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Growth and biocatalytic activities of aerobic thermophilic populations in sewage sludge

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Summary. Enzymatic activities of aerobic thermophilic microorganisms are described and investigated for the development and control of sewage sludge treatment processes in batch and fed-batch cultures. Proteolytic activity is the main enzymatic activity in an aerobic thermophilic sewage sludge treatment process. It has an optimum at 80° C and can be found also during growth on synthetic media. The activity is correlated with the increase in ammonium in the particle-free fraction and the values of the respiratory quotients during cultivation either in sewage sludge or in a synthetic medium. No other extracellular activities (lipase, amylase, pectinase and cellulase) were detected in the investigated sludge samples. Carbohydrates, lipids and other polymers were either not present in significant amounts or passed with only minor modifications through the treatment. Cultivations in sewage sludge were either oxygen or carbon limited. One strain able to excrete lysozyme was isolated. It might have a synergistic effect on the heat inactivation of pathogenic microorganisms (cryptic growth) although lytic activity remained very low. Two-thirds of the entire metabolic activity is due to degradation of insoluble matter. The utilization of particulate matter also has a positive influence on the efficiency of the process by reduction in dry matter and increase in water-removal properties. Even at extremely low aeration rates, the acidification effect was small. Only small amounts of isobutyrate, isovaleriate and 2-methylbutyrate were formed at extremely low aeration rates and caused an increase in the total volatile fatty acid content after 12 and 36 h cultivation time.

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Introduction

Sewage sludge is a very heterogeneous substrate. Its composition not only depends upon its origin but also on the system and efficiency of the waste-water treating plant. Primary and secondary sludge are products of waste-water treatment. Different processes are used to stabilize the sludge including the establishment of an anaerobic stage. In order to inactivate pathogenic microorganisms, an aerobic thermophilic treatment can be integrated as a pre-treatment stage.

Until now the activities of aerobic thermophilic microorganisms naturally present in sewage sludge either as spores or vegetative cells have been examined on synthetic or semi-synthetic substrates (Baier 1987; Hamer and Mason 1987) in order to evaluate potential metabolic pathways. These organisms, described as *Bacillus stearothermophilus* strains (Baier 1987) can metabolize a wide spectrum of substances and are also able to grow on intact yeast cells (Hamer and Mason 1987).

Only a little work has been done to evaluate the chemical, biological and physical composition of sewage sludge because of the complexity of this medium. Many potential organic materials, salts and trace elements are present in raw sludge (Koppe and Stozek 1986; Siegrist et al. 1988). The heavy metal content might even be toxic to microorganisms in sewage sludge (Tomlin and Forster 1988). Chemical parameters have a great influence on the growth of aerobic thermophilic microorganisms on such heterogeneous substrates. Basic information for the development and control of an efficient process has been obtained from the determination of limiting growth factors and the evaluation of substrates that are metabolized.

Materials and methods

Organisms

A mixed population of thermophilic bacteria present in sewage sludge was used for all experiments. These organisms were isolated, characterized and described as *B. stearothermophilus* strains (Baier 1987). The organisms develop spontaneously after heating to 65° C and aerating the sludge. The other organisms used were *Micrococcus luteus* (DSM 20030), *Escherichia coli* (DSM 1103), *Staphylococcus aureus* (DSM 1104), and *Pseudomonas aeruginosa* (DSM 1117).

Media

Sewage sludge. Sewage sludge was collected at the Werdhölzli waste-water treatment plant in Zürich, Switzerland, at 8° C in winter and 15° C in summer. This mixture of primary and secondary sludge was harvested before pasteurization so that no heat pretreatment was applied. Approximately 300 l sludge were collected for each series of tests in a pre-sterilized storage vessel (Bioengineering AG, Wald, Switzerland). The sludge was then tyndallized by heating rapidly to 35° C and maintaining this temperature for 2 h. After rapid cooling to −4° C the sludge was maintained at this temperature overnight. After 12 h a second heat treatment at 35° C for 2 h was applied and finally the sludge was cooled down to −2° C and maintained at this temperature with little agitation during the series of tests. At this temperature the sludge was at the freezing-point limit. The average dry matter content was 4.4% and the average organic matter content 65% of dry matter. This sludge was called untreated raw sludge.

Separation of soluble and non-soluble components of sewage sludge. Sewage sludge was centrifuged at 4° C and 8500 g 90 min. The supernatant was filtered through a glass microfibre filter (Whatman GF/A). The particulate, non-soluble components were resuspended for further analyses. Both components were utilized as a substrate for cultivation experiments.

Synthetic media. The cultivation medium was a modified *Bacillus* medium (Foerster 1983) with the following composition per 1 l water: glucose, 10 g; yeast extract, 0.1 g; KH_2PO_4, 0.5 g; $(NH_4)_2HPO_4$, 1 g; $MgSO_4$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $ZnSO_4 \cdot 7H_2O$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.02 g; $MnSO_4 \cdot H_2O$, 0.014 g. Cultivations on protein were performed by replacing glucose with soya protein (Purina PP 760; Protein Technologies International, Zaventem, Belgium). Cultivations for measurement of lytic enzyme activity or precultures of mesophilic strains were carried out in nutrient broth (Deutsche Sammlung für Mikroorganismen 1983).

Cultivation conditions

A fed-batch cultivation technique was employed in all experiments. Mean residence time was always 14 h. The necessary amounts of sludge were replaced by untreated sludge after distinct intervals which were varied. Original inocula were prepared by starting a batch culture with raw sludge which provided both substrates and microbial populations.

Bioreactors

Experiments were carried out in a 7-l compact loop reactor filled with 4 kg sludge. Degassed sludge had a density of 1030–1050 kg m^{-3} at 20° C. The bioreactor was equipped with an external foam breaker. A detailed description of the bioreactor is found elsewhere (Hess 1988). In addition to the standard equipment an activated charcoal filter was very efficient at reducing odour in the exhaust gas. The filter was positioned after the exhaust gas analysis unit.

Sterilization

Experience with thermophilic organisms (Baier 1987; Brock 1986) shows that sterilization at 121° C for 2 h may be necessary. In order to avoid any initial contamination with organisms not originating from the sludge, the reactor and storage vessels were sterilized under these conditions. Media other than sludge and peripheral equipment were sterilized at 121° C for 20 min.

On-line analyses

Sensors for temperature as well as pH and oxygen partial pressure (Ingold AG, Urdorf, Switzerland) were standard. Exhaust gas analysis was carried out for oxygen and carbon dioxide with a paramagnetic O_2- (Oxygor 6N; Maihak, Hamburg, FRG) and an infrared CO_2-analyser (Unor 4N; Maihak).

Off-line analyses

Proteolytic activity. Proteolytic activity was measured with different chromogenic substrates: Azocoll 100–250 and 80–150 mesh (Calbiochem; Behring Diagnostics, San Diego, Calif, USA); Azocasein (Sigma; St Louis, Mo, USA); Hide Powder Azure (Calbiochem). The best substrate found with standard enzyme Protease K type XI (Sigma P-0390) and with cultures was Azocasein. It showed better sensitivity compared to the other chromogenic substrates.

Supernatant (50 µl) centrifuged at 13000 *g* for 10 min or culture were added to 2.5 ml of 1% Azocasein suspension in 6.7×10^{-2} *M* phosphate buffer at pH 7 or boric acid/potassium chloride sodium hydroxide buffer at pH 9 and incubated at the desired temperature in a water bath. After 2 h, 1 ml of 10% trichloroacetic acid was added and the precipitate was centrifuged at 5000 *g* for 10 min. The absorption of the supernatant was measured against the blank at 376 nm. Standard calibrations were done with protease K type XI (Sigma) in concentrations between 2 and 20 munits ml^{-1}. All test tubes used were sterilized at 121° C for 20 min to remove residual proteolytic activity possibly deriving from detergents.

Ammonium concentration. Ammonium concentration was measured colorimetrically with the Berthelot reaction (Merck 1974). The absorption measured was linear in the range 10–50 mg l^{-1} ammonium.

Lipase activity. Lipase activity was determined by a method based on the catalytic action of lipase on an olive oil suspension and measurement of the liberated fatty acids (Tietz and Fiereck 1966).

Cellulase activity. Cellulolytic activity was measured with the chromogenic substrate cellulose azure (Calbiochem). Supernatant (50 µl) centrifuged at 13000 *g* for 10 min or culture were added to 2.5 ml of 1% cellulose azure suspension in 6.7×10^{-2} *M* phosphate buffer at pH 7 and incubated at the desired temperature in a water bath. After 2 h reaction was

stopped on ice and the mixture centrifuged at 5000 *g* for 10 min. The absorption of the supernatant was measured against the blank at 595 nm.

Amylase activity. Total amylase activity was measured with the chromogenic substrate amylopectin azure (Calbiochem) and the same standard method applied to the determination of cellulolytic activity.

Extraction of lysozyme. The pyridine method (Alderton et al. 1945) was modified and utilized in order to extract lysozyme from sewage sludge: 5 ml of sewage sludge were centrifuged at 5000 *g* for 10 min. The pellet was washed with 2 ml of 6.7×10^{-2} *M* phosphate buffer, pH 7, and centrifuged at 5000 *g* for 10 min. The pellet was washed three times with 2 ml of 5% aqueous pyridine solution. The centrifugate from the final washing step was treated twice with 1 ml of 5% sulphuric acid-pyridine solution at pH 5. All the supernatant fractions were collected and tested for lysozyme activity.

Activity of lysozyme. Complete sewage sludge, the particles and cell-free supernatant and the lysozyme extract (described above) were tested with three methods in order to detect lysozyme activity. The first method, using a fluorogenic substrate (Yang and Hamaguchi 1980) was modified as follows: 200 µl of test substance was pipetted into 800 µl of a 2.84×10^{-5} *M* solution of 4-methylumbelliferyl-N-acetyl-chitotrioside (Sigma M 5639) in phosphate buffer, pH 7, and incubated for 1 h at 42° or 65° C. The reaction was stopped by adding 2 ml glycine buffer at pH 12. The amount of 4-methylbelliferone was determined by measuring fluorescence at 450 nm (excitation at 360 nm). With standard egg lysozyme solutions the linearity of calibration solution was confirmed and the lowest detectable amount was found at 20 units.

The second method was based on measurement of the decrease in absorption of a suspension of *M. luteus* in the presence of lysozyme (Weisner 1985) without modification.

The third method was a modification of the lyso-plate method (Elliott et al. 1966) originally developed to detect low lysozyme activity in serum and urinary body fluids. The *M. luteus* was cultivated in 500 ml nutrient broth during 48 h at 30° C. After centrifugation at 8000 *g* for 10 min the cells were resuspended in 50 ml physiological sodium chloride solution (9 g l^{-1}). Nutrient agar was prepared and cooled after sterilization to 45° C. The cell suspension was mixed with agar and poured into petri dishes (20 ml suspension each). After solidification of the agar a 7-mm diameter slot was punched out; 80 µl of suspension or standard solution were transferred into the slot and after incubation at 30° or 65° C for 48 or 12 h, respectively, the diameter of the lytic area was measured. The activity of lysozyme was logarithmically proportional to the diameter and the lowest detectable amount was found at 20 units.

Volatile fatty acids. Volatile fatty acids (C2-C6) were determined using a gas chromatograph (Hewlett Packard, 5830 A) with 60/80 Carbopak C/0.3% Carbowax 20M/0.1% H_3PO_4 (Supelco, Bellefonte, PA, USA) at 125° C with N_2 at 60 ml min^{-1} and a flame ionisation detector. Sludge samples were acidified by mixing one volume of sample with one volume of 4% formic acid and then centrifuged for 10 min at 13000 *g*. The supernatant was filtered through a glass microfibre filter (Whatman GF/A) and, if necessary, diluted. Concentrations as low as 2 ppm could be detected by this method by injecting 1-µl samples; pivalic acid was used as internal standard.

Mass spectrometer. Analyses of gas composition in the storage vessel head space were done by sampling 1 l gas in a quadrupole mass spectrometer (PGA 100, Leybold Heraeus, Cologne, FRG).

Calculations

The oxygen uptake rate and carbon dioxide production rate were calculated from the data of the exhaust gas analysis and the gas flow rate using mass balancing techniques (Fiechter and von Meyenburg 1968). The integral oxygen uptake rate (OUR) and carbon dioxide production rate (CPR) over the time was calculated by step integration.

Results and discussion

Tyndallization of sludge

A very important precondition for experiments with a complex substrate like sludge is to maintain the substrate unchanged during a storage period of several weeks (i.e. during the series of experiments). A first batch was stored at −2° C without any pretreatment and used up during a 2-month experimental period: acetate, butyrate and propionate concentrations increased during this period and methane in the head space increased (Fig. 1); oxygen and carbon dioxide decreased. Mesophilic and thermophilic plate counts showed no significant changes. These changes in the concentrations of acetate in the storage vessel and of methane in the head space indicated that there was a low activity of psychrophilic microorganisms even at −2° C without any pretreatment. In order to avoid these changes and to avoid submitting the sludge to a relevant pretreatment (that could falsify the results) a soft

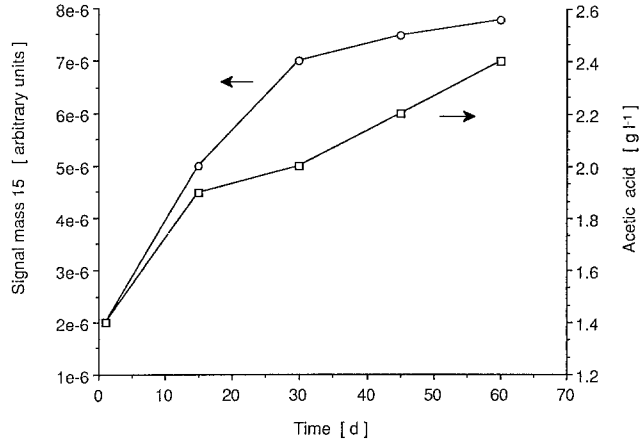

Fig. 1. Time course of concentrations of acetate in the storage vessel and methane (mass spectrometer signal m/z 15) in the head space during the storage period. No pretreatment had been applied to this sludge

inactivation method for psychrophilic microorganisms was developed. Tyndallization as described in Materials and methods was effectively applied. This method is, indeed, very efficient in inactivating obligately psychrophilic microorganisms so that storage over long periods is possible without any changes, as proved by analyses of the variables mentioned above (data not shown).

Cultivation of thermophilic microorganisms on sewage sludge

Batch and fed-batch cultivations were carried out under different cultivation conditions. Reproducibility of the cultures was controlled by fourfold replicates under identical conditions. All these experiments gave a similar time course of the OUR. Results obtained in a typical batch cultivation are shown in Fig. 2.

Foam formation was always detected starting in phase 3. Therefore, a mechanical foam breaker was necessary; chemical antifoam such as polypropyleneglycol was not efficient and beyond the scope of practical application. The external foam breaker (Hess 1988) was very efficient in destroying foam even at extremely high aeration rates (> 1 vvm).

During the decrease in OUR in phase 5 (i.e. after the increase of pO_2), different carbon sources, salts, trace elements and vitamins were pulsed to the culture in order to determine the type of limitation responsible for the decrease in this activity. The results are summarized in Table 1 and clearly show that the only limitation occurring was carbon limitation (oxygen was definitely not limiting because pO_2 was always more than 80% during these pulse experiments).

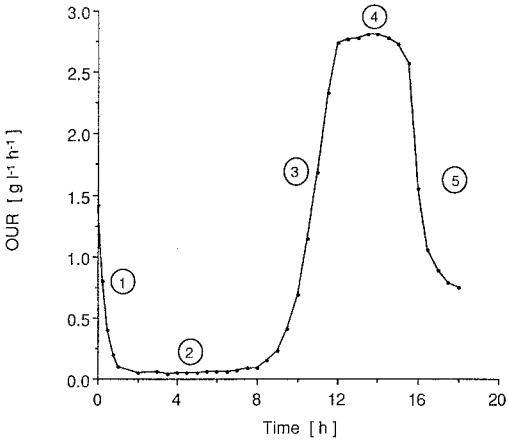

Fig. 2. Time course of the oxygen uptake rate (OUR) during cultivation of aerobic thermophiles in sewage sludge at 65° C, 0.5 vvm airflow, 1500 min^{-1} and pH 7. *Phase 1,* inactivation of non-thermophilic microorganisms; *phase 2,* lag phase of the thermophiles; *phase 3,* exponential growth of the thermophiles; *phase 4,* oxygen-limited phase; *phase 5,* carbon-limited phase with a decrease in the activity of thermophilic biomass and partial sporulation of thermophilic populations

Activities in sewage sludge and pure cultures

The pulse experiments indicated the presence of proteolytic activity because of growth on casein, gelatine and soya protein. This was demonstrated by measuring the activity of total proteolytic enzymes and also by isolating a mixed sub-population of thermophiles which grew on semi-synthetic media with protein as the sole carbon source. The proteolytic activity correlated with the increase in OUR, indicating a growth-associated production of the enzymes and correlated also with the increase of free ammonium in the culture. The respiratory quotient (RQ) had an average value of 0.82, which is typical for oxidative metabolism of proteins (Lentner 1981). Cultivations on the synthetic medium showed that the proteolytic activity was not cell wall bound. On

Table 1. Carbon source pulsed at a concentration of 4 g l^{-1} and salts, trace elements and vitamins pulsed at the concentrations described by Kenkel and Trela (1979) to thermophilic sludge cultures during the carbon-limited phase (phase 5)

Substance	Oxygen uptake rate immediately after pulsing
Glucose, fructose	+
Maltose	+
Sucrose, lactose	+
Xylose, ribose	+
Pyruvate, glycerol	+
Glutamic acid	+
Valine	+
Casein	+
Soya proteins	+
Gelatine	+
Starch	–
Pectin	–
Cellulose	–
Olive oil	–
Micrococcus luteus[a]	–
Salts	–
Trace elements	–
Vitamin medium D	–

[a] Suspension of 10.2 g l^{-1} wet weight

An increase in oxygen uptake rate (OUR) is marked as + response; no change in OUR is marked as – response

the other hand, analyses in the supernatant of sludge cultivations gave no detectable activity. This strongly indicates that the enzymes are excreted into the medium but are immediately absorbed by particles in the case of sludge cultivations.

The RQ value of all cultivations on sewage sludge showed an average value of 0.82 during exponential growth and the oxygen-limited phase. This value rose to 1.1 in the carbon-limited phase, indicating changes in metabolism (possibly mobilizing internal storage materials). Control cultures on the same semi-synthetic media with the same inocula but with glucose as sole carbon source showed an average respiratory coefficient of 1.05 which is typical for full oxidative growth on carbohydrates. The RQ values, proteolytic activity, pulse experiments with protein substrates and increase in free ammonium strongly indicate that the thermophilic populations preferentially utilize proteinaceous material as the carbon source.

Other polymers are present in sewage sludge and represent a potential substrate for thermophiles. Determinations of lipase, amylase, cellulase and pectinase activity, however, gave negative results with either the original methods described or after modification and adaptation of the analytical methods to the complex cultivation media. Figure 3 shows a time course of proteolytic activity and the RQ of one representative sewage sludge culture and Fig. 4 shows the proteolytic activity with respect to temperature and compared with a commercial protease.

The temperature dependence of proteolytic activity was measured in cultures on raw sludge during aerobic thermophilic treatment and in

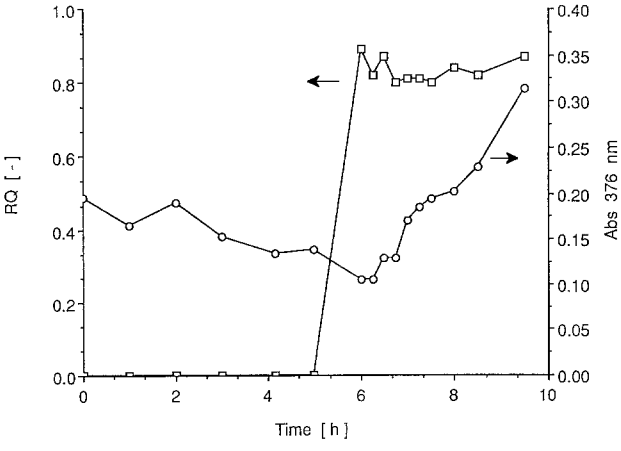

Fig. 3. Proteolytic activity absorption at 376 nm (Abs 376) and respiratory quotient (RQ) as a function of time during batch cultivation of sewage sludge at 65° C, 0.5 vvm, 1500 min^{-1}

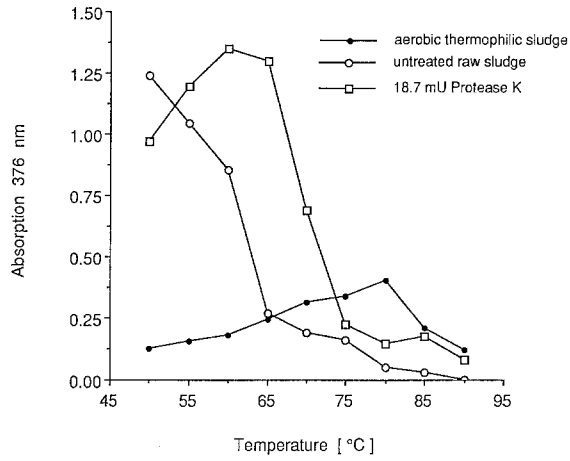

Fig. 4. Temperature dependence of proteolytic activity (Abs 376) for raw- and ATS-sludge and protease K; U = units

semi-synthetic media with soya protein. The proteolytic activity during the sludge treatment had an optimum at 80° C. This temperature dependence was also found in semi-synthetic media and is of interest when compared to the commercial enzyme, protease K, which shows a maximum at 60° C. Raw sludge had also a high proteolytic activity, especially at low temperatures, but at 80° C the activity was almost zero. Therefore, growth of the thermophilic populations could be followed by measuring protease activity at 80° C.

Lytic activity

One of the most important objectives of the aerobic thermophilic treatment of sewage sludge is the inactivation of non-thermophilic bacterial populations, especially of pathogens, viruses and parasite eggs. Thermophilic populations isolated from sewage sludge were able to grow in a semi-synthetic medium utilizing intact yeast cells as substrate (cryptic growth: Hamer and Mason 1987). The production of a lytic enzyme from induced *B. stearothermophilus* cultures has also been described (Welker and Campbell 1966). These observations stimulated the investigation of lysozyme production by mixed populations of *B. stearothermophilus* strains grown on sewage sludge.

Sewage sludge contains about 10^7–10^8 mesophilic colony forming units (cfu) per millilitre. Of course, a certain amount of non-active cells with an intact cell wall can also be present in the sludge. These cells could be utilized by the thermophiles as a substrate, but in order to do this the first obstacle is to digest the bacterial cell wall.

The enzyme responsible is expected to be a lysozyme (muramidase or endo-β-glucanase). However, assuming the maximum cell content in sewage sludge to be not greater than 10^9/ml (which is at least one order of magnitude greater than cfu found but including dead cells), an average particle size of 1×5 µm and 20% dry matter results in a total biomass not greater than 0.8 g l^{-1}. One may further assume that this can be completely utilized by the thermophiles during growth. Therefore, this amount cannot significantly contribute as a substrate when compared to an average content of 30 g l^{-1} of organic material in sewage sludge.

Three different methods were used in order to diminish the uncertainty of the measures due to the complicated substrate. All methods allowed the detection of minimal amounts of lysozyme with standards. The extraction of lysozyme from cultures of sewage sludge showed a recovery of 85%–90% when standard enzyme solutions were added. Lytic activity could not be detected, neither in sewage sludge, nor in the supernatants of cultures or in the extracts of sewage sludge (in all phases of thermophilic growth). The method using fluorogenic substrate could be used only after dilution of the sample because of the strong background fluorescence of raw sewage sludge. The increase in 4-methylbelliferone release was extremely low and not significant.

Mixed cultures of *E. coli, P. aeruginosa* and *S. aureus* were pulsed during the lag-, exponential- and carbon-limited phase of thermophilic cultures. The pulse concentrations were 10^8, 10^9 and 10^8 cfu ml^{-1}, respectively. Lytic activity was measured during the pulse experiments until inactivation of the pathogenic cells. Greatest increase in lytic activity was found when the pulses were applied in the carbon-limited growth phase (rather than in the other phases), but it was generally very low.

During these pulse experiments different populations of aerobic thermophilic strains were isolated and grown at 65° C on nutrient agar. After purification of the individual strains a screening of the pure strains for lysozyme production in lyso-plate agar was carried out. Among 43 different strains only one was positive. This indicated the presence of thermophilic bacteria with the capacity of releasing lysozyme during sludge treatment. However, the activity was so low that it could not be detected after extraction of the lysozyme from sewage sludge. Nevertheless, its contribution might be significant for the process. It can be speculated that the presence of lytic organisms growing at 65° C accelerates the heat inactivation effects on mesophilic cells.

Particulate and soluble substrates

Three parallel fed-batch experiments with the same inoculum were carried out on complete sewage sludge, on the soluble fraction, and on the resuspended particulate fraction of sludge in order to investigate the kind of components decomposed during treatment. The results clearly indicated that a selectively small amount (32%) of the thermophilic activity measured as the integral of the OUR was due to growth on soluble substrates. The main activity was bound to growth on particulate matter (68%). The sum of the integral OUR values from the cultivation with sludge particles and free supernatant was 95% of the integral OUR from cultivations on unseparated raw sludge and shows that no additional limitation was caused by the separation of the two fractions.

Production of volatile fatty acids in sewage sludge

The aerobic thermophilic treatment of sewage sludge can be formulated as (a) a full stabilization stage, or (b) a pretreatment prior to an anaerobic stage. In the latter case it would be advantageous to stimulate production of volatile fatty acids. Acetate, especially, could be directly metabolized by methanogenic bacteria in the following stage. Some authors have described aerobic thermophilic sludge as a possible "acidification stage" (Keller and Berninger 1984). In this study there was only a little production of acid found even at very low aeration rates.

Cultivations carried out at high aeration rates (1 vvm) and very low aeration rates (0.1 vvh) were analysed for the time course of volatile fatty acid concentrations. Under both conditions there was no production of acetate, propionate, *n*-butyrate and *n*-valeriate. These acids, if present in raw sludge, were fully metabolized during cultivation. Isobutyrate, 2-methylbutyrate and isovaleriate were produced during cultivation at very low aeration rates but were also utilized in the carbon-limited phase as substrates and disappeared totally.

The total acid content in the sludge increased only by 20% from the initial value in the first period of cultivation under oxygen limitation and reached the maximal value between 12 and 36 h

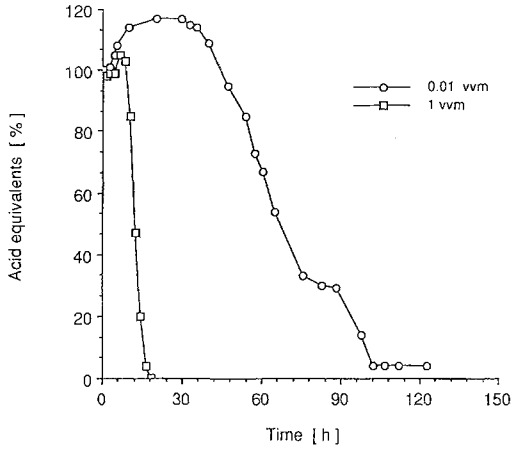

Fig. 5. Relative total fatty acid concentrations during batch cultivation at 500 min^{-1} at 0.1 vvm (absolute acid equivalent value at the start of cultivation; 45.2 mmol l^{-1}) and 1500 min^{-1} at 1 vvm (53.4 mmol l^{-1}), respectively. Both cultivations were at 65° C and pH 7

cultivation time (Fig. 5). The utilization of acids began after approximately 36 h and they totally disappeared after 100 h cultivation time. A low level of acid formation under severe oxygen limitation could be obtained depending on the cultivation time. The hydraulic retention time in the reactor is an important variable for process development.

Isomers of butyrate and valeriate were responsible for acidification. They are produced at the beginning of cultivation when other volatile fatty acids are not yet metabolized. The aerobic thermophilic populations are able to produce acetate in the presence of starch, mono- or disaccharides, but this condition is probably infrequent in sewage sludge so that the "acidification stage" (in the form of acetate production) represents an exception.

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