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POLYSACCHARIDES OF ACTIVATED SLUDGE PRODUCED BY THE TREATMENT OF TANNERY EFFLUENTS WITH SEWAGE

I. EXTRACTION AND ISOLATION

A suitable procedure for extraction of polysaccharides from activated sludge, produced by the treatment of tannery wastes combined with sewage, was chosen basing on the literature data and verified. The procedure is based on the classical Westphal method which consists in action of a homogeneous phenol–water mixture under heat condition. The method was slightly modified for the given material and requirements. A procedure of treatment with trichloroacetic acid was worked up in order to remove proteins from the obtained aqueous solution of polysaccharides. Polysaccharides precipitated from aqueous solutions by the ethanol–aceton mixture appear as light brown flocs. The yield lies between 0.75 and 1.5% by wt. of the sludge dry mass and varies with quality of the extracted sample. The whole procedure of extraction and preparation takes into account the chemical characterization of obtained polysaccharides which will be dealt with in the next communications.

1. INTRODUCTION

The heterogenous population of microorganisms used for wastewater purification can be characterized, according to COACKLEY [2], as a highly hydrated polymeric gel. From the viewpoint of elementary composition, the activated sludge biomass may be defined by empirical formulae $C_5H_7NO_2$ or $C_7H_{10}NO_3$ [15] or — in the presence of phosphorus — as $C_{118}H_{170}O_{51}N_{17}P$. Of other elements sodium, potassium, and — to a less extent — also calcium can be considered. The activated sludge dry matter consists of a number of biopolymers present in the microbial cells as a building material, reserve matter or as metabolites. PITTER [14] collected data from various references. Activated sludge contains 32–41% of proteins, which prevail over other organic matter. Polysaccharides and lipides, considered as one group, appear in approximately the same proportion. When comparing the given data, it is necessary to remember that the quantitative composition of

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the activated sludge may to a considerable extent depend on the composition of the supplied wastewaters as well as on the technological parameters of the activated sludge process. COACKLEY studied the polysaccharides in the activated sludge [2] and came to the following conclusions:

the mixed microbial culture in activated sludge flocs produces a great amount of polysaccharides;

aerobic conditions are substantial for the production of extracellular polysaccharides;

the polysaccharides have a strong affinity to water; therefore the properties of separate compounds and groups, together with a great number of possible positions for formation of hydrogen bonds, are the factors which influence the behaviour and condition of activated sludge flocs;

so far at least 20 monosaccharidic components of the activated sludge have been found; most of them can, however, be classified among 3 groups which have the glucose, mannose and galactose configurations.

The same paper [2] contains the preliminary results of the laboratory research. Chromatographic analysis of the extracted polysaccharide allowed to determine 85% of glucose, 2% of mannose, 4% of xylose, 4% of ribose, and 5% of rhamnose. Experiments in which the substrate was changed have shown that the proportions of the monosaccharides remained unchanged.

WALLEN and DAVIS [22] analyzed an acidic hydrolyzate of biopolymer extracted from activated sludge by hot water. The gas chromatography of trimethyl silane derivatives identified the peaks of glucose (40%), mannose (13%), and rhamnose (20%), and probably also of galactose and xylose. The paper chromatography identified glucose, hexosamine, maltose, and lactose. ANDERSON and MCCOY [1] identified pentoses and hexosamines in the hydrolyzed extracts of natural flocs of the activated sludge. FRIEDMAN et al. [4] isolated fibrillary polymer similar to cellulose which contained only glucose cross-linked in the 1,4 position. MITCHELL and NEVO [11] found that polysaccharides isolated from the flocs mucilage of the activated sludge caught by sand filters contained mostly glucose, galactose, and glucuronic acid. NISHIKAWA and KURIYAMA [12] described an interesting method of the activated sludge extraction followed by the separation of extracted polysaccharides and deoxyribonucleic acid. Their method is based on the use of 0.5 M Na_2CO_3. FORSTER [3] isolated the surface polymers from activated sludge flocs (centrifugation) and after hydrolysis he identified galactose, fructose, mannose, and glucuronic acid; the presence of aminosaccharides were not stated. NOVÁKOVÁ [13] and Van GILS [21] accomplished a direct hydrolysis of the activated sludge (without foregoing extraction) in order to identify monosaccharidic components. The extraction of polysaccharides from activated sludge and the identification as well as the determination of monosaccharidic components were investigated by GRÜNWALD [5, 6], TELETZKE [19], and TAKIGUCHI [18].

The importance of study on polysaccharides lies in the fact that it allows to find a correlation between the accumulation of polysaccharides (especially of extracellulary ones)

and the flocculation ability of sludge flocs. This relation was first found by McKINNEY [10]. TENNEY and STUMM [20] agreed with this opinion and found that polysaccharides were the main factor influencing the sedimentation behaviour of the activated sludge. FORSTER [3] found, however, no relation between the content of polysaccharides and the sedimenting properties of the activated sludge, while STEINER et al. [17] and MITCHELL [8] supported the statement on the importance of polysaccharides as far as the properties of activated sludge flocs and the inhibition of the flocculation process are concerned. In our work an attempt has been made to give basic and primary chemical characteristics of polysaccharides contained in the activated sludge produced in the treatment of tannery wastes in combination with sewage. Its character is particularly determined by the leather production. For this purpose a suitable method for extraction and preparation of polysaccharides was chosen and verified. The methods allowing to obtain polysaccharides from the biological material described in the literature, modified for the given conditions and the given type of activated sludge are the subject of this first communication. The next communications will describe the process and results of analytical and preparative separation of the investigated polysaccharides by the gel permeation chromatography (GPC) allownig to estimate molecular weights and their distributions. The GPC is followed by the identification of monosaccharide components by the methods of paper chromatography, quantitative elementary analysis and estimation of functional groups by the IR spectroscopy analysis of individual fractions.

2. EXPERIMENT AND RESULTS

2.1. EXTRACTION OF POLYSACCHARIDES FROM ACTIVATED SLUDGE

Activated sludge obtained in the biological treatment of tannery effluents and sanitary sewage (2 : 1) was studied in pilot plant conditions. The experiments in such conditions were not carried before.

The following sections include only reference sources which are connected directly with the selected extraction method and its modification; the work is based on thorough literature perusal.

For the extraction of polysaccharides from the given type of activated sludge the WESTPHAL method [23] was chosen after as eries of experiments on the effects of homogeneous mixture phenol–water in heat conditions. This method is recommended in the literature as the most effective one for the extraction of complex polysaccharides from bacterial material. The method was slightly modified to match our conditions and needs.

When treating bacteria by liquid phenol, the somatic proteins are transferred to the solution, consequently, the cell structure is so changed that during the subsequent extraction by aqueous agents, most polysaccharides are transferred to the solution. In order to achieve a complete extraction of polysaccharides, this two-stage extraction must be several times repeated. Later on, WESTPHAL [24] showed that the extraction could be sim-

plified by shaking the bacteria directly for several minutes in the emulsion of phenol and water in equal volumes at a low temperature (5–10°C). The mixture subject to centrifugation is separated to the upper aqueous layer, middle phenolic layer, and insoluble residue. The aqueous phase contains the total amount of undegraded polysaccharides and nucleic acids (the process A, ref. 21). This process was applied by GRÜNWALD et al. [5] to extract the polysaccharides from the microorganisms of the model activated sludge; the maximum yields were as high as 2.2% (with respect to the dry matter). In another work GRÜNWALD [6] first separated extracellular polysaccharides (a part of crusts and mucilages) from the microorganisms of the activated sludge by centrifugation at high revolutions and by applying the A process he extracted and isolated from the cell wall intercellular polysaccharides alone. The obtained isolates (white flakes) were hydrolized by cation exchanger and the monosaccharides determined qualitatively and quantitatively by means of a thin-layer chromatography with spectrophotometric detection. The isolated products were not deprived of proteins (no protein tests have been performed). However, the polysaccharides extracted by means of phenol–water cold emulsions contain often a varying amount of strong-bonded proteins [23] (polysaccharide–proteine complexes). At the temperatures exceeding 68°C, phenol can be mixed with water in any proportion. When being cooled, the homogeneous mixture separates into two layers: the upper aqueous layer (saturated with phenol) and the lower phenolic layer (saturated with water). If the bacterial material is similar to the product of extraction with a homogeneous mixture of equal phenol and water volumes at 65–68°C, a rapid destruction of cells occurs and a great proportion (as much as 40%) of the bacterial mass is transferred to the solution. The cooling to 5–10°C and centrifugation yields an aqueous layer, phenolic layer, and undissolved residue (the process B, ref. 21).

According to literature sources, concerning the extraction in process B, the aqueous phase contains only polysaccharides and nucleic acids and does not include proteins. In our case, however, the protein tests of aqueous phase obtained by the application of the mentioned process were alwyas positive. Therefore, it was necessary to find a suitable method for protein-removal from the obtained aqueous phase.

The literature contains a number of such methods for aqueous solutions of saccharides. Several methods were tested and verified. We used first the known method with barium hydroxide and zinc sulphate according to SOMOGY. The protein-removal effect was excellent, but it was accompanied with a high loss of saccharide biopolymers. Further, we examined the SEWAGE method which is recommended in the modern literature [16] as one of the mildest methods. It appeared, however, that this technique eliminated only small amounts of proteins. In our case it should be repeated many times, but then a significant loss of extracted polysaccharides would occur. As further possibilities the protein-removal by means of bentonite [7], sulphosalicylic acid, and trichloroacetic acid were verified.

The protein-removal effects of all above-mentioned substances were compared basing on chromatographic records obtained from the gel permeation chromatography (the next report). The samples of the aqueous phase, deprived of proteins by all the methods being

verified, were put together with the sample of protein-containing aqueous phase into columns filled by gel. For comparative reasons a single deproteinization was successively performed by means of papaine, a vegetable enzyme. The results revealed univocally that protein-removal effects of trichloroacetic and sulphosalicylic acids are comparable with the enzymatic method. The use of bentonite resulted in the loss of extracted polysaccharides. Finally, we selected the protein-removal by trichloroacetic acid with the simultaneous use of the SEWAGE method.

2.2. PROCEDURE

A fresh suspension of the activated sludge, taken from the pilot plant, is first cleared of a coarse suspended solids by filtering it through a Büchner funnel which does not include a filtration paper. Then, it is centrifuged for 7 min (3 500 rpm). The water is drawn off and the equal volume of acetone is added. After a 15-min occasional agitation, the acetone suspension is centrifuged for 7 min (3 500 rpm). Then, a similar washing with 0.02% solution of sodium azide in distilled water (bacteriostat) follows with the subsequent centrifugation under carefully kept conditions (7 min, 3 500 rpm). This reproducibly obtained sludge is collected and kept at the temperature of about 0°C.

A dose of about 20 g of sludge (referred to the dry matter) is suspended in 350 ml of distilled water preheated to 65–68°C, then 350 ml of 90% phenol solution (65–68°C) are added and the mixture is agitated in a mixer for 2 min. After 15 min of rest (under occasional agitation at the temperature of 65–68°C) the mixture is cooled to the temperature of about 5°C. The produced emulsion is centrifuged at the same temperature for 30 min at 3 500 r.p.m. Consequently, 3 layers are formed: aqueous layer, phenolic layer, and undissolved residue. The aqueous phase is carefully drawn off and the second and third layers are subject to the repeated operation at 65–68°C with another 350 ml sample of water, as described above. The mixing of both aqueous extracts results in a raw aqueous solution of saccharidic biopolymers. The raw aqueous solution is still strongly turbid and its colour is due to the phenol residues. It is deproteinized by the addition of trichloroacetic acid (in amount allowing to obtain approx. 8% solution), chloroform (0.2 of the raw aqueous solution volume), and 1-butanol (0.2 of the added chloroform volume). The mixture is shaken for 30 min, the precipitated proteins are centrifuged (10 min, 350 rpm), and the obtained strong-opalescent aqueous solution is coloured by phenol to a light-brown shade. The solution is dialyzed for 5 to 7 days against the 0.02% solution of sodium azide in distilled water (before the gel permeable chromatography system) to eliminate the protein-removal agents, phenol residues, and extracted low-molecular bacterial mass. The above process is not uniform and differs depending on whether the extracted polysaccharides were isolated solely as the total extract or their solution was analyzed and preparatively separated by the gel permeable chromatography method.

All references, concerning the phenolic extraction of bacterial polysaccharides, mention the isolation of the extract in total. The obtained product, described as a high-molecular water-soluble lipopolysaccharide, containing a low proportion of lipoidal com-

ponent, is used for various biological purposes, e.g. for preparation of a highly effective pyrogen [25], and some attempts have been made to identify and determine quantitatively monosaccharidic components after their hydrolysis [9].

2.3. ISOLATION OF THE TOTAL EXTRACT

The colour of the resulting solution is not alwyas identical. It may depend on the varying quality of tannery effluents (variable amounts of the tanning agents and other dyestuffs). We tried to eliminate the tanning agents from the resulting solution by means of a polyamide pulver which, as is generally known, does not absorb saccharides. However, the gel-permeation chromatograms and the practically unchanged colour of the solution did not allow to identify the loss of any substance.

The dialyzed opalescent solution, light to brown colour, is thickened in a vacuum evaporator at 40°C to the volume of about 30 ml and is precipitated by the addition of 6-fold volume of alcohol–acetone (1 : 1) mixture and the saturated solution of sodium acetate in acetone (1/30 of the volume of the added precipitation mixture). The extracted polysaccharides precipitate in form of light–brown flocks. The isolate is centrifuged, washed with alcohol and acetone and dried in vacuum. The yield ranges within 0.75–1.5% per weight of sludge dry matter and varies depending on the quality of the analyzed sample.

The isolated polysaccharides are strongly hygroscopic. In order to obtain a perfectly dried specimen, it should be several times dried in vacuum above P_2O_5. Weighing requires the use of closed weighing bottles (during a 5-min free storage, the weight of a specimen increased by 3.3%).

3. CONCLUSIONS

The paper describes detail proceedings for the isolation of saccharidic biopolymers from activated sludge produced by the biological treatment of tannery effluents combined with sewage. The general extraction principles recommended in the literature were modified to match the given problem of preparative separation and identification of polysaccharides by chromatographic methods. The chromatographic methods and the results obtained will be included in the next paper.

REFERENCES

[1] ANDERSON R., MCCOY E., *Floc–forming bacteria from activated sludge*, Bacteriol. Proc. RT 8, p. 162, 1963.

[2] COACKLEY P., *Some aspects of activated sludge*, Process. Biochem., Vol. 4 (1969), p. 27.

[3] FORSTER C. F., *Separation of activated sludge using natural and synthetic polymers*, Effl. Water Treatment J., Vol. 11 (1971), p. 363.

[4] FRIEDMAN B. A., DUGAN P. R., PFISTER R. M., RAMSEN C. C., *Fine structure and composition of the zoological matrix surrouding Zooglea ramigera*, J. Bacteriol., Vol. 96 (1968), p. 2144.

[5] GRÜNWALD A., BARTEČKOVÁ H., *The synthesis of polysaccharides in activated sludge* (in Czech), Scientific Paper of the Institute of Chemical Technology, Technology of Water, Prague, F 18, 5, 1973.
[6] GRÜNWALD A., *The determination of some basic elements of cell polysaccharides isolated from activated sludge* (in Czech), Scientific Papers of the Institute of Chemical Technology, Technology of Water, Prague, F 18, 23, 1973.
[7] GREGOR M., ČIČEL B., *Bentonite and its using* (in Czech), SAV, Bratislava 1969.
[8] HARRIS R. H., MITCHELL R., *Inhibition of the flocculation of bacteria by bio-polymers*, Water Res., Vol. 9 (1975), p. 993.
[9] LÜDERITZ O., WESTPHAL O., *Über bakterielle Reizstoffe*. II. *Mit.*: *Qualitative und quantitative papierchromatographische Bestimmung der Zuckerbausteine eines hichgereiningten Polysaccharid-Pyrogens aus Colibakterien*, Z. Naturforschg., Vol. 76 (1952), p. 548.
[10] MCKINNEY R. E., *Staining bacterial polysaccharides*, J. Bacteriol., Vol. 66 (1953), p. 453.
[11] MITCHELL R., NEVO Z., *Effect of bacterial polysaccharide accumulation on infiltration of water through sand*, Appl. Microbiol., Vol. 12 (1964), p. 219.
[12] NISHIKAWA S., KURIYAMA M., *Studies on mucilage in activated sludge*, J. Ferment. Technol. (Japan), Vol. 46 (1968), p. 381.
[13] NOVÁKOVÁ E., *The saccharidic storage compounds in activated sludge*, M. Sc. thesis, Institute of Chemical Technology, Technology of Water, Prague 1971.
[14] PITTER P., *Chemistry of water* (in Czech), Vol. 2, p. 100, SNTL, Prague 1974.
[15] PORGES N., HOOVER S., *Biological treatment of sewage and industrial wastes*, Vol. 1, Reinhold, New York 1956.
[16] STAUB A. M., *Removal of proteins*, [in:] WHISTLER R. L., *Methods in carbohydrate chemistry*, Vol. V. *General polysaccharides*, Academic Press, New York, London 1965.
[17] STEINER A. E., MCLAREN D. A., FORSTER C. F., *The nature of activated sludge flocs*, Water Res., Vol. 10 (1976), p. 25.
[18] TAKIGUCHI Y., *Purification of the mucilage in activated sludge and its physico-chemical properties*, J. Ferment. Technol., Vol. 50 (1972), p. 331.
[19] TELETZKE G. H., GITCHEL W. B., DIDDAMS D. G., HOFFMAN C. S., *The components of activated sludge and products*; *wet oxidation by air*, J. Water Pollut. Contr. Fed., Vol. 39 (1967), p. 994.
[20] TENNEY M., STUMM A., *Chemical flocculation of microorganisms in biological waste treatment*, J. Water Pollut. Contr. Fed., Vol. 37 (1965), p. 1370.
[21] Van GILS G., *Bacteriology of the activated sludge*, Publ. Health Inst., Hague 1973.
[22] WALLEN L. L., DAVIS E. N., *Biopolymers of activated sludge*, Environ. Sci. Technol., Vol. 6 (1972), p. 161.
[23] WESTPHAL O., JANN K., *Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure*, [in:] WHISTLER R. L., *Methods in carbohydrate chemistry*, Vol. V. *General polysaccharides*, Acad. Press., New York, London 1965.
[24] WESTPHAL O., LÜDERITZ O., BISTER F., *Über die Extraktion von Bakterien mit Phenol/Wasser*, Z. Naturforschg., Vol. 76 (1952), p. 148.
[25] WESTPHAL O., LÜDERITZ O., EICHENBERGER E., KEIDERLING W., *Über bakterielle Reizstoffe*. I. *Mitt.*: *Reindarstellung eines Polysaccharid-Pyrogens aus Bacterium coli*, Z. Naturforschg., Vol. 76 (1952), p. 536.

WIELOCUKRY OSADU CZYNNEGO WYTWARZANE PRZEZ OCZYSZCZANIE ŚCIEKÓW GARBARSKICH ZE ŚCIEKAMI KOMUNALNYMI

I. EKSTRAKCJA I IZOLACJA

Na podstawie danych literaturowych wybrano i sprawdzono odpowiednią procedurę ekstrakcji z osadu czynnego wielocukrów wytwarzanych przez oczyszczanie ścieków garbarskich połączonych ze ściekami komunalnymi. Procedura jest oparta na klasycznej metodzie Westphala, która polega na działaniu

homogenicznej mieszaniny fenolu z wodą w podwyższonej temperaturze. Metodę tę nieco zmodyfikowano dla danego materiału oraz warunków. Opracowano procedurę deproteinizacji za pomocą kwasu trójchlorooctowego w celu usunięcia białek z otrzymanego wodnego roztworu wielocukrów. Wielocukry strącone z roztworów wodnych mieszaniną etanolu z acetonem mają postać jasnobrązowych kłaczków. Wydajność waha się od 0,75% do 1,5% suchej masy osadu i zmienia się wraz z jakością ekstrahowanej próbki. Cała procedura ekstrakcji i przygotowania uwzględnia chemiczną charakterystykę otrzymanych wielocukrów. Będzie ona przedmiotem następnego artykułu.

POLYSACCHARIDE IM BELEBTSCHLAMM EINER MISCHUNG VON KOMMUNAL- UND GERBEREIABWÄSSERN

I. EXTRAHIERUNG UND ISOLIERUNG

Literaturhinweise dienten zur Nachprüfung der Extraktion von Polysacchariden die aufgrund der Biosynthese des Belebtschlammes einer Mischung von Kommunal- und Gerbereiabwässern produziert werden. Die Methode stützt sich auf der klassischen Methode von Westphal, bei der mit einem homogenen Phenol–Wasser–Gemisch bei erhöhten Temperaturen gearbeitet wird. Diese Methode wurde für das angeführte Medium modifiziert. Die Proteinabnahme erfolgt durch Anwendung von Trichloressigsäure; die Ausscheidung der Eiweißstoffe geschieht aus der wässerigen Lösung der Polysaccharide. Polysaccharide werden anschliessend mit einer wässerigen Äthanol–Azeton–Mischung als hellbraune Flocken ausgefällt. Die Ergiebigkeit schwankt zwischen 0,75 und 1,5% der Schlammtrockensubstanz und ändert sich gemäß der Qualität der extrahierten Probe. Die gesamte Behandlungsweise der Vorbereitung und Extraktion, nimmt die chemische Charakteristik der entstehenden Polysaccharide in Betracht, was im nächsten Beitrag eingehend erörtert wird.

ПОЛИСАХАРИДЫ АКТИВНОГО ИЛА, ПОЛУЧЕННЫЕ ПУТЁМ ОЧИСТКИ КОЖЕВЕННЫХ СТОЧНЫХ ВОД, СОЕДИНЁННЫХ С КОММУНАЛЬНЫМИ СТОЧНЫМИ ВОДАМИ

I. ЭКСТРАКЦИЯ И ИЗОЛЯЦИЯ

На основе литературных данных была выбрана и проверена соответствующая процедура экстракции из активного ила полисахаридов, полученных путём очистки кожевенных сточных вод, соединённых с коммунальными сточными водами. Процедура основана на классическом методде Вестфаля, который заключается в гомогенном действии смеси фенола с водой при повышенной температуре. Разработана процедура депротеинизации (освобождения от белков) с помощью трихлоруксусной кислоты для удаления белков из полученного водного раствора полисахаридов. Полисахариды, осаждённые из водных растворов смесью этанола с ацетоном, имеют форму светло-коричневых хлопьев. Выход колеблется от 0,75% до 1,5% сухой массы осадка и изменяется с качеством экстрагируемой пробы. Вся процедура экстракции и подготовки учитывает химическую характеристику полученных полисахаридов. Она будет предметом следующей статьи.