REDOX POTENTIAL AND OXYGEN CONCENTRATION (pO₂) AS CRITERIA FOR THE VALUATION OF THE EFFECTIVENESS AND FOR THE APPLICATION OF DISINFECTANTS

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ABSTRACT

Microbial process control is essential for economic sugar production. In practice, indirect methods for the determination of the microorganisms have been accepted. However, measurable results are produced only if considerable damage has occurred. This paper has examined whether the change in the redox potential is useful for the control, at the earliest possible moment, of microbial infection in the process of sugar manufacture. Yet the main subject of this work is the development and comparison of two methods intended for the estimation of disinfectants. These methods are the determination of the change in both the oxygen consumption and the redox potential. These changes are dependent upon the number and activity of bacteria. A substantial advantage of these methods is the possibility to adapt the testing conditions (temperature 70°C, pH=6.0 regulated, etc.) to the application conditions of the disinfectants in the process (cane diffuser). The lethal concentration was determined only graphically as yet. The comparison of the effectiveness was accomplished for 8 disinfectants with different active groups.

INTRODUCTION

Due to its composition, sugar cane juice offers excellent conditions for microbial growth. As a result, large quantities of sucrose are destroyed and microbial metabolic products are formed that cause difficulties in the ensuing stages of the sugar manufacturing process and reduce the quality of the produced sugar. Thus for an economic sugar production a microbial process control is essential. In principle, a series of methods is available for the determination of the number of micro-organisms (Table 1). In practice, indirect methods for the determination of the micro-organisms are preferred. The decrease in the pH-value, as well as the increase in the concentration of lactic acid¹⁻³, nitrite⁴⁻⁶, invert sugar and gums, serve as indicators for microbial infections (Table 2). However, the quantitative amount of these infections cannot be estimated by the above mentioned indica-
tors. That is to say, the concentration of microbial metabolic products is dependent upon the bacteria species, the temperature, the partial pressure of oxygen and the composition of the medium. Furthermore, the usual methods for the determination of infections only then give measurable results if considerable damage has occurred.

**TABLE 1** Direct methods for the determination of infections.

<table>
<thead>
<tr>
<th>Biomass</th>
<th>volumetrical</th>
<th>gravimetrical</th>
<th>turbidimetrical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct determination of germ number</td>
<td>Thoma hemocytometer</td>
<td>microscopic membrane filter (direct method)</td>
<td>coulter counter</td>
</tr>
<tr>
<td>Culture procedure</td>
<td>plate count with poured plates</td>
<td>membrane filtration</td>
<td>spread with glass spatula (Drigalski)</td>
</tr>
</tbody>
</table>

**TABLE 2** Indirect methods for the determination of infections.

<table>
<thead>
<tr>
<th>Carbon dioxide formation</th>
<th>volumetrical</th>
<th>photometrical</th>
<th>electrochemical</th>
</tr>
</thead>
<tbody>
<tr>
<td>Consumption of oxygen</td>
<td>radiometrical</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$^{14}$CO$_2$ formation with glucose $^{14}$C</td>
<td>enzymatical</td>
<td>photometrical</td>
<td>gas-chromatographical</td>
</tr>
<tr>
<td>Metabolic products (e.g. nitrite, lactic acid, pyruvic acid, gums, invert sugar, pH-value, acetone)</td>
<td>enzymatical</td>
<td>photometrical</td>
<td>gas-chromatographical</td>
</tr>
<tr>
<td>Redox indicators</td>
<td>electrochemical</td>
<td>photometrical</td>
<td></td>
</tr>
</tbody>
</table>
Even if strictest cleanliness is observed, often infections cannot be avoided during milling or extraction and in the ensuing stages of the manufacturing process in tanks and pipes and other equipment. Therefore the use of disinfectants is essential. In the paper at hand, some disinfectants of different chemical structure are to be examined as to their use at an application temperature of 70°C, signifying their effectiveness against thermophilic bacteria. Their occurrence during cane sugar processing has been confirmed3. In order to establish the effectiveness of disinfectants, it is customary to determine the survival rate with solid agar plate experiments. However, it has been recognized that for thermophilic bacteria that grow at a temperature of 70°C, the change from a submersed to a surface culture often shows a lethal effect and this occurs without the addition of disinfectants. Moreover, the metabolic activity of these bacteria may cause the pH value of the medium to fall so rapidly that the lethal effect occasioned by the decrease in the pH value is far greater than that which is brought about by the disinfectants. Consequently, it is not recommendable to incubate thermophilic bacteria with disinfectants in a shaker without having regulated the pH-value. Thus it proved necessary to look for new methods for the determination of the effectiveness of biocides.

According to our examinations, both the update of oxygen and the change in the redox potential, appear to be suitable for testing the effectiveness of disinfectants. These also seem to be methods for infection control i.e. for defining the moment of disinfectant application.

**EXPERIMENTAL PROCEDURE**

**Consumption of oxygen**

Apart from carbon sources, nitrogen sources, salts, etc., oxygen is the main growth factor for aerobic micro-organisms. Dependent on the oxidation state of the medium8 and on the growth phase, the bacterial oxygen consumption rises — the partial pressure of oxygen falls in the medium respectively — as the number of cells increases (Fig. 1). Under defined conditions, the amount of oxygen consumed per gram of microbial dry substance is a constant \( Q_{O_2} \). If the reaction vessel of a measurement system is filled with an oxygen saturated solution, the addition of a bacterial suspension causes a definite decrease in the partial pressure of oxygen. This decrease, which is measured polarographically, depends on the number of physiologically active bacteria.

For our examination, bacteria (treated as described in the section after the next) in a concentration of 1.3 mg dry substance, are transferred to a reaction vessel (7.8 ml; magnetic stirrer) with polarographic oxygen electrodes, which is filled with a nutrient medium (10 g/l Standard 1 nutrient broth, 10 g/l sucrose, pH 6.0, saturated with synthetic air) and kept at a temperature of 70°C. As the disinfectants are examined, these are added to the nutrient medium in a defined concentration. The change in the partial pressure of oxygen that now occurs is plotted.
FIGURE 1. Redox potential in batch culture (schematic). Optical density (OD) and partial pressure of oxygen (pO$_2$) as a function of time.

The oxygen analyzer and the pO$_2$ electrodes of Messrs. Bachofen, Reutlingen (F. R. Germany), are employed as measuring devices.

The measurement of the redox potential is more suitable for the estimation of germs and germinating spores than the testing of specific metabolic products or the increasing optical density (Fig. 3). In a sterile medium, a constant redox potential appears when the pH-value and the partial pressure of oxygen are both constant. At the commencement of the bacterial growth the value of the redox potential goes down. This may be ascertained far earlier than, for instance, the increase in the optical density or the drop in the pH-value. During the stationary phase (batch culture) or within the steady state (continuous culture) the redox potential is constantly low. It is very sensitive to slight changes in the physiological state of the culture. This high sensitivity can be applied, for instance, for regulating continuous fermentative processes.

In the method suggested, we use the change in the redox potential — the amount of which depends on the number of active cells — to determine the lethal effect of disinfectants. The measurement is carried out in a reaction vessel of 50 ml volume, with a magnetic stirrer, at a temperature of 70°C. The reaction vessel is filled with a nutrient medium (10 g/l Standard 1 nutrient broth, 10 g/l sucrose,
pH 6.0, aerated by nitrogen). This also contains the disinfectants that are to be tested in varying concentrations. Furthermore, a known quantity of bacteria (24 mg dry substance) is given into the reaction vessel. The change in the redox potential that appears is plotted. Here the redox electrodes (Type 4805) of Messrs. Ingold, Frankfurt/Main, were used, together with the mV-Meter of Messrs. Knick, Berlin.

**Propagation and preparation of micro-organisms**

The examinations which are described here were conducted with stems of the thermophilic bacterium *B. stearothermophilus*, which had been isolated from the extraction equipment. In order to obtain reproducible results, bacteria were employed from continuous cultures (fermenter) with defined growth conditions (fermenter volume 1600 ml; 70°C, pH 6.0; rate of flow 280 ml/h). The medium for the aerobic fermentation (bacteria employed for measurement of oxygen consumption) consists of 10 g/l Standard I nutrient broth (Merck AG), 10 g/l sucrose and 0.1 ml/l polyethylene glycol as antifoaming agent, which is aerated by pressure air (aeration rate 3 l/min). For the micro-aerophilic fermentation (bacteria used for the determination of the redox potential) the medium contains: 10 g/l Standard I nutrient broth, 10 g/l sucrose and 0.13 g/l sodium nitrate. The bacterial suspension was subjected to an abrupt cooling from 70°C to 0°C. Subsequently, it was centrifuged and washed with buffer solution. It was possible to keep the bacterial cells treated in this manner for 4 hours in an ice-bath, without any evidence of a lethal effect. On the other hand, when exposed to room temperature, the cells died.

**Metabolical-physiological tests**

In order to find a method suitable for infection control, we examined the metabolism of the initial phase of bacterial growth. Thereby we determined the concentration of nitrite (sulphanilic acid, test strips), lactic acid (enzymatically), pyruvate (enzymatically) and other organic acids (gas-chromatographically); the increase in the optical density (photometrically), the change in the partial pressure of oxygen (polarographically) and the redox potential.

**RESULTS AND DISCUSSION**

**Valuation of disinfectants**

The disinfectants which have been tested in these examinations contain different active substances that are shown in Table 3. Both criteria the change in the partial pressure of oxygen and in the redox potential are employed to obtain a statement concerning the effectiveness of the different active groups. The lethal concentration of a disinfectant is determined by the change in the partial pressure of oxygen and in the redox potential in several reaction batches with increasing concentrations of this disinfectant.
The decrease in the partial pressure of oxygen, which occurs in the nutrient medium depends on the cell number (Fig. 2). At a constant cell quantity (dry substance) the altered decrease in the partial pressure of oxygen indicates the effect of the disinfectant-containing medium. If the partial pressure of oxygen is plotted against time, a typical inactivation kinetic is obtained (Figs. 5 and 6). From the course of the inactivation kinetic one may infer the different rates of effect (reaction rate). Whilst quaternary ammonium compounds show an effect that commences immediately, yet progresses slowly (Fig. 5), iodophore possesses an effect that begins decelerated but develops rapidly (Fig. 6).

The process of sugar manufacture requires an immediate effect. For that reason, the effect of the disinfectants is determined after a reaction period of 7 minutes, according to the plotted curve. That concentration of a disinfectant, for which no change can be ascertained in the partial pressure of oxygen after a reaction period of 7 minutes, is declared the lethal concentration. As a characteristic value, the concentration of the disinfectant, which kills a bacterial mass of 1.3 mg dry substance per reaction volume of 170 mg/l, is listed in Table 3.

**TABLE 3.** Active substances and lethal concentrations of disinfectants.

<table>
<thead>
<tr>
<th>Active substance</th>
<th>Concentration of active substance (%)</th>
<th>Lethal concentration (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>by $pO_2$ method for 170 mg/kg bacteria</td>
<td>by redox method for 480 mg/kg bacteria</td>
</tr>
<tr>
<td>thiocarbamate</td>
<td>40</td>
<td>130</td>
</tr>
<tr>
<td>cresol-sulphuric acid mixture</td>
<td>35</td>
<td>400</td>
</tr>
<tr>
<td>biguanide</td>
<td>50</td>
<td>6</td>
</tr>
<tr>
<td>polymere</td>
<td>37</td>
<td>1200</td>
</tr>
<tr>
<td>formaldehyde</td>
<td>2</td>
<td>64</td>
</tr>
<tr>
<td>iodophore</td>
<td>50</td>
<td>11</td>
</tr>
<tr>
<td>quaternary ammonium compound</td>
<td>25</td>
<td>66</td>
</tr>
<tr>
<td>cationic substance</td>
<td>40</td>
<td>106</td>
</tr>
</tbody>
</table>

For the present, we have limited ourselves to a graphical evaluation. A mathematically calculated characteristic factor for the bacteria killing, in dependence upon disinfectants and concentration, is being sought.
FIGURE 2. Oxygen consumption after the addition of different cell quantities (mg dry substance, d.s., in the reaction volume) without disinfectants.

FIGURE 3. Bacillus stearothermophilus in batch culture (schematic). Optical density and redox potential as a function of time.
FIGURE 4. Redox potential after the addition of different cell quantities (mg dry substance, d.s., in the reaction volume) without disinfectants.

FIGURE 5. Oxygen consumption after the addition of a quaternary ammonium compound (mg/kg) at a constant cell quantity (1.3 mg in the reaction volume).
Similar results are obtained by measuring the redox potential. Under defined conditions the value of the redox potential in a solution depends upon the cell number (Fig. 4). If the initial number of cells is constant, the decrease in the redox potential depends upon the lethal effect, i.e. on the concentration of the biocide. Thus the redox potential describes the number of surviving cells. If the redox potential is plotted against time, a typical inactivation kinetic or curve is also obtained, as in the case of the determination of the partial pressure of oxygen. Fig. 7 shows the effect of various concentrations of a disinfectant containing a quaternary ammonium compound as the active substance in the presence of a constant bacterial number (24 mg dry substance per 50 ml reaction volume). Without the disinfectants, a sharp decrease in the redox potential may be seen. In the batches that contain a higher concentration of disinfectants, the decrease in the redox potential is weaker. That concentration of the disinfectant, in which after a reaction period of 15 minutes no further decrease in the redox potential may be noted, is considered a lethal concentration. It is converted to 480 mg/kg bacterial mass (Table 3). Some disinfectants were not estimated with the redox potential, as these also cause a change in the redox potential in the absence of bacteria. If one relates the lethal concentrations of the same disinfectants — obtained from these two methods — to an equal bacterial mass, one will find similar values for the lethal concentrations. For the chosen test conditions it was necessary to apply a higher concentration of the disinfectants than otherwise indicated, in order to achieve a complete bacterial killing. These conditions were deliberate so that they would come
close to those existing in the manufacturing process. The killing was determined after 7 to 15 minutes respectively and not — as frequently done — after 60 minutes (as the reaction period increases, the lethal concentration decreases). The disinfectants were tested on bacteria being in optimum growth conditions. (3) For some disinfectants the chosen temperature and pH-value have not been in their optimum range of effectiveness.

![Redox potential graph](image)

**FIGURE 7.** Redox potential after the addition of a quaternary ammonium compound (mg/kg) at a constant cell quantity (24 mg dry substance in the reaction volume).

Obviously the lethal concentrations of disinfectants that have been noted in Table 3, cannot be the only selection criterion for their application in the process. To make the decision, the concentration of the active substance in the disinfectant, its price, pH and temperature optimum, the hazards involved during handling, its aggressiveness towards materials and the appearance of toxic residues in products and by-products must also be taken into consideration. Yet another selection criterion should be the eventual formation of bacterial resistance against a disinfectant.

These investigations had the aim of comparing the effectiveness of the active substances of disinfectants under the circumstances as they exist in the process and to develop a method that characterizes the physiological status of bacteria, making a ready statement possible.
Applicability of the redox potential for infection control

In the introduction the various possibilities to detect infections have already been described. However, in practice mainly indirect methods are used (e.g. the determination of formed metabolic products; Table 2).

Considering the metabolic products that are suitable for infection control, it showed that their formation and composition are specific for the species and depend on physico-ecological conditions (pH, $pO_2$, redox potential). Furthermore, the infection is a mixed culture.

As is evident from Figs. 8 and 9 (these are batch cultures of *B. stearothermophilus*, subjected to micro-aerophilic or aerobic conditions) the beginning of bacterial growth (both under micro-aerophilic conditions and aerobic conditions) may be determined by the decreasing redox potential approximately 20 minutes earlier than by the rise in the optical density, the drop in the pH value or the formation of lactic acid and nitrite.

**FIGURE 8.** Lactic acid, nitrite, redox potential, and growth (optical density) in a batch culture under aerobic conditions (aerated).
Nevertheless, it must be mentioned that the redox potential is influenced by the pH-value, the temperature and the partial pressure of oxygen. The influence of the oxygen on the measurement of the redox potential may prove to be an alternative method for the determination of lactic acid, nitrite and the pH-value, in the process. This will have to be verified later.

**FIGURE 9.** Lactic acid, nitrite, redox potential, and growth (optical density) in a batch culture under micro-aerophilic conditions (non-aerated).

**FIGURE 10.** Formation of lactic acid ——— and nitrite ——— and change in the redox potential during a continuous culture depending upon aeration.
REFERENCES


