The effect of sulfide on α-glucosidases: Implications for starch degradation in anaerobic bioreactors

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Abstract

Membrane associated α-glucosidase activity was investigated in a methanogenic bioreactor (MR) and a biosulfidogenic bioreactor (SR). Temperature and pH optima studies showed temperature optima of 50 °C and pH optima of 8.0 for the α-glucosidases from both the MR and SR. Sulfide (at a concentration of 150 mg l^{-1}) resulted in the complete loss of all α-glucosidase activity in both the MR and SR. β-Glucosidase activities in our bioreactors were previously shown to be stimulated in the presence of sulfide. α-Glucosidases, in contrast, are inhibited by sulfide. This differential effect of sulfide on α-glucosidase and β-glucosidase activities is highlighted and is of crucial consequence to the respective degradation and utilization of starch and cellulose substrates in natural anaerobic environments and anaerobic bioreactors specifically designed for the accelerated digestion of wastewater sludge under biosulfidogenic conditions.

1. Introduction

Sulfate reducing bacteria (SRB), through the process of sulfate reduction to form sulfide, have proven to be valuable microorganisms in the bioremediation of acid mine drainage, as well as in dramatically increasing the rates of hydrolysis and solubilization of primary sewage sludge by enzymatic action (Pletschke et al., 2002, Whiteley et al., 2002a, Whiteley et al., 2002b, Whiteley et al., 2003a and Whiteley et al., 2003b). SRB are obligate anaerobes making use of inorganic sulfate as an electron acceptor during an ATP driven reaction (Pletschke et al., 2002). SRB utilize the carbon source provided by the hydrolytic and acidogenic bacteria, because they are unable to produce the hydrolytic enzymes necessary for protein, carbohydrate and lipid hydrolysis. Although substantial progress has been made into understanding the effect that sulfide has on enhanced sludge solubilization with respect to increased enzymatic activity, the underlying enzymology remains obscure.

Wastewater sludge consists mainly of microorganisms surrounded by large macromolecules (Nielsen et al., 1992 and Raunkjear et al., 1994). Microorganisms utilize extracellular enzymes to hydrolyze these large macromolecules to monomers, which are then easily assimilated for their cellular metabolism. These extracellular enzymes can originate from influent sewage or from microorganism secretions into the surrounding environment (Frølund et al., 1995). The enzymes are membrane bound, dissolved in water or found absorbed to a surface other than that of the producers (Chróst, 1991).

The enzymatic hydrolysis of large substrates to their smaller monomers is the rate-limiting step in anaerobic digestion, due to the fact that enzymatic hydrolysis is a relatively slow procedure. The rate of hydrolysis will be affected by differing electron acceptor conditions (Goel et al., 1998). Conflicting reports on the hydrolysis rates occurring under the different electron acceptor conditions are available. Some reports conclude an elevated hydrolysis rate under aerobic conditions (Dold et al., 1980 and Henze and Mladenovski, 1991), while others conclude that similar hydrolysis rates occur under anaerobic conditions (Kristenten et al., 1992, Mino et al., 1995 and San Pedro et al., 1995). Under anaerobic conditions significant sludge floc matrix degradation occurs due to increased enzymatic hydrolysis of sludge proteins and carbohydrates (Nielsen et al., 1996). The presence and activity of α-glucosidases and in some cases, β-glucosidases, has been demonstrated in several systems, including an activated sludge sequencing batch reactor (Goel et al., 1998), in an activated sludge floc matrix (Frølund et al., 1995), activated sludge and anaerobic hydrolysis sludge from a pilot scale plant (Nybroe et al., 1992), biofilms (Roth and Lemmer, 1994), and whole and dispersed activated sludges (Cadoret et al., 2002). Cadoret et al. (2002) reported that 5% of the α-glucosidase activity was associated to the extracellular polymeric substances (EPS) from the flocs.

The primary aim of this study was to confirm the presence and to determine the activity of α-glucosidases in the sludge obtained from the SR (containing both SRB and "methane producing prokaryotes") and a control MR (containing only "methane producing prokaryotes"). pH and temperature optima for the α-glucosidases were determined. Results for α-glucosidase activity in the bioreactors in this study were compared to results previously obtained for β-glucosidase activity under similar conditions of high sulfate concentration (Watson et al., 2004). In addition, we also assessed the effect of selected divalent metal ions on the activity of the α-glucosidases in the bioreactor, as these compounds are often present in anaerobic sludge bioreactors.

2. Material and methods

2.1. Reactor design and sludge collection

Two different bioreactor systems were used during the course of this study, a 20 l SR that was continuously stirred, and a 10 l MR. The continuous bioreactors were set up as described by Whiteley et al. (2002b). Primary sludge was collected from the anaerobic digester at the Grahamstown Municipal Sewage Works, Grahamstown, South Africa. Two reactors were set up in glass tanks at room temperature (25 ± 2 °C) as follows: The MR (10 l), seeded with a 10% inoculum of methanogenic sludge (also obtained from the anaerobic digester at the Grahamstown Municipal Sewage Works, Grahamstown, South Africa) served as a control reactor, and was used to monitor the normal hydrolysis of polymeric compounds in primary sludge to methane in the absence of sulfate reducing prokaryotes and sulfate. The SR (20 l) served as the experimental reactor and was seeded with a 10% inoculum of sulfate reducing prokaryotes. The sulfidogenic reactor was fed with primary sludge (sieved through a 2 mm mesh sieve, and diluted to a COD (chemical oxygen demand) of 2000 mg l^{-1}, sulfate was added to a concentration of 2000 mg l^{-1}, i.e. a $COD : SO_4^{2-}$ ratio of 1:1 was maintained). The hydraulic retention time (HRT) was maintained at 2 d (mean organic loading rate of 0.0588 kg COD l^{-1} d^{-1}), and the overflow from the SR was allowed to settle by gravity to yield particulate material (containing a high number of sulfate reducing prokaryotes. This particulate material was pumped back to the reactor once a week to minimize the washing out and subsequent loss of sulfate reducing prokaryotes from the SR.

The VSS (volatile suspended solids) determination of the sludge was determined as described in Watson et al. (2004).

2.2. Enzyme assays

A modified version of the α-glucosidase assay as described by Goel et al. (1998) was used. An aliquot of 0.5 ml 0.2 M Tris–HCl (pH 7.6) was added to 0.25 ml of 0.1% (w/v) *p*-nitrophenyl-α-D-glucopyranoside, and the reaction was initiated by the addition of 0.25 ml of sludge. The reaction tube was incubated at 37 °C for 60 min and the reaction terminated by boiling of the samples for 5 min. The samples were centrifuged (3000*g*, 1 min) at room temperature (20 ± 2 °C). An aliquot of 0.25 ml was removed from the assay mixture for microtiter plate analysis (PowerWave$_x$, Bio-Tek Instrumentation, Inc.) at 410 nm. Suitable controls were performed where the sludge was substituted with boiled sludge to remove all enzyme activity. All enzyme activities were expressed as μmol min^{-1} g VSS^{-1}.

2.3. Fractionation studies

MR and SR sludge samples (10 ml) were centrifuged (1811*g*, 10 min) at 20 ± 2 °C. The supernatant was decanted and the pellet was washed three times with 10 ml distilled water and sonicated (Virtis Sonicator, power output of 37 W in ice for 2 intervals of 30 s each) followed by a final centrifugation step. The samples taken directly from the bioreactors, as well as all supernatants, pellets, sonicated supernatants and sonicated resuspended pellet samples were assayed for α-glucosidase activity.

2.4. pH and temperature studies

The effect of pH and temperature on α-glucosidase was determined using the re-suspended pellet fractions from both MR and SR. Sludge (10 ml) from both bioreactors was centrifuged (3000*g*, 10 min) at 20 ± 2 °C and re-suspended in 10 ml distilled water. For the determination of the temperature optimum, 0.25 ml of re-suspended pellet fraction was added to 0.5 ml of 0.2 M Tris–HCl (pH 7.6) and 0.25 ml of substrate and incubated at varying temperatures (25–75 °C) for 1 h. The samples were boiled for 5 min to terminate the reaction. In order to determine the pH optimum, 0.25 ml of the re-suspended pellet samples were added to 0.25 ml of substrate and 0.5 ml buffer of varying pH. The following range of buffers (0.1 M) was used: sodium acetate (pH 4–4.5), sodium phosphate (pH 6–6.5), Tris–HCl (pH 7–9), carbonate/bicarbonate (pH 9.5–10). The samples were incubated at 37 °C for 60 min and the enzymatic reactions terminated by boiling the samples for 10 min. The samples were centrifuged (3000*g*, 1 min) at 20 ± 2 °C and microtiter plate analysis of the enzymatic reaction at 410 nm was performed. Suitable controls were performed for each temperature and pH, by substituting the sludge containing the enzyme with boiled sludge (denatured enzyme). MR and SR sludge was incubated at 4 and 50 °C over a 3 h period to determine the temperature stability of the α-glucosidases. Samples (0.25 ml) were removed at 10 min intervals and the α-glucosidase assay was performed.

2.5. Effect of sulfate, sulfite, and sulfide on α-glucosidase activity

The effect of sulfate, sulfite and sulfide (up to 1000 mg l^{-1}) on α-glucosidase activity in MR and SR sludge was investigated. The effect of sulfide was further investigated between 0 and 200 mg l^{-1}. The effector (0.125 ml) was pre-incubated with 0.125 ml sludge from either the MR or SR for 30 min at 37 °C, before the subsequent addition of 0.5 ml buffer and 0.25 ml substrate to initiate the reaction. As before, suitable controls for each test sample were performed, substituting the sludge with boiled sludge. Suitable controls were also performed to account for the effect of the sulfate, sulfide, and sulfite on the α-glucosidase assay components by substituting each of the sludge components with buffer. Sulfate, sulfite and sulfide were reacted with the sludge samples in the dark with minimal headspace to prevent oxidation.

2.6. Effect of heavy metals on α-glucosidase activity

Stock concentrations of divalent metal ions such as iron, zinc, magnesium, lead, copper and nickel were diluted with 0.1 M Tris–HCl (pH 7.6) to obtain final soluble metal concentrations of 0–250 mg l^{-1}. The effect of these metal ions on α-glucosidase activity was determined, by pre-incubating 0.125 ml of the metal ion solution with 0.125 ml of sludge obtained from either the MR or SR for 30 min at 37 °C. The reaction was then started by the addition of 0.5 ml buffer and 0.25 ml of substrate. After termination of the reaction by boiling, the samples were centrifuged (3000*g*, 1 min) at 20 ± 2 °C and the absorbance was monitored in a microtiter plate reader at 410 nm. Suitable controls were performed to assess the effect of the metal ions on the α-glucosidase assay components and reaction pH by replacing the sludge with 0.125 ml of buffer.

3. Results and discussion

3.1. Fractionation studies

α-Glucosidase activity in the MR was highest in the pellet (2.11 μmol min^{-1} g VSS^{-1}) compared to the highest activity observed in the SR sonicated resuspended pellet (1.66 μmol min^{-1} g VSS^{-1}). Taking into consideration the volume of sludge used from both reactors for each fractionation procedure, the highest total activity measured in both the MR and SR sludge was localized in the pellet fraction (10.6 μmol min^{-1} g VSS^{-1} and 7.5 μmol min^{-1} g VSS^{-1}, respectively). These results indicated that the α-glucosidases are membrane bound enzymes in the MR and SR, which are mainly present in the pellet fraction. Sonication resulted in the release of the α-glucosidases into the supernatant. However, some activity was lost during the sonication due to denaturation of the enzymes. Watson et al. (2004) previously also reported that the β-glucosidase activity was mainly localized in the pellet fraction for both MR and SR sludge samples, an indication that β-glucosidases were also membrane bound.

3.2. pH optima studies

The effect of pH on the α-glucosidases was determined over a pH range of 4–10. α-Glucosidases in both the MR and SR sludges exhibited a pH optimum of 8.0. The MR and SR were operated at a pH of approximately pH 6–7 and pH 7–8, respectively. The bioreactors were therefore well suited for maximal α-glucosidase production and optimal enzymatic activity. The temperature optima for MR and SR α-glucosidases were determined between 25 and 70 °C. α-Glucosidases in both the MR and SR exhibited a temperature optimum of 50 °C (see Fig. 1). Both α-glucosidases in the MR and SR samples rapidly lost activity above 50 °C, which compared favorably with the results obtained for the β-glucosidases (Watson et al., 2004). α-Glucosidase activity was stable for up to 3 h of incubation at both 4 and 50 °C in both the MR and SR. This indicated to the fact that α-glucosidases were very stable for extended periods of time over a wide temperature range up to 50 °C. Apparent activation energies for the α-glucosidases obtained from the MR and SR were calculated (using Arrhenius plots) to be 14.729 and 23.159 kJ mol^{-1}, respectively. Kumar and Satyanarayana (2003) reported an activation energy of 43 kJ mol^{-1} for a raw starch-hydrolyzing, thermostable, neutral α-glucosidase from *Thermomucor indicae-seudaticae*.

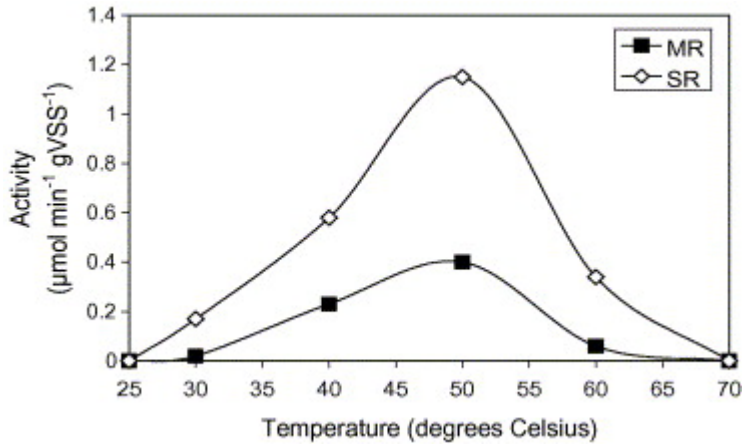

Fig. 1. Temperature optimum profile of α-glucosidase activities in the MR and SR sludge samples.

3.3. The effect of sulfate, sulfite and sulfide on α-glucosidase activity

MR α-glucosidase activity was maximal in the absence of sulfate and the activity generally decreased as the concentration of the sulfate was increased (Fig. 2a). Sulfate therefore appeared to be an inhibitor of α-glucosidase activity. Sulfite at an optimal concentration of 600 mg l^{-1} sulfite led to a 4.5-fold increase in MR α-glucosidase activity compared to when no sulfite was added (Fig. 2b). Sulfide was a potent inhibitor of MR α-glucosidase activity (Fig. 3). All MR α-glucosidase activity was lost upon the addition of 150–200 mg l^{-1} sulfide (Fig. 3), and we propose the hypothesis that sulfide blocks the enzymatic action of α-glucosidase by reacting with the α(1 → 4)-glucosidic bond on the maltose ring that is required for cleavage by α-glucosidases. This hypothesis is supported by the following evidence: inhibition of α-glucosidase activity by sulfide was reversed by the displacement of sulfide from the substrate by incremental addition of the substrate. A similar pattern was observed for SR sludge where the sulfate also caused the inhibition of SR α-glucosidase activity at values of 1000 mg l^{-1} (Fig. 2a). Sulfite had little or no effect on SR α-glucosidase activity (Fig. 2b). This variation in α-glucosidase activity does not seem to be related to the concentration of sulfite. This observation may, in part, be explained by the fact that sulfite is rapidly removed and converted to sulfide, after it has been produced by the action of the enzyme APS reductase. The addition of sulfide (150–200 mg l^{-1}) again resulted in a complete loss of all SR α-glucosidase activity and proved to be a strong inhibitor of SR α-glucosidase activity (Fig. 3). In this previous study, an optimal sulfide concentration of 600 mg l^{-1} sulfide acted as a major activator of β-glucosidase activity (Watson et al., 2004). Sulfide levels in our SR normally attain a steady state concentration of around 600 mg l^{-1}.

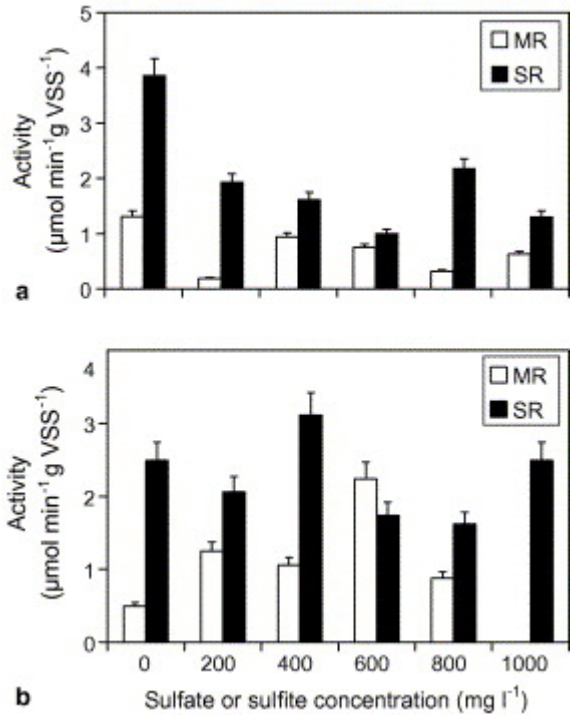

Fig. 2. The effect of (a) sulfate and (b) sulfite on α-glucosidase activity. Sulfate or sulfite (0.125 ml of varying sulfate or sulfite concentration) was pre-incubated with 0.125 ml sludge from either the MR or SR for 30 min at 37 °C, before the subsequent addition of 0.5 ml buffer and 0.25 ml substrate to initiate the reaction. Values represent means ± SD (n = 3).

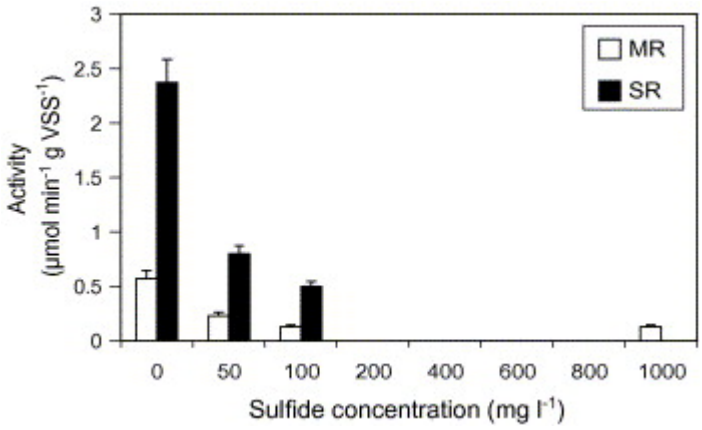

Fig. 3. The effect of sulfide on α-glucosidase activity between 0 mg l^{-1} and 1000 mg l^{-1}. Sulfide (0.125 ml of varying sulfide concentration) was pre-incubated with 0.125 ml sludge from either the MR or SR for 30 min at 37 °C, before the subsequent addition of 0.5 ml buffer and 0.25 ml substrate to initiate the reaction. Values represent means ± SD (n = 3).

3.4. The effect of divalent metal ions on α-glucosidases

All metal ions tested showed an inhibitory effect on both MR and SR α-glucosidase activities (Fig. 4a and b). A sharp decrease in activity was generally observed at 50 mg l^{-1}, while the degree of reduced activity remained relatively constant between 50 and 250 mg l^{-1} of metal ion. The most potent inhibitors of the α-glucosidases in both the MR and SR sludge samples proved to be copper, zinc and lead, with lead leading to the greatest reduction in α-glucosidase activity- a decrease of 66.6% and 70.3% in the activity for MR and SR sludge, respectively (at a concentration of 50 mg l^{-1}). Magnesium was the least inhibitory to α-glucosidase activity, leading to a 28% and 37.5% decrease in MR and SR α-glucosidase activities, respectively. The effect of Fe^{2+}on α-glucosidase could not be assessed, as it was essentially insoluble in the medium and interfered with the assay and its components. Iron (Fe^{3+}) is often used as a flocculation agent and in the presence of sulfide is reduced from $Fe^{3+}$to Fe^{2+} to form FeS (Nielsen et al., 1992).

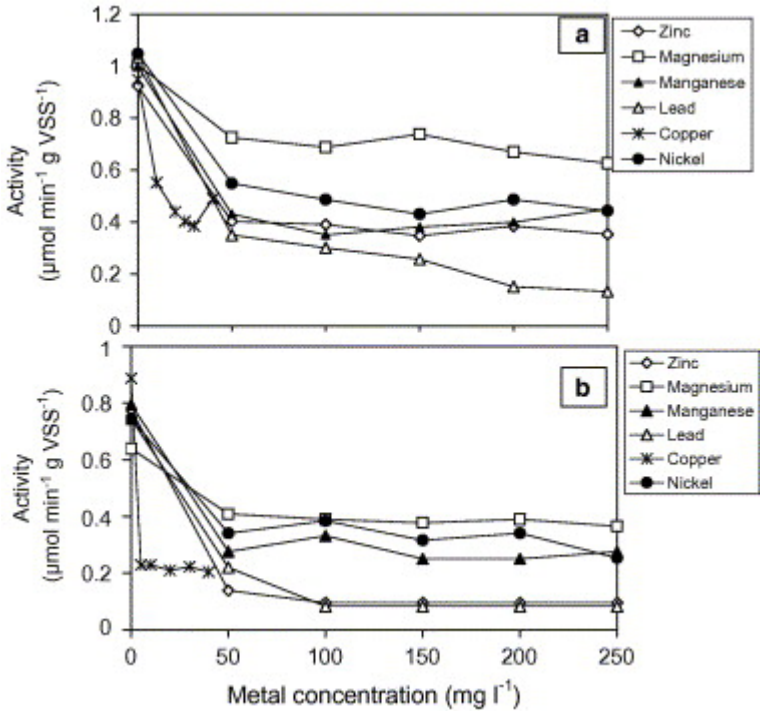

Fig. 4. (a) The effect of divalent metal ions on (a) MR α-glucosidase activity and (b) MR α-glucosidase activity between 0 and 250 mg l^{-1}. The divalent metal ions (0.125 ml of varying concentration) were pre-incubated with 0.125 ml sludge from the MR or SR for 30 min at 37 °C, before the subsequent addition of 0.5 ml buffer and 0.25 ml substrate to initiate the reaction. Values represent means ± SD (n = 3).

4. Conclusions

The polysaccharides cellulose and starch are found in abundance in nature, and are both hydrolyzed to the monosaccharide glucose. Cellulose, the structural polysaccharide of plant cell walls, is a linear polymer of β (1 → 4)-linked D-glucose residues. In contrast, starch, the food storage polysaccharide of plants contains a mixture of the linear α(1 → 4)-linked glucan α-amylose and the α(1 → 6)-branched and α(1 → 4)-linked glucan amylopectin (Voet and Voet, 2004). The strong β-glucosidic bonds, which are present in cellulose, protect the cellulose from hydrolysis by animals and thus cellulose constitutes a large portion of the waste products found in sewage sludge. The cellulose is degraded by the exoglucanases, endoglucanases and the β-glucosidases secreted by the hydrolytic or fermentative bacteria. An increase in sludge solubilization under anaerobic (sulfidogenic) conditions can be related back to the enhanced enzymatic rates of hydrolysis. β-Glucosidase was postulated to be a key enzyme in the increased hydrolysis observed under biosulfidogenic conditions, as it most probably reflects the proportional availability of starch and cellulose as substrates in our anaerobic bioreactors specifically designed for the accelerated treatment of waste water sludge. We therefore propose that the sulfide inhibition of the α-glucosidases is not significant enough to affect the sludge solubilization rates under sulfidogenic conditions, but rather that the solubilization of sludge is increased, due to the stimulatory effect of sulfide on the activity of the β-glucosidases.

Newton et al. (1998) investigated the ecological and physiological effects of the sulfate reducing bacterium *Desulfovibrio desulfuricans* on other intestinal organisms in anaerobic chemostats, and reported that the metabolic activities of saccharolytic organisms were altered in a sulfate reducing bacterium chemostat (sulfide concentration of 265 mg l^{-1}). These altered metabolic activities included the synthesis of a number of hydrolytic enzymes involved in carbohydrate breakdown, such as α-galactosidase, α-glucosidase, β-galactosidase and several other mucinolytic enzymes. Saccharolytic organisms growing in the fermenter appeared to utilize more starch and less galactose-containing polymers, which correlated with the observed glycosidase activities. In our study; however, the anaerobic organisms appeared to produce enzymes capable of hydrolyzing both cellulose (α-glucosidase) and starch (β-glucosidase) substrates. However, the sulfide produced (up to 600 mg l^{-1}) was highly inhibitory to the α-glucosidases produced.

The actual mechanism by which sulfide is able to activate the activity of the β-glucosidases (whilst inhibiting the activity of the α-glucosidases) is not clear at present. Presumably, the sulfide molecule is able to participate in a chemical reaction with the α(1 → 4) bonds in maltose (thereby preventing enzymatic access to the maltose), but not with the β(1 → 4) bonds in cellulose. Previously, Watson et al., 2004 and Whiteley et al., 2002b have proposed a hypothetical model that favors the direct activation (or inactivation) of the glucosidase(s) and other hydrolases on the floc surface. This, in turn, results in the increased (or decreased) rates of reduction of the overall integrity of the floc structure, exposing more or less substrate for these enzymes, leading to further synergy between the sulfide and the enzymes. Further studies will focus on a study of the purified enzymes in the absence and presence of sulfide as activator or inhibitor.

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