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The relative effects of some elements on the DNS method in cellulase assay

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ABSTRACT: For evaluating the relative effects of some polluting salts on the measurement of cellulase activity assayed by 3,5-dinitrosalicylate (DNS) this study was done. Glucose and cellulase solutions have been treated with salts. Exoglucanase and endoglucanase were assayed by 3,5-dinitrosalicylate reagent. Measurement of reducing sugar by DNS indicator in the presence of Ca^{+2}, Ba^{+2}, Fe^{+3}, Mn^{+2}, Pb^{+2}, Fe^{+2}, Ag^{+1}, Zn^{+2}, Co^{+2}, and Al^{+3} salts overestimated and in the presence of Mg^{+2}, Cu^{+2}, Cd^{+2}, and Hg^{+2} salts underestimated the real contents. The intensity of DNS color and/or the reducing power of glucose increased by decreasing radius of hydrated cation of alkaline-earth elements (from Mg^{+2} to Ba^{+2}) in glucose solution. Exoglucanase and endoglucanase activities increased in the presence of Na^{+}, K^{+}, Ca^{+2}, Ba^{+2} and Mn^{+2} salts and decreased in the presence of NH_4^{+} and Mg^{+2} salts. Among the trace elements studied, Fe^{+3}, Pb^{+2}, Fe^{+2}, Ag^{+1}, Zn^{+2}, Co^{+2}, and Al^{+3} were the most effective inhibitors of cellulase activity. These ions inhibited exoglucanase more than endoglucanase. The effect of most ions on cellulase activity may be related to negative or positive effect of them on the method of cellulase assessment. So, for a better data interpretation in the study of ion effects on the soil enzyme activity, both the effects of ions on the method of enzyme assay, and the effects of ions on the enzyme activity should be studied. @JASEM

Cellulase is an enzyme system that degrades cellulose and releases reducing sugars as the end products. The system consists of endo-l,4-B-glucanase (EC3.2.1.4), exo-I,4-B-glucanase (EC 3.2.1.91) and B-D-glucosidase (EC 3.2.1.21). Endo- α -1,4-glucanase (1,4-α-D-glucan-4-glucan-hydrolase) has the role of randomly degrading α-1,4-glucosidic bonds from the middle of the cellulose molecule. It does not attack cellobiose but hydrolyzes cellodextrines and substituted celluloses, for instance carboxymethylcellulose. Its specificity is moderate, different sub-types having various affinities for different length oligosaccharides. Exo-β-1,4-glucanase (1,4- β -D-glucan cellobiohydrolase) cuts step by step cellobiose units from the non-reducing end of cellulosic chains. It hydrolyses the cellodextrines but not the cellobiose. Its substrate specificity is rather high enabling it to degrade more than 80% from the crystalline cellulose while its degree of activity is different for different microorganisms.

α-Glucosidase (α-D-glucoside glucohydrolase) hydrolyzes both cellobiose and cellooligosaccharides in glucose. It is not able to degrade either cellobiose or cellodextrines with high molecular weigh but favorites this process by removing cellobiose and this way, diminishing the cellobiose accumulated in the medium. Such accumulation could inhibit, by feed back, the activity of endo- and exoglucanase. The capacity of degrading the natural cellulose involves the biosynthesis of the whole enzyme system (Nevalainen and Penttil, 1995; Lee and Fan, 1980).

Soil enzymes were found to discriminate between land management practices and to evaluate waste disposal on lands, and therefore appear to be useful for monitoring changes in soil over time (Dick, R.P., 1997; Bandick and Dick, 1999; Badiane et al, 2001). There is considerable documentation on the activities of many enzymes inhibited by trace elements. The activities of the enzymes investigated include α -glucosidase (Tyler, 1974), urease (Tyler, 1974; Tabatabai, 1977), phosphatase (Tyler, 1974; Juma and Tabatabai, 1977), arylsulfatase (Al-Khafaji and Tabatabai, 1979), nitrate reductase (Fu and Tabatabai, 1989), cellulase (Deng and Tabatabai, 1995), and cellulase and α -glucosidase (Geiger et al, 1998). In some studies salts and trace elements are added to soils, after time of equilibration, enzyme activities are assayed. In some other studies enzyme activity are assayed in salts or trace element affected soils. However there are many factors in soils can change both the results of colorimetric determination, and enzyme activity.

Dinitrosalicylic acid, potassium ferric hexacyanid (Prussian blue) and Somogyi-nelson (molybdenum blue) are three common methods generally used for colorimetric determination of reducing sugars. The later two methods are more sensitive than first one. However many of trace element e.g. Ag^{+1}, Ba^{+2}, Cd^{+2}, Cr^{+3}, Cu^{+2}, Fe^{+3}, Hg^{+2}, Zn^{+2} and ... can effect on their results (Deng and Tabatabai, 1994a,b). Dinitrosalicylic acid method is more suitable for measuring high concentration of reducing sugars in solutions. It is not sensitive as high as potassium ferric hexacyanid (Prussian blue) and Somogyi-nelson

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(molybdenum blue) methods. This study was done to evaluate the relative effects of some environmental polluting elements on the activities of cellulase assayed by 3,5-dinitrosalicylate reagent (Mandel and Weber, 1969; Miller, 1959). Our main objective, was to demonstrate the disruptive effects of common and trace elements on both the colourimetric determination of reducing sugars, and cellulase activity.

MATERIAL AND METHODS

For study the effect of elements on the ability of glucose in reducing of DNS, a solution with 1.5 gl^{-1} glucose in citrate buffer (pH=4.8) was prepared. Except the blank solution, glucose solution was treated with common and trace element salts. The final concentration of salts added to citrate buffer solution of glucose was 0.04 N (~EC=4 dSm^{-1} that is near to EC_e of saline soils). This low pH inhibits salts to precipitate. Cations of Na^+, K^+, NH_4^+, Ca^{+2}, Mg^{+2}, Ba^{+2} were added as the chloride, sulfate, bicarbonate, nitrate, phosphate, molibdate or borate. The trace elements Fe^{+2}, Mn^{+2}, Hg^{+2}, Pb^{+2}, Co^{+2}, and Al^{+3} were added as the chloride; Fe^{+2}, Mn^{+2}, Zn^{+2}, Cu^{+2}, Al^{+3}, Cd^{+2} and Ag^{+1} were added as the sulfate; Pb^{+2}, and Co^{+2} as the nitrate. After 30 min of equilibration, the ability of glucose in reducing of DNS in the presence of these elements was assayed by addition of 3,5-dinitrosalicylate reagent (Miller, 1959). The result of glucose measurement in salt treated solutions was compared with the result of glucose measurement in the blank solution untreated with salts.

Fluca prepared cellulase (with 1.4 Uml^{-1} activity) was used in this study. The concentration of cellulase was 0.2 gl^{-1} in citrate buffer solution (pH=4.8) . Same as glucose solution, enzyme solution was treated with common and trace elements. Final concentration of salts added to cellulase solution was also 0.04 N. After 30 min of equilibration, cellulase activity was assayed. The result of cellulase assessment in salt treated solutions was compared with the result of cellulase assessment in the blank solution untreated with salts.

Endoglucanase (CMCase, EC 3.2.1.4), and exoglucanase or cellobiohydrolase (FPase, EC 3.2.1.91) were assayed for the release of reducing sugar from carboxymethyl cellulose (CMC) and filter paper (Whatman No.1), respectively. The reaction mixture for reducing sugar assay contained (total volume 2ml) 0.5 ml enzyme solution, 1.5 ml buffer 0.05 M citric acid (pH 4.8), and 0.05 g substrate. After 1-hour incubation at 50 ^oC, the reaction was stopped by the addition of 2 ml of 3,5-dinitrosalicylate reagent. The resulting mixture was boiled for 15 minutes and reducing sugar content measured by absorbance at 575 nm. CMCase and FPase activities were expressed as micromoles of glucose released per minute (international unit) per ml of culture extract (Mandel and Weber, 1969; Miller, 1959).

The Results of glucose measurement and cellulase assessment obtained from salt-treated solutions were compared with that obtained from their respective blank solutions (untreated with salts). The percentage of overestimate or underestimate of glucose concentration and the percentage inhibition of cellulase activity by each element was calculated from (S-B)/B*100, where S is the glucose concentration or cellulase activity of salt-treated solution and B is the glucose concentration or cellulase activity of the untreated solution. Results reported here are averages of duplicate assays.

RESULTS AND DISCUSSION

Results of the effect of common salts on cellulase assays are shown in Table 1. Results showed that, with few exceptions, most of the salts studied influenced the DNS method in the assay of cellulase activity. The intensity of DNS color and/or the reducing power of glucose in this method increased by addition of salts of Na^+, K^+, and especially Ca^{+2} and Ba^{+2}. In contrast, the intensity of DNS color decreased by addition of NH_4^+ and surprisingly Mg^{+2} salts. The intensity of DNS color increases by increasing the radius of alkaline-earth elements from magnesium to barium. So, dependent on soil salinity and type of salts in soil, the measured soil cellulase activity may be misrepresented. Measurements of reducing sugar in the presence of Ca^{+2} and Ba^{+2} salts by DNS indicator with a more intense color, overestimated and in the presence of Mg^{+2} salt with a less intense color, underestimated the real contents.
Salts with same cation and different anions had a different effect on the intensity of DNS color changed by glucose. From table 1 it can be concluded that negative effect of anions is in the order of $MoO_4^{=}$< $SO_4^{=}$< Cl^-< HCO_3^-< NO_3^-< $HPO_4^{=}$< $B_4O_7^{=}$. Exoglucanase activity increased in the presence of Ca^{+2} and Ba^{+2} salts, but decreased in the presence of Na^+, K^+, NH_4^+ and Mg^{+2} salts. Deng and Tabatabai, (1995) by treating three different soil with trace elements have shown that Ba^{+2}, Co^{+2}, Mn^{+2}, Ni^{+2}, and W^{+4} against other trace elements inhibited cellulase activity very low. However these results (as shown before) may be related to the effect of these cations on the methods of cellulase assessment. Unfortunately, these effects are not separated by colorimetric methods.

Endoglucanase activity has increased in the presence of Na^+, K^+, Ca^{+2}, and Ba^{+2} salts and decreased in the

presence of NH_4^+ and Mg^{+2} salts. Generally, ions inhibited endoglucanase lower than exoglucanase.

Table 1 Effect of different salts on determination of glucose and cellulase activity *

Salts	Percentage of inaccuracy in glucose determination	Percentage inhibition of cellulase activity	
		Exoglucanase	Endoglucanase
NaCl	3.5	-20.1	11.9
KCl	0.0	-17.3	20.5
$MgCl_2$	-27.7	-24.6	-21.3
$CaCl_2$	30.7	14.6	29.8
$BaCl_2$	43.4	41.9	28.3
NH_4Cl	-13.8	-26.9	-15.3
Na_2SO_4	6.9	-22.5	0.0
K_2SO_4	0.0	-31.2	8.7
$MgSO_4$	-20.8	-31.7	-25.7
$CaSO_4$	27.7	-11.0	18.4
$(NH_4)_2SO_4$	-6.9	-28.7	-21.3
$NaNO_3$	-6.9	-16.9	13.2
KNO_3	3.5	-20.2	12.6
$Mg(NO_3)_2$	-20.8	-34.8	-22.7
$Ca(NO_3)_2$	-17.3	-36.1	13.1
NH_4NO_3	-24.2	-37.7	-14.9
Na_2HPO_4	-12.5	-17.6	-20.7
K_2HPO_4	-12.5	-16.9	-3.7
$NaHCO_3$	-3.5	-28.2	-18.6
Na_2MoO_4	6.9	-23.2	-10.8
$Na_2B_4O_7$	-59.9	-60.1	-34.4

**Each data was calculated by (S-B)/B*100, in which: B is glucose concentration or cellulase activity measured by DNS method in the blank solution of glucose or cellulase (untreated with salts), and S is glucose concentration or cellulase activity measured by DNS method in the sample solution of glucose or cellulase (treated with a salt).*

In saline soils exoglucanase may be produce by microorganisms, but soil salinity may has a higher effect on exoglucanase activity than endoglucanase activity. The relative effectiveness of the trace elements in inhibition of cellulase activity varied considerably, depending on the type of trace element applied. Among the trace elements studied, Cu^{+2}, Cd^{+2}, and Hg^{+2} had negative effect on the intensity of DNS color and/or the reducing power of glucose. Against them, Fe^{+3}, Mn^{+2}, Pb^{+2}, Fe^{+2}, Ag^{+1}, Zn^{+2}, Co^{+2}, and Al^{+3} had positive effect on the intensity of DNS color and/or the reducing power of glucose (table 2).

Exoglucanase and endoglucanase activities only in the presence of Mn^{+2} salts increased. In Deng and Tabatabai (1995) studies, Mn^{+2} was the least inhibitor of cellulase activity assayed by the Prussian blue method. However a part of inhibitory effect may be related to the specific effect of elements on the method of enzyme assessment. Among the trace elements studied, Fe^{+3}, Pb^{+2}, Fe^{+2}, Ag^{+1}, Zn^{+2}, Co^{+2}, and Al^{+3} were the most effective inhibitors of cellulase activity. Sensitivity of exoglucanase to trace elements was higher than that of endoglucanase. Although, inhibition percentages of Hg^{+2}, Cu^{+2}, and Cd^{+2} were relatively high but these may be related to negative effect of them on the method of cellulase assessment.

Table 2- Effect of trace elements on determination of glucose and cellulase activity *

Salts	Percentage of inaccuracy in glucose determination	Percentage inhibition of cellulase activity	
		Exoglucanase	Endoglucanase
$FeCl_3.6H_2O$	90.6	-53.6	-42.4
$MnCl_2.4H_2O$	46.0	42.7	16.8
$HgCl_2$	-79.1	-88.0	-73.0
$PbCl_2$	2.3	-27.2	-29.7
$CoCl_2.6H_2O$	16.0	-49.7	-6.4
$AlCl_3$	3.9	-60.9	-43.8
$FeSO_4.7H_2O$	52.5	-17.9	-6.8
$MnSO_4.4H_2O$	69.5	63.1	37.8
$ZnSO_4.7H_2O$	16.9	-64.5	-20.2
$CuSO_4.5H_2O$	-24.0	-77.1	-39.9
$Al_2(SO_4)_3.18H_2O$	3.9	-65.7	-54.8
$3CdSO_4.8H_2O$	-17.5	-53.1	-46.1
Ag_2SO_4	27.4	-52.9	-43.4
$Pb(NO_3)_2$	59.8	-52.2	-45.4
$Co(NO_3)_2.6H_2O$	16.0	-39.5	-7.7

**Each data was calculated by (S-B)/B*100, in which: B is glucose concentration or cellulase activity measured by DNS method in the blank solution of glucose or cellulase (untreated with salts), and S is glucose concentration or cellulase activity measured by DNS method in the sample solution of glucose or cellulase (treated with a salt).*

Deng and Tabatabai, (1995) have reported that the degree of inhibition of soil cellulase activity by most of the trace elements studied was much less than those reported for inhibition of urease (Tabatabai, 1977). The lack of complete inhibition of cellulase activity in solution by metal ions, especially Ag^{+1} and Hg^{+2}, suggests that functional groups other than sulfhydryl groups are involved in the active sits of enzymes (exoglucanase and endoglucanase). It has been reported that at the active sites of these enzymes there are hydrophobic amino acid (like tryptophan or tyrosine) residues. They are not as readily complexed with trace metals as those involving sulfhydryl groups.

Conclusion: Measurement of reducing sugar in solution by DNS indicator in the presence of some ions overestimated and in the presence of some other ions underestimated the real contents. Results showed that the intensity of DNS color and/or the reducing power of glucose increased by increasing radius of alkaline-earth cations. So, dependent on soil salinity and type of cation or anion of a salt, assessment of cellulase activity by colorimetric methods may be misrepresented. Exoglucanase and endoglucanase

activities in the presence of some ions (i.e. Na^{+}, K^{+}, Ca^{+2}, Ba^{+2}, and Mn) increased and in the presence of many other ions decreased. Fe^{+3}, Pb^{+2}, Fe^{+2}, Ag^{+1}, Zn^{+2}, Co^{+2}, and Al^{+3} were the most effective inhibitors of cellulase activity. Generally the effect of ions on exoglucanase activity was more intensive than endoglucanase activity.

Generally, ions had a noticeable effect on the intensity of DNS color and/or the reducing power of glucose which can affect the cellulase activity determined. So, in the colorimetric determination of cellulase activity in a salt treated solution the percentage inhibition may be related to negative effect of ions on the method of assessment. Unfortunately, the salt effects on cellulase activity and the method of assessment can not be separated by colorimetric methods. If one ion can change the properties of sugars, it will change the cellulase activity determined by every colorimetric method. So, in the study of the effect one ion on the enzyme activity, knowing the effect of ion on the method of enzyme assay is very important for data interpretation.

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