tap water was used to provide trace elements and the medium was sterilised by autoclaving without glucose before inoculation). During bioreactor cultivations, oxygen was supplied through pulse additions of H_2O_2. In certain cultivations, 5 mM ascorbic acid (vitamin C) and/or 1 mM tocopherol (vitamin E) were added to the standard medium, either initially or in pulses along with H_2O_2. However, the total concentration of antioxidants added were the same whether they were added initially or in pulses. For tocopherol addition, it was dissolved initially in DMSO, added, and vigorously agitated at 1200 rpm, before inoculation, to ensure solubility and hence, availability to cells. Since vigorous agitation was not employed during the cultivation to maintain shear levels, the availability of tocopherol to the cells during pulse additions is suspect. Also, care was taken to ensure negligible volume change on addition of H_2O_2, ascorbic acid and/or tocopherol.

3. Analyses, reagents and equipment

The cell concentration was measured using a spectrophotometer (JASCO, Japan) through cell scatter at 600 nm, using a calibration curve of cell concentration versus optical density. The number of colonies on plates were counted under a magnifying glass. The xanthan concentration was determined through dry weight measurement after isolation through alcohol precipitation followed by centrifugation at 10 000 × *g* for 45 min [10]. The extracted xanthan gum was dissolved in de-ionised water to make 0.1% w/w xanthan gum solutions, and viscosities of such solutions (not the broth viscosities) were measured using a Brookfield viscometer at 30 °C.

To measure oxygen centred radicals formed, a spin trapping technique [11] was employed. A quartz liquid-cell accessory was used for the sample placement and the same volume (250 μl) of different samples was used in all ESR analyses. Care was also exercised to maintain the required concentrations of the species in the liquid-cell accessory. The free radical concentration is proportional to the area under the absorption curve [12]. The area under the absorption curve was obtained by double integration of the derivative spectrum obtained, using computer programs. The free radical concentrations were obtained by comparison with concentrations of known standards: KO_2 for superoxide radicals and H_2O_2 with 0.1 mg ml^{-1} $FeCl_2$ for hydroxyl radicals [13].

Hypochlorous acid was generated and quantified from sodium hypochlorite (SRL, India) and only freshly prepared HOCl was used [14]. The spin trap, dimethylpyrolline-*N*-oxide (DMPO) (Sigma) was used to arrest the ROS, for ESR analysis.

The bioreactor (Vaspan Industries, India) employed was a 2 l (1 l working volume) stirred tank reactor with two Rushton turbine impellers, rpm and temperature controls. The DO and pH data were obtained on-line using probes (Bela Instruments, India) through a data acquisition and control system (SCR Elektroniks, India) interfaced with a PC. The ESR spectrometer (Varian E-3) was used for obtaining the ESR spectra.

3.1. Treatment procedures

Cells in the required growth phase, at a concentration of 2 g l^{-1}, were resuspended in 0.05 M phosphate buffer, taken in several 25 ml shake flasks. The flasks were thoroughly cleaned before use, including a sulphachromic acid wash. Freshly prepared hypochlorous acid, the concentrations of which were determined by iodometry, were added to the cultures in three stages. Each stage consisted of a 60 s HOCl exposure after which the cells were removed from the HOCl containing treatment medium by centrifugation. Then, the cells were incubated in normal growth medium for 40 min in the dark, at 30 °C, with gentle shaking. The concentrations of HOCl used for exposure at the beginning of each stage were: 0.66 mmol per g cell (I stage), 4.65 mmol per g cell (II stage) and 20 mmol per g cell (III stage). After the third incubation, the free chlorine left in the flasks was quenched by using sterile sodium thiosulphate. Culturable bacteria were assayed by plating on LB agar and colonies were counted after 10 h of incubation.

The live colonies were isolated and grown in shake flasks placed in a shaker-incubator at 30 °C, in the same growth medium mentioned previously. The growth experiments with the treated cells were also conducted in the 1 l bioreactor at 30 °C.

All experiments described were repeated at least twice.

4. Model for intracellular ROS level

The experimental results showed that the specific intracellular ROS level plays an important role in the fermentation, and therefore, an attempt is made in this section to develop a predictive model for intracellular ROS concentration/specific level. While reactions reported in the literature for the initiation and termination of the free radical species of interest are considered, the focus in this first attempt is on total radical concentrations rather than on levels of individual radical species. This 'lumped' approach to free radical species permits some lumping of reactions as well, thereby, reducing the number of unknown parameters in the model.

The effect of known antioxidants, ascorbate (Vit C^-) and tocopherol (ArOH) have been considered in the model. When present, they generate antioxidant free radicals (Vit $C^{-\bullet}$ and $ArO^\bullet$) by reaction with ROS of interest ($OH^\bullet$, $O_2^{-\bullet}$, $ROO^\bullet$). The antioxidant free radicals are more stable than the ROS of interest, because, the unpaired electron can be de-localised into the aromatic ring structure [2]. Thus, the rate of termination of the lumped radicals is expected to be different in the presence of the antioxidants. Further, the effect of ascorbic acid on the oxidation-state of iron in the cell is explicitly addressed in the model.

4.1. Initiation reactions

The initiation reactions considered were:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + OH^\bullet \quad (1)$$

$$H_2O_2 + Cu^+ \rightarrow Cu^{2+} + OH^- + OH^\bullet \quad (2)$$

$$O_2 + Fe^{2+} \rightarrow Fe^{3+} + O_2^{-\bullet} \quad (3)$$

$$ROOH + Fe^{3+} \rightarrow Fe^{2+} + H^+ + ROO^\bullet \quad (4)$$

4.2. Termination reactions

$$OH^\bullet + OH^\bullet \rightarrow H_2O_2 \quad (5)$$

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \quad (6)$$

where, SOD is superoxide dismutase.

$$HOO^\bullet + HOO^\bullet \rightarrow H_2O_2 + O_2 \quad (7)$$

$$HOO^\bullet + O_2^{-\bullet} + H^+ \rightarrow H_2O_2 + O_2 \quad (8)$$

$$OH^\bullet + HOO^\bullet \rightarrow H_2O + O_2 \quad (9)$$

$$O_2^{-\bullet} + OH^\bullet \rightarrow OH^- + O_2 \quad (10)$$

Clearly, some of the reactions above (for example, the enzyme mediated reaction Eq. (6)) are governed by mechanisms of their own. However, the overall rate constants for these reactions can be obtained from literature as Table 1 shows.

When vitamins C and E are present in the medium, they interact with the radical species of interest through reactions such as the following, and thereby influence the relative abundance of various ROS in the free radical pool:

$$\text{Vit } C^- + O_2^{-\bullet} + H^+ \rightarrow H_2O_2 + \text{Vit } C^{-\bullet} \quad (11)$$

$$\text{Vit } C^- + OH^\bullet \rightarrow H_2O + \text{Vit } C^{-\bullet} \quad (12)$$

$$ArOH + O_2^{-\bullet} \rightarrow HO_2^- + ArO^\bullet \quad (13)$$

$$ArOH + ROO^\bullet \rightarrow ROOH + ArO^\bullet \quad (14)$$

The rates of initiation for the hydroxyl, superoxide and peroxyl radicals can be written as:

$$r_{ini,OH^\bullet} = k_1[H_2O_2][Fe^{2+}] + k_2[H_2O_2][Cu^+] \quad (15)$$

$$r_{ini,O_2^{-\bullet}} = k_3[O_2][Fe^{2+}] \quad (16)$$

$$r_{ini,ROO^\bullet} = k_4[ROOH][Fe^{3+}] \quad (17)$$

If the total ROS concentration of interest ($=[OH^\bullet]+[O_2^{-\bullet}]+[ROO^\bullet]$) is represented as $[Rad^\bullet]$, then, the total rate of initiation is given by:

Table 1
Parameter values used in the model; the subscripts for k refer to the corresponding equations

Parameter	Value	Source/basis
k_1	76 M^{-1} s^{-1}	[2]
k_2	10^9 M^{-1} s^{-1}	[2]
k_3	7.0×10^8 M^{-1} s^{-1}	[18]
k_4	2.6×10^7 M^{-1} s^{-1}	[19]
k_5	6.0×10^9 M^{-1} s^{-1}	[20]
k_6	1.9×10^9 M^{-1} s^{-1}	at physiological pH [21]
k_7	8.0×10^5 M^{-1} s^{-1}	[22]
k_8	8.5×10^7 M^{-1} s^{-1}	At physiological pH [21]
k_9	1.5×10^{10} M^{-1} s^{-1}	[23]
k_{10}	10^{10} M^{-1} s^{-1}	[24]
k_{11}	2.7×10^5 M^{-1} s^{-1}	at physiological pH [2]
k_{12}	1.2×10^{10} M^{-1} s^{-1}	[25]
k_{13}	4.5×10^3 M^{-1} s^{-1}	[26]
k_{14}	5.1×10^5 M^{-1} s^{-1}	[27]
Cu_i	1.25×10^{-6} M^{-1} s^{-1}	Estimated from [16]
Fe_i	3×10^{-5} M	Estimated from [16]
$[H_2O_2]$	0.1 M	Sriram and Sureshkumar, 2000
$[O_2]$	5 μM	Sriram and Sureshkumar, 2000
ROOH	3.1×10^{-4} g per g cell	Estimated from [16]
R	4	Assumed, based on information from [2]
α	10 M^{-1}	From error minimisation
β	70 M^{-1}	From error minimisation
γ	4.54×10^3 M^{-1}	From error minimisation

$$r_{ini} = k_1[H_2O_2][Fe^{2+}] + k_2[H_2O_2][Cu^{+}] + k_3[O_2][Fe^{2+}] + k_4[ROOH][Fe^{3+}] \quad (18)$$

The dominant mechanism for radical termination (quadratic termination) would involve interactions between two radicals, either of the same type or of different types. In view of the paucity of rate constant information for all the possible reactions, and to restrict the number of unknown parameters, a quadratic termination rate law for the lumped radical species was assumed;

$$r_{ter} = k_{av}[Rad^{\bullet}]^2 \quad (19)$$

where, k_{av} is an average termination rate constant. A reference to Table 1 shows that the termination rate constants for quadratic termination for different radical types differ greatly; in a detailed model that treats the individual radical types separately, the value of k_{av} would be expected to depend strongly on circumstances that change the composition of the radical pool. In the present lumped model, such effects are accounted for, through a scaling factor that modifies the overall order of magnitude and other factors, which specifically account for the effect of substances such as vitamin C and E, as detailed below.

In addition to the effect on the composition of the radical pool, some other factors need to be considered in connection with vitamins C and E. It is known that vitamin C can reduce Fe^{3+} to Fe^{2+} [2], and thus make Fe^{2+} available for ROS generation by the initiation reactions listed above. Thus, when vitamin C is present, the ratio of Fe^{2+} to Fe^{3+} may be expected to be higher than otherwise. The concentration of iron species in the cell is, however, influenced by many other cellular processes in which these species participate. Therefore, it was assumed that vitamin C alters the ratio of Fe^{2+} to Fe^{3+}, while maintaining the total Fe pool unaltered. Thus;

$$\frac{[Fe^{2+}]}{[Fe^{3+}]} = (1 + \alpha \text{Vit C})R; \quad [Fe^{2+}] + [Fe^{3+}] = [Fe_i] \quad (20)$$

where, R stands for the ratio of Fe^{2+} to Fe^{3+} in the absence of vitamins C and E.

As mentioned above, the presence of ascorbic acid and/or tocopherol can be expected to alter the composition of the free radical pool. In the lumped model, where a single rate constant is used to treat the termination rates, it is, therefore, expected, that the value of k_{av} will reflect such changes. For example, as reactions (Eqs. (11)–(14)) shows, vitamin E would reduce the concentration of the less reactive peroxide radical, thus making the radical pool richer in the more reactive species. On the other hand, vitamin C reduces the concentration of the more reactive hydroxyl radical, thus making the radical pool richer in the less reactive species. It can, therefore, be expected that the value of k_{av} will decrease in the presence of vitamin C, while the effect of vitamin E would be to enhance it. Here, such effects of these antioxidants are represented by writing;

$$k_{av} = k_{av}^0 \exp(-\beta \text{Vit C}) \exp(\gamma \text{ArOH}) \quad (21)$$

a functionality which ensures that the termination rate constant reverts to the normal value k_{av}^0 in the absence of vitamins C and E. For k_{av}^0, in the absence of information on the relative abundance of the different types of radicals, a scaled average of the values reported for the individual termination reactions listed above has been used.

Applying the commonly used pseudo-steady state approximation to the total radical concentration, the rates of initiation and termination were equated and the resulting equation solved to obtain the total radical concentration, [Rad•]. Table 1 lists values of the rate parameters as well as concentrations used as inputs to the calculation. All parameters could be obtained or estimated from information in the literature, except for the constants α, β and γ, which are constructs of the model. These latter values were, therefore, chosen by minimising the error between experimental and simulated values, in the cases when vitamin C or E was present alone. Subsequently, these same values were used to predict the [Rad•] in situations where vitamin C and E were both present simultaneously. The equations were solved using a C program on a Pentium III PC.

5. Results and discussion

5.1. Types of intracellular ROS detected

DMPO was used to trap free radicals for measurement. DMPO is a nitrone which binds the radical to its α-carbon to form adducts which give hyper-fine splits characteristic of the bound radical, in the ESR derivative spectrum [15]. Analyses of the spectra obtained showed that hydroxyl radicals (four splits), and in certain cases, superoxide radicals (three splits), were formed in the intracellular space when *X. campestris* cells were grown in culture with H_2O_2 as the oxygen source. The ROS in the log phase (2.5 h) and stationary phase (50 h) of the various cultivations were quantified.

The ESR determinations showed that one type of radical usually dominated the ROS population in a sample; the other types were not detectable. Superoxide radicals were found only in certain cultivations involving the untreated cells; in the log-phase when grown in standard medium, and both in the log and stationary phases when grown with vitamin E. Hydroxyl radicals were found in all other cases.

The discussion in the following sections does not differentiate between ROS types. However, the lumping

of ROS species does not significantly reduce the interpretation of the results presented, because the less reactive superoxide radicals were found in lower specific levels than the more reactive hydroxyl radicals. The relative reactivities were deduced from the rate constants for reactions of superoxide and hydroxyl radicals with various compounds [2].

5.2. Intracellular ROS level and its effect on growth

The growth curves of untreated cells when H_2O_2 was used as the oxygen source in cultivations, with and without antioxidants, are presented in Fig. 1. The total amount of H_2O_2 added and the initial cell concentrations used were the same in all cultivations reported in this paper. The maximum cell concentrations was the highest (1.75 g l^{-1}) in the cultivation with 1 mM tocopherol (all antioxidant concentrations are initial concentrations), which was 59% higher than that obtained when the untreated cells were grown in standard medium. The lowest maximum cell concentration (0.8 g l^{-1}) was obtained in the cultivation with 5 mM ascorbic acid. However, when a mixture of 5 mM ascorbic acid and 1 mM tocopherol were present, the maximum cell concentration was comparable with that obtained in standard medium.

A logistic equation [16];

$$\frac{dX}{dt} = k\left(1 - \frac{X}{X_s}\right)X \quad (22)$$

was used to fit the growth curves, and the simulations are also shown in Fig. 1. The corresponding maximum cell concentration for each of the cases shown, was chosen as the X_s parameter and the growth rate constants were found through least squares analyses. Growth rate constants, ROS types and total ROS levels measured in the log phase of growth, in the cultivations carried out are summarised in Table 2. In general, it is seen that the growth rate constants were higher when the ROS levels were lower.

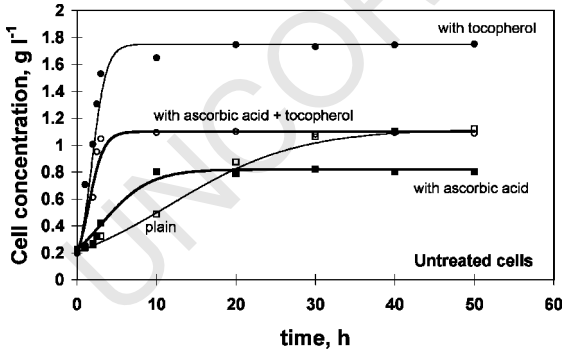

Fig. 1. Growth curves obtained when untreated cells were grown with H_2O_2 as the oxygen source. Points refer to experimental data and lines are simulated profiles.

It is interesting that the presence of ascorbic acid increased the intracellular free radical level by 20% compared with that obtained in standard medium, whereas, a decrease was expected, because ascorbic acid was expected to scavenge the free radicals formed. The possible reason for the unexpected increase is the reduction by ascorbate of Fe^{3+} to Fe^{2+}, which can further react with H_2O_2 to produce hydroxyl radicals [2]. Also, the presence of tocopherol alone or in combination with ascorbic acid, resulted in a significant decrease in the total intracellular ROS level; it was only about 11% of that obtained in standard medium. Also, there was a very significant (8.8-fold) increase in growth rate constant when tocopherol alone or in combination with ascorbic acid, was present. Whether this large increase is due to the antioxidant action of tocopherol alone or a combination of antioxidant action and some other growth-promoting action, is unclear.

When the antioxidants were added in pulses along with H_2O_2 to untreated cells, such that the total concentration of antioxidants added was comparable with that in the cultivations described above, the increases in growth rate were less significant: 0.23 h^{-1} with ascorbic acid, 0.27 h^{-1} with tocopherol and 0.25 h^{-1} with a combination of ascorbic acid and tocopherol compared with 0.13 h^{-1} in standard medium. Also, the differences in maximum cell concentrations achieved were not very significant. Since the antioxidants were added in pulses, their effective concentration at any time point was much less than the earlier cultivations where the entire amount was added as a bolus in the beginning. Also, the availability of tocopherol to the cells is suspect because vigorous mixing conditions could not be employed during pulse addition when cells were present. Therefore, the increases in growth rates were less marked compared with the cultivations discussed earlier.

The model developed in the earlier section was used to simulate the specific intracellular ROS levels obtained when H_2O_2 is used as the oxygen source, and also when either ascorbic acid and/or tocopherol was present in the medium during the H_2O_2-based cultivations. The parameter values used for the simulation are presented in Table 1. The simulated values are shown in Table 3 along with the experimental values and it can be seen that the model simulates the experimental data well. The values of the parameters, α, β and γ, obtained during the simulations were used to predict the specific ROS level when both, vitamin C and E, are present simultaneously. As can be seen from Table 3, the predicted value is in good agreement with the experimental value.

The growth curves of HOCl-treated cells when H_2O_2 was used as the oxygen source in cultivations, with or without antioxidants, is presented in Fig. 2 and the growth simulations using the same logistic equation as above is also presented in the same figure. The

Table 2
Log phase ROS type/levels and growth rate constants in cultivations with untreated and HOCl-treated cells

Cultivation in	Untreated cells		HOCl-treated cells	
	ROS (mmol per g cell)	Growth rate (h^{-1})	ROS (mmol per g cell)	Growth rate (h^{-1})
Standard medium	2.09±0.05 superoxide	0.13	2.30±0.08 hydroxyl	0.15
+Vit C	2.50±0.04 hydroxyl	0.33	2.56±0.10 hydroxyl	0.27
+Vit E	0.22±0.01 superoxide	1.10	0.55±0.02 hydroxyl	0.95
+Vit C+Vit E	0.25±0.01 hydroxyl	1.05	1.15±0.03 hydroxyl	0.90

The initial concentration of ascorbic acid employed was 5 mM and the concentration of tocopherol employed was 1 mM.

Table 3
Experimental and simulated or predicted values of intracellular ROS levels with untreated cells

Type of cultivation	Experimental value (mmol per g cell)	Simulated/predicted value (mmol per g cell)
With aeration in standard medium	0.08±0.01	0.08
With H_2O_2 in standard medium	2.09±0.05	2.09
With H_2O_2 in standard medium+Vit C	2.50±0.04	2.49
With H_2O_2 in standard medium+Vit E	0.22±0.01	0.22
With H_2O_2 in standard medium+Vit C+Vit E	0.25±0.01	0.26

The experimental ROS value with aeration was reported earlier [7].

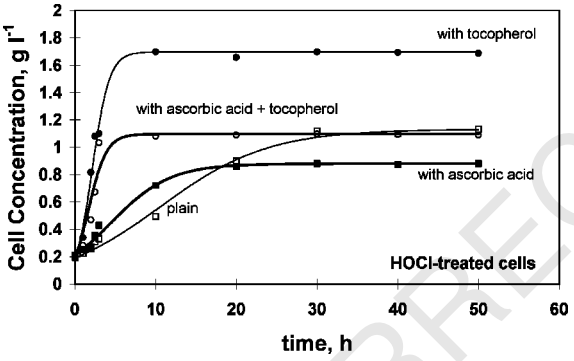

Fig. 2. Growth curves obtained when HOCl-treated cells were grown with H_2O_2 as the oxygen source. Points refer to experimental data and lines are simulated profiles.

maximum cell concentration was highest in the cultivation with tocopherol, as obtained in the cultivation with untreated cells. Also, the variation in maximum cell concentration obtained with the type of cultivation was similar to that obtained with untreated cells. In addition, the ROS levels and the corresponding growth rate constants, presented in Table 2, show that the correlation between ROS levels (log phase) and growth rate is qualitatively similar to the case of the untreated cells.

Irrespective of the type of cells (untreated or HOCl-treated), a reasonable correlation is seen between the growth rate and the log phase intracellular ROS level (obtained from all the cultivations), as shown in Fig. 3. The increase in intracellular ROS level is seen to result in a decrease in the growth rate, which can be represented in a linear fashion as: growth rate = −0.39(intracellular ROS level)+1.18.

5.3. Effect of intracellular ROS levels on specific xanthan gum productivity

Specific xanthan gum levels and ROS types obtained in cultivations in which, H_2O_2 pulses were employed for oxygen supply, in the presence and absence of antioxidants, are given in Table 4; all xanthan gum levels mentioned were obtained at the same time (50 h). With untreated cells, the maximum specific xanthan gum level (3.78 g per g cell) was obtained when grown in standard medium The presence of ascorbic acid significantly reduced the specific gum level to 49% of that obtained in standard medium and, although the presence of tocopherol, either alone or in combination with ascorbic acid, improved the specific gum level, it was lower than that obtained in standard medium. Similar trends were obtained from cultivations in which the antioxidants

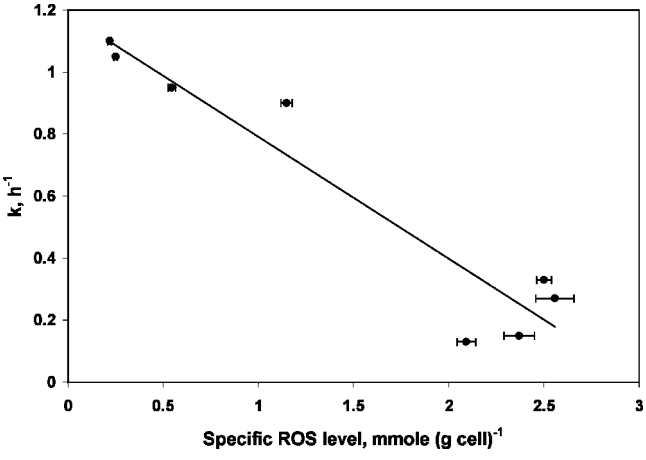

Fig. 3. Correlation between growth rate and specific ROS level in the log-phase.

Table 4
Stationary phase ROS type/levels and specific xanthan gum levels in cultivations with untreated and HOCl-treated cells

Cultivation in	untreated cells		HOCl-treated cells	
	ROS, mmol per g cell	Xanthan gum, g per g cell	ROS, mmol per g cell	Xanthan gum, g per g cell
Standard medium	0.19±0.02 hydroxyl	3.78±0.20	0.34±0.03 hydroxyl	7.00±0.30
+Vit C	1.01±0.07 hydroxyl	1.88±0.20	1.27±0.06 hydroxyl	2.25±0.10
+Vit E	0.13±0.01 superoxide	2.86±0.30	0.40±0.03 hydroxyl	6.40±0.20
+Vit C+Vit E	0.28±0.02 hydroxyl	3.47±0.20	0.59±0.05 hydroxyl	4.70±0.20

The initial concentration of ascorbic acid employed was 5 mM and the concentration of tocopherol employed was 1 mM.

were added in pulses along with H_2O_2, although the reduction extents were lower.

As expected [8], HOCl treatment significantly improved the specific xanthan gum production in all cultivations (Table 4), although the increase varied from 25%, in the cultivation with ascorbic acid, to 124%, in the cultivation with tocopherol. Also, the cultivation with tocopherol provided higher specific xanthan productivity (6.4 g per g cell) than the cultivation with a combination of tocopherol and ascorbic acid (4.7 g per g cell), which was different from that observed with untreated cell cultivations.

The data presented above indicate that the presence of antioxidants does not improve the specific xanthan gum productivity of cells, both untreated and HOCl-treated. However, the overall gum concentration in a cultivation depends on specific gum productivity and the cell concentration and, therefore, it is not surprising that the presence of antioxidants, especially tocopherol, increased the overall xanthan gum concentrations (4.86 g l^{-1} with untreated cells to and 11.01 g l^{-1} with HOCl treated cells) compared with the cultivations in the standard medium (4.16 g l^{-1} with untreated cells and 7.84 g l^{-1} with HOCl-treated cells).

The relationship between the ROS levels in the stationary phase and specific xanthan gum levels (obtained from all cultivations), presented in Fig. 4,

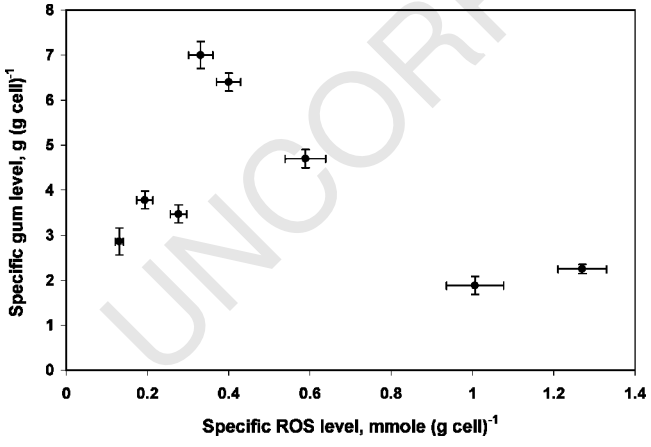

Fig. 4. Correlation between specific xanthan levels and specific ROS level in the stationary phase.

interestingly, suggests that an optimum level exists for the intracellular ROS concentration, at which the specific gum production reaches a maximum value. In the present case, this optimum is seen to be about 0.33 mmol per g cell. Possibly, below this optimal level, the genetic mechanism for free radical mediated increase in xanthan gum production [8] is not fully utilised and above the optimal level, other deleterious effects of ROS such as oxidation of cellular components play a role in decreasing productivity.

5.4. Rheological characteristics of xanthan gum

The importance of xanthan gum in many of its application areas is due to its ability to impart a high viscosity and a shear thinning behaviour to its solutions. The non-Newtonian rheological behaviour of xanthan gum solutions is well known [17], and it can be expressed as a power law relationship:

$$\tau = K|\dot{\gamma}|^n; \quad n < 1 \tag{23}$$

Here τ is the shear stress and $\dot{\gamma}$ is the shear rate. The apparent viscosity, η, at any shear rate, $\dot{\gamma}$ is, therefore, given by:

$$\eta = \frac{\tau}{|\dot{\gamma}|^{n-1}} \tag{24}$$

The degree of shear thinning depends on the value of 'n', the power law index. The value of 'n', the larger the decrease in viscosity for a given increase in shear rate. In this study, the rheology of xanthan gum produced by using the H_2O_2 based oxygen has characterised the supply strategy, with and without antioxidants.

The viscosities of solutions of xanthan gum at 0.1% w/w in water were determined at different shear rates (calculated from the rpm employed) and the results are presented in Table 5. Both at highest and lowest shear rates, gums produced by HOCl-treated cells in H_2O_2-based and conventional cultivation showed the maximum viscosity and gums from cells grown with vitamin C showed the minimum viscosity. Also important is the change in viscosity with shear rate, which depends on the parameter 'n' in the constitutive equation. Logarithmic plots of viscosity versus shear rate were made

Table 5
Apparent viscosities, power law index, n, and flow consistency index, K, of xanthan gum extracted from various cultivations at different shear rates or rpm

Rpm	6	12	30	60		
Shear rate (s^{-1})	7.92	15.84	39.60	79.20		
Cultivation	Apparent viscosity (cP)				n	ln K
Untreated (aeration)	230.0	133.0	65.0	39.5	0.234	7.011
HOCl (aeration)	550.0	188.0	89.0	47.5	0.140	7.628
Untreated (H_2O_2)	250.0	150.0	61.0	35.0	0.130	7.355
HOCl (H_2O_2)	360.0	200.0	92.0	50.0	0.144	7.660
Untreated (H_2O_2+vit C)	150.0	95.0	40.0	25.0	0.200	6.700
HOCl (H_2O_2+vit C)	200.0	120.0	60.0	34.0	0.200	6.896
Untreated (H_2O_2+vit E)	265.0	140.0	61.0	40.0	0.190	7.227
HOCl (H_2O_3+vit E)	275.0	148.0	64.0	45.0	0.194	7.241
Untreated (H_2O_2+vit (C+E))	210.0	117.5	36.0	34.5	0.212	6.957
HOCl (H_2O_2+vit (C+E))	195.0	102.5	56.0	32.0	0.222	6.860

Untreated refers to untreated cells; HOCl refers to HOCl treated cells; aeration implies that oxygen supply was through aeration in the cultivation; H_2O_2 implies that oxygen supply was through need-based pulse additions of H_2O_2; when not mentioned explicitly, the cultivation was carried out in standard medium; when vit C or vit E or vit (C+E) are mentioned, the cultivations were carried out with 5 mM vitamin C or 1 mM vitamin E or a combination of 5 mM vitamin C and 1 mM vitamin E, respectively.

and the values of 'n' and 'K' calculated from the slopes and intercepts of such plots are also tabulated in Table 5. The values of n (<1) clearly show the shear thinning nature of the gum solutions. The larger the value of 'n' (i.e. the closer n is to 1), the less is the shear thinning behaviour. Thus it may be seen that cultivations with hydrogen peroxide (untreated cells) give gums with higher shear thinning characteristics. The HOCl-treated cells give similar gums even in conventional cultivation. It may also be noted that the gum produced by HOCl-treated cells gives higher viscosity solutions in general. Interestingly, the differences in characteristics between gum produced by untreated cells and that produced by HOCl-treated cells are narrowed down by the presence of the antioxidants employed. This probably happened because the SoxRS regulon responsible for the improvement in xanthan gum productivity [8], was activated to a lesser extent in the presence of the antioxidants.

Attempts were made to correlate the rheological characteristics of the xanthan gum produced during the various cultivations with the ROS levels in the intracellular space and/or in the medium. Since the gum is produced inside the cells and secreted out, the final characteristics may reflect an influence of mechanistic aspects (intracellular) or degradation/modification after secretion (say, due to ROS in the medium) or both. However, no clear correlations were obtained. Further work, probably on molecular weight and its distribution, is required to understand ROS effects on gum quality.

6. Conclusions

Intracellular ROS levels during the log and stationary phases in the cultivations of untreated and HOCl-treated *X. campestris* cells (oxygen supply was through H_2O_2 pulses), with and without antioxidants (ascorbic acid and tocopherol), were quantified using spin traps and ESR spectroscopy. The intracellular ROS levels depended on the presence of antioxidants. Further, the observed differences in the growth rate constants and the specific xanthan gum levels were correlated with the specific ROS levels. An optimal value of intracellular specific ROS level was found to exist for specific xanthan gum levels. In addition, a model based on fundamental ROS intracellular reactions was developed to simulate and predict the intracellular ROS levels in the various cultivations of untreated cells. There was a good agreement between the model predicted and experimental values of intracellular ROS. The rheological properties of xanthan gum varied with the conditions of the cultivation.

7. Nomenclature

ArOH	tocopherol (vitamin E)
$ArO^{\bullet}$	tocopherol radical
Cu_i	intracellular copper
Fe_i	intracellular iron
k	growth rate constant (s^{-1})
k^0_{av}	scaled average of the rate constants for the termination reactions ($M^{-1}\,s^{-1}$)
k_{av}	scaled average of the rate constants for the termination reactions in the presence of ascorbic acid and/or tocopherol ($M^{-1}\,s^{-1}$)
k_x	second order rate constant for the reaction, x ($M^{-1}\,s^{-1}$)
K	flow consistency index (kg $(m\,s^{2-n})^{-1}$)
n	power law index
$O_2^{-\bullet}$	superoxide radical

$OH^{\bullet}$	hydroxyl radical
$r_{ini,x}$	initiation rate of species, x (M s^{-1})
$r_{ter,x}$	termination rate of species, x (M s^{-1})
r_{ini}	initiation rate of $Rad^{\bullet}$ (M s^{-1})
r_{ter}	termination rate of $Rad^{\bullet}$ (M s^{-1})
R	ratio of the concentrations of Fe^{2+} to Fe^{3+}
$Rad^{\bullet}$	sum of the ROS species of interest, $OH^{\bullet}$, $O_2^{-\bullet}$ and $ROO^{\bullet}$
$ROO^{\bullet}$	peroxyl radical
ROS	reactive oxygen species
SOD	superoxide dismutase
t	time (s^{-1})
Vit C^-	Ascorbate
Vit	ascorbic radical (semihydroascorbate)
$C^{-\bullet}$	
$[x]$	concentration of intracellular species, x (M)
X	cell concentration (g l^{-1})
X_s	parameter in the logistic model (g l^{-1})
α	constant in the model for intracellular ROS level (M^{-1})
β	constant in the model for intracellular ROS level (M^{-1})
γ	constant in the model for intracellular ROS level (M^{-1})
$\dot{\gamma}$	shear rate (s^{-1})
η	apparent viscosity (cP)
τ	shear stress (N m^{-2})

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