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# Activities of a Cellulase of the Termite, Ametermes Eveuncifer (Silverstri) Soldier: Clue to Termites Salt Intolerance

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**Abstract**
Table salt which contains predominantly NaCl is both toxic and lethal to termites and is therefore used to control the insect traditionally. In an attempt to find out a scientific explanation for this treatment and possibly design a pesticide for the destructive insect, we carried out some tests on the effects of NaCl (table salt), some other chloride and sodium salts on some important enzymes produced by termites. At 0.1mM concentration, all the chloride salts inhibited all the enzymes. Acid phosphatase and arginase were however mildly inhibited. Interestingly, some chloride salts were more potent than NaCl the conventional pesticide. The greatest inhibition was by the chlorides of mercury (81%), manganese (78%), and sodium (76%). The inhibitory effect was more on cellulolytic enzymes; β-glucosidase and cellulase than on detoxifying enzymes; 3-MST and rhodanese. Again, all the sodium salts tested inhibited cellulase drastically with most of them more potent than NaCl. Thus both the sodium and chloride ions contributed immensely to the inhibition. Form these discoveries, one of the chloride salts of mercury, manganese and sodium or a combination of at least two can be used as a pesticide for termites. Almost all the sodium salts tested or a combination of at least two can also be used.
**Keywords:** cellulase, cellulose, termites, salts, insects, enzyme.

## I. INTRODUCTION

Termites are a group of social insects: they live in a nest or colony which is typically found underground, often near a tree stump, wood pile or other source of 'food', using cellulose as a food resource, and they play a key role in decomposing dead plant tissue in natural ecosystems. (Gay and Calaby, 1970; Smith, 2007). Phylogenetically, termites can be classified into two subgroups: lower termites, which are termites from Mastotermitidae, Hodotermitidae, Kalotermitidae, Rhinotermitidae and Serritermitidae, and higher termites, which are termites from Termitidae (Edwards and Mill, 1986). Termites are among the most significant wood-feeders on the earth. Cellulose, a main structural constituent of plants, is the major nutritional component for wood-feeding termites. While investigating cellulose digestion of termites; cellulase has been purified from many species of termite such as Nasutitermes takasagoensis (Shiraki), Nasutitermes exitiosus and Nasutitermes walkeri, Amertermes eveuncifer Silvestri (Tokuda et al., 1997; Tokuda et al., 1999; Tokuda et al., 2000; Tokuda et al., 2004; Tokuda et al., 2005; Tokuda and Watanabe, 2007; Ezima et al., 2012; Fagbohunka 2011). Termites are a good example of 'symbiosis': they provide a home (their lower intestines) to protozoa and/or bacteria, who, in return, convert the wood into products usable by the termite. However, it has been found that flagellate-harboring termites produce their own cellulases in the salivary glands (Watanabe et al., 1997; Watanabe et al., 1998; Watanabe et al., 2001), while their intestinal flagellates also produce cellulases that show different amino acid sequences from the termite cellulases (Watanabe et al., 2001). It has also been shown recently that termites do not necessarily depend on symbiotic bacteria to process cellulose. They secrete their own cellulases, mainly endo-β-1, 4-glucanase and β-1, 4-glucosidase (Tokuda and Watanabe, 2007).

Cellulase is a class of enzymes that catalyze the cellulolysis (or hydrolysis) of cellulose by end-wise cleavage of the cello-oligosaccharide to produce glucose. It is a multi-enzyme complex containing at least 3 major components: Endoglucanase (EC 3.2.1.4) also called endo-1,4-β-glucanase, Exoglucanase (EC 3.2.1.91) also called Cellobiohydrolase, or exo-1, 4-β-glucanase, Cellobiase (EC 3.2.1.21) also called β-Glucosidase (Emert et al, 1974; Klyosov et al., 1986; Klyosov, 1990). These 3 vital enzyme components are both enzymatically and physically distinct and all play essential roles in the overall process of converting cellulose to glucose in a synergistic way. (Klyosov et al., 1986; Klyosov, 1990). Cellulase is a complex system of enzyme that works synergistically to attack native cellulose by hydrolyzing the β-1, 4 linkages of cellulose to glucose units. (Emert et al., 1974). It is a metabolic enzyme and the cellulase system is required to digest vegetable matter whose major constituent is cellulose.

The activities of termites could be destructive and undesirable sometimes. Thus they need to be controlled in such cases. Many methods have been designed locally and scientifically for termite control. The discovery of termite genes has led to the determination of the three-dimensional structure of the enzyme, which will hopefully lead to the development of environmental-friendly inhibitors of these enzymes that can be impregnated into wood to inhibit termites' activity (Nakashima et al., 2002a; Nakashima et al., 2002b). Termites,

such Ametermes eveuncifer Silverstri require cellulase to efficiently digest cellulose for survival. Enzymatic hydrolysis of cellulose to glucose occurs by the action of cellulase, a mixture of the three major classes of enzymes including endo-1, 4-β-glucanases, exo-1,4-β-glucanases, and β-glucosidase. Inhibitors developed against any of these cellulase system enzymes would be a potential termite treatment avenue. Traditionally, table salt which contains predominantly NaCl, both toxic and lethal to termites, is used to control the insect. To our knowledge, there has not been any scientific reason proposed to explain this phenomenon. Salts containing cation and anion will either inhibit or activate enzyme activity, it is suspected that either Na or Cl ion or both might be inhibiting or totally deactivating the activities of some crucial enzyme in termites thereby killing them. Inhibition in metabolizing cellulose might be sufficient to starve termites. In an attempt to find out a scientific explanation for the table salt control of termite and possibly design a pesticide for the destructive insect, we carried out some tests on the effects of NaCl, some other chloride and sodium salts on some important enzymes produced by termites.

## II. MATERIALS AND METHODS

### Reagents and Apparatus

All the reagents used in this research were of analytical grades and were obtained from Bio-Rad Laboratories, Richmond, California, U.S.A., Pharmacia AB, Uppsala, Sweden, Sigma Chemical Company Limited, St. Louis, Mo, U.S.A., Pierce Chemical Company, Rockfold, Illinois, U.S.A., BDH Chemicals Limited, Poole, England., from Eastern Kodak, Company, Rochester, N.Y., U.S.A., Glass distilled water was used for all preparations of solutions and all pH measurements were made at $25^0$C using the standard pH meter Radiometer, Copenhagen.

Apparatus used include, top load weighing balance (Mettler PN1210), pH meter (Mettler MP200) and UV/VIS Spectrophotometer (Cecil 2041), Ultracentrifuge (Beckman Optimal LE-80K Ultracentrifuge), mortar and pestle.

### Enzyme Purification of Crude Extract

Termite soldiers were washed and rinsed well with distilled water. The termites were gently homogenized with a pre-frozen warring blender or mortar and pestle in an ice bath using acid washed sand. An aliquot of 10mM sodium acetate buffer, pH 5.0, containing 1mM EDTA, was added intermittently in the ratio of 5:1 (v/w); buffer/termites, while homogenizing.

The mixture collected was centrifuged at 15,000rpm for 15minutes with a bench centrifuge at room temperature. The supernatant was collected and stored in a refrigerator. The supernatant was salted out by bringing the crude extract to 70% (w/v) saturation with Ammonium sulfate.

### Enzyme Assays for Cellulase

Cellulase activity toward CM-cellulose was measured by the appearance of reducing end groups in solution of CM-cellulose. The assay mixture consisted of 0.5ml of 1% (w/v) CM-cellulose (in 10mM sodium acetate buffer, pH 5.0 containing 1mM EDTA) and 0.1ml of the enzyme solution. This was incubated at $40^0$C for 30 minutes. After incubation, 0.4ml of water and 1.0ml of combined copper reagent containing alkaline solution of $CuSO_4$ buffered with a (carbonate-bicarbonate mixture containing Sodium potassium tartarate) were added according to the method of Hurst and his coworkers (Hurst et al., 1977). The above mixture was heated at $100^0$C for 20 minutes and allowed to cool to room temperature. Arsenomolybdate reagent (containing concentrated $H_2SO_4$ and sodium arsenate) (1.0ml) was added and mixed immediately and thoroughly to dissolve all precipitated $Cu_2O$. For easy absorbance reading, 7.0ml of distilled water was added to dilute mixture which was thoroughly mixed by covering the tube with parafilm and inverting it 3 to 4 times. The absorbance was read at 540nm. A unit of cellulase activity in this work is defined as the amount of enzyme that produces a change of an absorbance at 540nm of 0.10 under the conditions defined (Hurst et al., 1977).

### Assay for β-Glucosidase

β-Glucosidase activity was determined by the method of Umezurike (1976). The reaction mixture which was made up of 2.0ml of 1mM o-nitrophenyl-β-D-glucopyranoside (in 10mM sodium acetate buffer, pH 5.0 containing 1mM EDTA) and 0.1ml of enzyme solution was incubated for 30 minutes at $40^0$C. After incubation, 10 ml of 0.1M sodium carbonate was added to 0.5ml of the assay mixture. The nitrophenol released was measured by its absorbance at 400nm. One unit of β-glucosidase activity was defined as that amount of enzyme needed to liberate 1µmol of nitrophenyl per minute under the conditions of the assay.

### Assay for 3-Mercaptopyruvate sulphurtransferase

3-MST was assayed according to the spectrophotometric method of Agboola et al, (2008). The 3-MST measurement was based on calorimetric determination of thiocyanate formed in the reaction. The reaction mixture contained 1.05ml of 0.38M Tris-HCl buffer, pH 9.0, and 0.15ml of 0.3M mercaptopyruvate, 0.15ml of

0.5M KCN and 0.03ml of enzyme solution in a final volume of 1.5ml. The reaction was initiated after the addition of the enzyme solution.

After 15 min, 0.1ml of 38% formaldehyde was added to terminate the reaction. The colour was developed by the addition of 0.5ml of ferric nitrate reagent. The absorbance was measured at 460nm. The control contained no salt. One enzyme unit is the amount of enzyme that catalyses the formation of 10µmol of thiocyanate under the assay conditions.

**Assay for Rhodanese**

The method of Fagbohunka et al, (2004) was employed to measure rhodanese activity. The reaction mixture consisted of 0.5ml of 50mM Borate buffer, pH 9.4; 25mM KCN, 25mM $Na_2S_2O_3$, 0.09ml distilled water and 0.01ml of enzyme in a total volume of 10ml. The reaction was stopped by the addition of 0.5ml of 15% formaldehyde and 1.5ml of ferric nitrate solution (i.e. Sorbo reagent). The absorbance was then read at 460nm. The unit was expressed in rhodanese unit (RU). One rhodanese unit was taken as the amount of enzyme which under given the given conditions produced an O.D reading of 1.08 at O.D 460nm.

**Assay for Arginase**

Arginase activity was determined by the measurement of urea produced by the reaction with Ehrlich's reagent (Agboola et al, 2008). The reaction mixture contained, in final concentration, 0.1M arginine solution, 1.0mM Tris-HCl buffer, pH 9.5 containing 1.0 mM $MnCl_2$ and 0.05ml of the enzyme preparation in a final volume of 1.0ml. the was incubated for 10 minutes at $37^0$C. the reaction was terminated by the addition of 2.5ml Erhlich reagent (which contains 2.0g of p-dimethylaminobenzaldehyde in 20.0ml of concentrated hydrochloric acid and made up to 100ml by adding distilled water). The optical density was taken after 20 minutes at 450nm. The urea produced was estimated from the urea curved prepared by varying the concentration of urea between 0.1µmol and 1.0µmol. a graph of optical density against urea concentration was plotted. A unit of arginase activity is the amount of the enzyme that will produced 1µmol of urea per minute at $37^0$C at pH 9.5.

**Assay for Acid Phosphatase**

Acid phosphate activity towards p-nitrophenyphosphate (substrate) was measured by the appearance of reducing end group in solution of p-nitrophenyphosphate (Fagbohunka et al, 2005). The mixture was incubated for 3-4 minutes at $25^0$C and allowed to cool to room temperature. The absorbance was read at 300nm. A unit of activity is defined as the amount of enzyme that would liberate 1.0 µmole of p-nitrophenyl phosphate per minute under the assay conditions.

**Termites Control Strategy**

The effects of Chloride salts such as NaCl, $MnCl_2$, $SnCl_2$, $CoCl_2$, $NiCl_2$, $NH_4Cl$, $BaCl_2$, $ZnCl_2$ and sodium salts on the activities of the enzyme was determined with varied concentrations of 0.1mM, 0.2mM, 0.5mM and 1.0mM. The activities were measured based on the assay techniques for the enzymes as earlier described (Agboola et al., 2008).

## III. RESULTS

**Enzyme Purification Steps**

The results of the purification procedure are summarized in Table II. The purification procedure yielded a cellulase with a specific activity of 14.10 Units/mg of protein.

**Table I: Summary of the Purification Steps of Cellulase from the Termite (Ametermes Eveuncifer Silverstri) Soldier**

| Extract | Volume (ml) | Total Activity ( Units) | Total protein | Specific Activity (Units/mg) | Yield (%) | Purification Fold |
|---|---|---|---|---|---|---|
| Crude Extract | 1000.00 | 105,000.00 | 34,170.00 | 3.07 | 100.00 | 1.00 |
| 70% Ammonium sulphate | 560.00 | 96,150.00 | 13,580.00 | 7.08 | 91.60 | 2.30 |

**Termite Control**

The action of various chloride and salts on various termite enzymes was investigated. The results are presented in Tables II & IX. All the chloride salts tested have an inhibitory effect of at least 70% on all the enzymes even at a low concentration of 0.1mM. Interestingly too, it was observed that some chloride salts were more potent than sodium shloride (table salt), the conventional pesticide. The greatest inhibition was by the chlorides of mercury, manganese, magnesium and sodium in decreasing order. The greatest effect was on β-glucosidase and cellulase, the major metabolic enzymes of this organism.

To ascertain whether it was the chloride ion and not the sodium ion that is responsible for this effect,

the action of some sodium salts on cellulase was also tested. Most of the sodium salts tested inhibited cellulase more than NaCl.

**Table II: Percentage Inhibition of all Enzyme Activities by NaCl Salt**

| ENZYME | 0.1 mM | 0.2 mM | 0.5 mM | 1.0 mM |
|---|---|---|---|---|
| B-Gucosidase | 84.42 | 84.54 | 85.92 | 87.18 |
| Cellulase | 76.00 | 83.00 | 93.00 | 100.00 |
| 3-MST | 56.59 | 57.25 | 57.69 | 58.62 |
| Rhodanese | 29.04 | 38.56 | 49.67 | 59.43 |
| Arginase | 12.86 | 13.87 | 17.43 | 28.17 |
| Acid Phosphatase | 8.25 | 12.95 | 22.12 | 37.20 |

**Table III: Percentage Inhibition of Cellulase Activity by Chloride Salts**

| SALTS | 0.1 mM | 0.2 mM | 0.5 mM | 1.0 mM |
|---|---|---|---|---|
| $HgCl_2$ | 81 | 83 | 89 | 98 |
| $MnCl_2$ | 78 | 88 | 99 | 100 |
| NaCl | 76 | 83 | 93 | 100 |
| $CoCl_2$ | 75 | 78 | 83 | 91 |
| $NH_4Cl_2$ | 74 | 81 | 90 | 100 |
| $ZnCl_2$ | 73 | 81 | 90 | 100 |
| $BaCl_2$ | 72 | 77 | 87 | 100 |
| $SnCl_2$ | 72 | 73 | 76 | 82 |

**Table IV: Percentage Inhibition of B-Glucosidase Activity by Chloride Salts**

| SALTS | 0.1 mM | 0.2 mM | 0.5 mM | 1.0 mM |
|---|---|---|---|---|
| $NH_4Cl_2$ | 85.93 | 86.78 | 86.88 | 87.00 |
| $HgCl_2$ | 85.77 | 85.77 | 87.36 | 87.95 |
| $MgCl_2$ | 85.25 | 85.67 | 86.00 | 86.40 |
| $SnCl_2$ | 85.00 | 85.73 | 87.18 | 87.78 |
| NaCl | 84.42 | 84.54 | 85.92 | 87.18 |
| $BaCl_2$ | 84.04 | 84.30 | 84.60 | 84.75 |
| $CoCl_2$ | 83.62 | 83.31 | 84.81 | 85.37 |
| $MnCl_2$ | 79.39 | 79.39 | 80.11 | 83.25 |
| $ZnCl_2$ | 71.72 | 80.89 | 84.81 | 84.91 |
| $NiCl_2$ | 65.96 | 83.21 | 83.37 | 84.99 |

**Table V: Percentage Inhibition of Acid Phosphatase Activity by Chloride Salts**

| SALTS | 0.1 mM | 0.2 mM | 0.5 mM | 1.0 mM |
|---|---|---|---|---|
| $BaCl_2$ | 60.70 | 42.82 | 44.83 | 35.91 |
| $NiCl_2$ | 44.60 | 39.74 | 33.50 | 31.13 |
| $MnCl_2$ | 33.00 | 46.93 | 61.28 | 63.38 |
| $SnCl_2$ | 32.87 | 36.22 | 44.16 | 49.02 |
| $NH_4Cl_2$ | 30.42 | 32.20 | 34.97 | 67.03 |
| $ZnCl_2$ | 12.58 | 19.94 | 34.92 | 38.36 |
| NaCl | 8.25 | 12.95 | 22.12 | 37.20 |

**Table VI: Percentage Inhibition Of 3-Mercaptopyruvate Sulphur Transferase Activity By Chloride Salts**

| SALTS | Concentration | | | |
|---|---|---|---|---|
| | 0.1 mM | 0.2 mM | 0.5 mM | 1.0 mM |
| NaCl | 56.59 | 57.25 | 57.69 | 58.62 |
| $CoCl_2$ | 50.00 | 51.00 | 51.30 | 56.70 |
| $ZnCl_2$ | 46.40 | 51.80 | 56.10 | 58.10 |
| $NiCl_2$ | 42.60 | 47.90 | 49.80 | 54.00 |
| $NH_4Cl_2$ | 40.40 | 41.70 | 45.70 | 53.90 |
| $BaCl_2$ | 37.40 | 37.50 | 42.90 | 45.60 |
| $SnCl_2$ | 25.00 | 28.90 | 30.60 | 34.60 |
| $MnCl_2$ | 0.00 | 4.90 | 12.60 | 16.50 |

**Table VII: Percentage Inhibition of Rhodanese Activity by Chloride Salts**

| SALTS | Concentration | | | |
|---|---|---|---|---|
| | 0.1mM | 0.2mM | 0.5mM | 1.0mM |
| $ZnCl_2$ | 67.75 | 69.50 | 71.33 | 74.38 |
| $NiCl_2$ | 57.35 | 55.01 | 20.55 | 14.93 |
| $CoCl_2$ | 56.24 | 57.48 | 66.32 | 68.34 |
| $MnCl_2$ | 54.29 | 57.41 | 65.80 | 69.73 |
| $NH_4Cl_2$ | 44.86 | 38.30 | 33.22 | 28.20 |
| $SnCl_2$ | 37.58 | 39.08 | 56.76 | 57.22 |
| NaCl | 29.04 | 38.56 | 49.67 | 59.43 |

**Table VIII: Percentage Inhibition of Arginase Activity by Chloride Salts**

| SALTS | Concentration | | | |
|---|---|---|---|---|
| | 0.1 mM | 0.2 mM | 0.5 mM | 1.0 mM |
| NaCl | 12.86 | 13.87 | 17.43 | 28.17 |
| $MnCl_2$ | 12.56 | 13.62 | 16.92 | 28.00 |
| $NH_4Cl_2$ | 11.51 | 15.82 | 25.21 | 34.18 |
| $BaCl_2$ | 03.89 | 04.74 | 11.42 | 18.02 |
| $CoCl_2$ | 01.44 | 13.03 | 16.92 | 21.40 |
| $SnCl_2$ | 00.34 | 16.92 | 23.00 | 35.59 |
| $ZnCl_2$ | 00.00 | 13.62 | 16.92 | 35.59 |
| $NiCl_2$ | 00.00 | 05.84 | 11.42 | 18.02 |

**Table IX: Effect of Sodium Salts on Cellulase from the Termite Armetermes eveuncifer Silverstri**

| SALTS | Concentration | | | |
|---|---|---|---|---|
| | 0.1 mM | 0.2 mM | 0.5 mM | 1.0 mM |
| $NaNO_3$ | 100 | 100 | 100 | 100 |
| $Na_2CO_3$ | 100 | 100 | 100 | 100 |
| $NaH_2PO_4$ | 94.5 | 96.5 | 97.9 | 97.2 |
| $NaHCO_3$ | 87.3 | 92.7 | 95.9 | 100 |
| $Na_2WO_4$ | 81.9 | 93.8 | 100 | 100 |
| $Na_2SO_4$ | 80.77 | 47.12 | 8.65 | 92.31 |
| NaCl | 76 | 83 | 93 | 100 |
| $Na_3AsO_3$ | 73.1 | 85.5 | 100 | 100 |
| $Na_2HPO_4$ | 71.8 | 68.9 | 100 | 100 |
| $NaCH_3COOH$ | 0.67 | 2.0 | 93.3 | 100 |

## DISCUSSION

The test for the effect of NaCl on the six enzymes showed that the activity of β-glucosidase and cellulase were the most inhibited even at low as 0.1mM concentration (Table II). Interestingly, this result is not so much different from what was obtained for snail cellulase in our previous work (Agboola et al., 2008). It had been reported that sodium chloride had an inhibitory effect on cellulolytic ability of 16 species of Aspergillus isolated from salt-affected areas of Punjab, Pakistan (Malik et al., 1980) and other enzymes such as alkaline protesase (Alabi and Daini, 2009). The feeding to termites of wood, antecedently soaked in aqueous solutions of various chemicals (NaCl, $CaC1_2$, $HgC1_2$, KCl, etc.) had been reported to result in killing both host and parasite (Cleveland, 1923). 3-MST and rhodanese were moderately inhibited. This however is different from the results obtained for snail enzymes as only the metabolic enzymes were totally inhibited by the salts while the detoxifying enzymes were only slightly inhibited (Agboola et al., 2008). Acid phosphatase and Arginase activities were only slightly inhibited at this concentration. This also is not so much different from what was obtained for snail cellulase in our previous work (Agboola et al., 2008).

β-glucosidase and cellulase are cellulolytic and are known to be involved in energy metabolism while 3-MST and rhodanese play secondary roles such as detoxification. It can be suggested that the toxicity of salts to termite stems from the effect of the salts on its key enzymes of both metabolism and detoxification. The inhibition of termite cellulase and β-glucosidase, the important enzymes in the overall metabolism of the termite (which feeds exclusively wooden materials) which requires enzymes of the cellulase complex (of which β-glucosidase is a member) and Rhodanese and 3-MST which are involved in CN detoxication in animal system could be lethal to the organism. Though the activities of the latter enzymes do not have direct bearing on the energy metabolism of animals, they also play a major role in detoxifying the termites of the toxic cellulolytic materials they feed on. Moreover, the activities of arginase and acid phosphatase were inhibited to varying

degrees by these salts as may be expected of normal interaction of enzymes with salts. Inhibition of the metabolic enzymes by NaCl indicates the organism may not be able to metabolise wood even if it feeds on it. Inhibition of the detoxifying enzymes by NaCl also suggests the organism may not be able to detoxify the toxic cellulolytic materials they feed on. These definitely will result in the death of the organism. If at 0.1mM, this enormous harvoc is done by NaCl, one can only imagine the degree of the harm the undiluted NaCl which is poured on termites to control it locally will do on the organism.

To ascertain whether the enzymes can also be inhibited by other chloride salts; same concentrations of different chloride salts were tested on the six enzymes (Tables III-VIII). Interestingly, $HgCl_2$ and $MnCl_2$ inhibited cellulase 81% and 78%, respectively at 0.1mM which is in fact higher than 76% the inhibition by NaCl at the same concentration. All other enzymes were also inhibited. It has been reported however that cellulases are inhibited in the presence of heavy metals including Hg and Mn (Mawadza et al., 2000). Therefore, it is not surprising that these two salts have a high percentage of inhibition than that of NaCl.

Also, to find out whether the inhibition was due to the chloride or sodium ion, same concentrations of different sodium salts were tested on cellulase. It was clearly revealed that all the sodium salts tested inhibited cellulase drastically with most of them more potent than NaCl (Table IX). Thus both the sodium and chloride ions contributed to the inhibition. Interestingly, this result is also different from what was earlier obtained for snail cellulase in our previous work (Agboola et al., 2008). However, though both the sodium and chloride ions contribute to the lethal action of Sodium Chloride on termites, it was observed that Sodium salts rather than Chloride salts were more potent as inhibitors.

As for the design of a pesticide for termites, one of the chloride salts of mercury, manganese, magnesium and sodium or a combination of at least two can be used. Also, almost all the sodium salts tested in this work or a combination of at least two can also be used.

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