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MICROBIAL EXTRACELLULAR ENZYME DETECTION ON AGAR PLATES BY MEANS OF FLUOROGENIC METHYLUMBELLIFERYL-SUBSTRATES

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ABSTRACT - A rapid and sensitive method to detect the extracellular enzymatic activity of bacteria colonies grown on agar plates is described. Selective agar media supplemented with protein, starch, chitin, Tween-80, etc. are conventionally used to detect biochemical properties of bacteria. It has been experimentally demonstrated with bacteria pure cultures that fluorogenic Methylumbelliferyl (MUF) -substrates are excellent substrate analogues for normally occurring polymers. Based on MUF-substrate hydrolysis the new method provides reliable qualitative estimates of extracellular enzymatic properties of bacteria within minutes using pure cultures as well as agar plates prepared for colony counts.

Key words: microbial extracellular enzyme detection, methylumbelliferyl-substrates, agar plate.

RÉSUMÉ - Une méthode rapide et sensible de détection de l'activité enzymatique extracellulaire de colonies bactériennes se développant sur milieu gelosé est décrite. Des milieux sélectifs enrichis en protéines, amidon, chitine, Tween 80, etc. sont généralement utilisés pour détecter les propriétés biochimiques des bactéries. Il a été démontré expérimentalement sur cultures pures de bactéries, que les substrats au Methylumbelliferyl fluorogénique sont d'excellents analogues des substrats polymériques courants. Basée sur l'hydrolyse des substrats MUF cette nouvelle méthode fournit des estimations qualitatives fiables sur les propriétés des enzymes extracellulaires des bactéries. Le résultat est obtenu en quelques minutes en utilisant des cultures pures aussi bien que des colonies ensemencées sur agar.

Mots clés: détection d'enzymes microbiennes extracellulaires, substrats Methylumbelliferyl, milieu gélosé.

INTRODUCTION

Microbial hydrolysis of high molecular weight organic matter plays an important role in the substrate turnover of aquatic environments. It is assumed that high molecular weight organic matter is not directly utilized by bacteria but undergoes enzymatic hydrolyzation to low molecular weight substances before incorporation.

Many microbiologists have tried to identify bacteria on the basis of "physiological groups" for a more biochemically oriented analysis of the composition of a bacterial population. Sieburth (1971) and Kjelleberg and Hakansson (1977) have investigated the distribution of bacterial physiological groups in the sea on the basis of selective agar media supplemented with starch, protein, other polymers or Tween-80. In freshwater lakes Jones (1971) showed a seasonal fluctuation in separate populations of protease-, amylase- and lipase-producing bacteria. This methodological approach was developed, modified and also extensively used by many other microbiologists (Fred and Waksman, 1928; ZoBell, 1946; Sierra, 1957; Skerman, 1967; Holding and Collee, 1971). However, the selective media technique is rather time consuming and zones of enzymatic activity around colonies may very possibly overlap (e.g for amylase). Consequently, incubation times for plates must be shortened, and results cannot be directly compared to standard colony counts in every case.

Alternative approaches for the qualitative detection of extracellular enzymatic properties of bacteria are rare in the literature. Sizemore and Stevenson (1970) developed a marine agar-milk double-layer plate technique for the detection of proteolytic marine bacteria colonies. Paoni and Arroyo (1984) described a method which makes use of chromatogenic substrates (p-nitrophenyl-2-acetamido-2-deoxy-α-D-galactopyranoside) in detecting glycosidase activity of bacteria colonies on agar plates. Although these methods offer some advantages in comparison with the selective media approach, it is somewhat inconvenient to prepare double-layer agar plates. Furthermore, the quantitative assessment of chromatogenic reaction products (p-nitrophenol) is less precise than that of their fluorogenic counterparts (e.g. 4-methylumbelliferone) (Taylor *et al.*, 1977, Pettersson and Jansson, 1978).

Quantitative estimates of enzymatic activities of bacteria in aquatic environments have been made by several authors (e.g. Kim and ZoBell, 1974; Little *et al.*, 1979). Recently some investigators have applied fluorogenic substrates in highly sensitive methods suitable for direct *in situ* measurements of bacterial extracellular enzymatic activities (Hoppe 1983, Somville and Billen, 1983).

Among these fluorogenic substrates the butyryl ester of 7-hydroxy-4-methylcoumarin was employed to identify esterase-positive fungal colonies on mixed culture plates (Pancholy and Lynd, 1971). Littel and Hartman (1983) reported using a large number of fluorogenic substrates in order to differentiate species of fecal *Streptococci*. Now that quantitative estimates of biochemical activities of bacteria (e.g. polymeric hydrolysis by extracellular enzymes) can be made with high precision, the lack of adequate methods for the qualitative determination of "physiological groups" and single colony enzymatic properties is more apparent than ever.

In this report we describe a method for the detection of microbial extracellular enzymatic activity on agar plates with fluorogenic MUF-substrates. In addition, comparative studies of the hydrolytic activity of bacterial pure cultures on selective agar plates and enzymatic activity in a liquid medium supplemented with MUF-substrates are described.

Material and Methods

Bacteria inoculation

Natural water samples were diluted with sterile isotonic sea water and aliquot parts of dilutions were transferred to ZoBell 2216 E agar plates (spread plate method). Plates were incubated for 2 weeks at 20°C in the dark after which standard colony counts were obtained. The plates were subsequently used for enzyme detection procedures as described below. In addition, 10 colonies were randomly selected from these agar plates and each served to inoculate 7 agar plate parallels, which supplied material for 7 subsequent enzyme detection treatments. After colonies had developed on these plates, they were treated for extracellular enzyme production in the same manner as the standard agar plates.

Substrate preparation

7 fluorogenic substrates were used for the qualitative detection of extracellular enzymatic activities of bacteria. For example Methylumbelliferyl-α-D-glucopyranoside was used for the α-glucosidase of bacteria and so on as listed in Tab. 1. To detect the lipase two substrates were selected in order to check the difference between these two substrates (MUF-butyrate and MUF-heptanoate). A fluorogenic substrate consists of a fluorescent fluorophore (MUF) linked to an organic or inorganic substrate molecule (Fig. 1). The

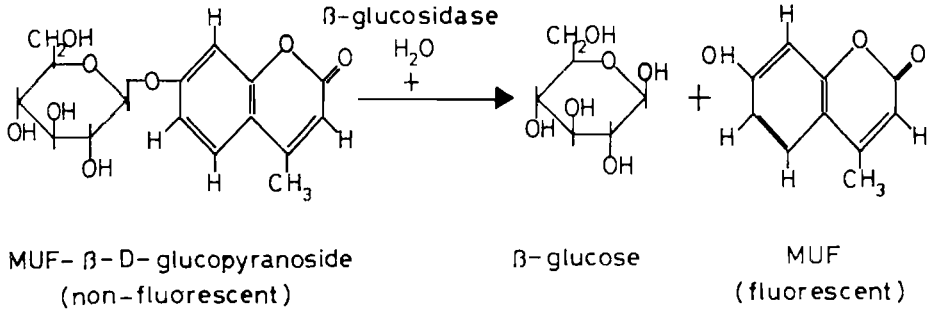

Figure 1 : The molecular structure of MUF-β-D-glucopyranoside MUF-β-D-glucopyranoside (nonfluorescent) is hydrolyzed by β-glucosidase into equimolar concentrations of β-glucose and free MUF (fluorescent). The concentration of free MUF is measured at 445 NM under 364 NM exitation in a spectrofluorimeter.

complex is nonfluorescent until the specific fluorophore-substrate bond is uncoupled by enzymatic hydrolysis. Equimolar quantities of the organic/inorganic compound and the fluorophore of the complex are set free during hydrolysis. Fluorophore (MUF) displays maximal fluorescence under illumination with long wavelength UV light (Exitation max. 365 nm). Stock solutions of MUF-substrates (5 mM l^{1-}) were prepared in methylcellosolve (ethyleneglycolmonomethylether; $C_3H_8O_2$) and stored at -25°C in the dark. Before the experiment stock solutions were diluted to 0.1 mM l^{1-} in various buffer systems. (working solutions), which were adjusted to the optimal pH for the desired enzymatic reaction (Table 1).

Substrate	Tested enzyme	Buffer system
4-Methylumbelliferyl-α-D-glucopyranoside	α-glucosidase	Tris/ HCl (ph 7.4)[1]
4-Methylmbelliferyl-β-D-glucopyranoside	V β-glucosidase	Tris/ HCl (pH 7.4)[1]
4-Methylumbelliferyl-N-acetyl-B-D-glucosaminide	N-acetyl-β-glucosaminidase	Phosphate/Citrate [2] (pH 4.95)
L-Leucine-4-methyl-7-coumarinylamide Hydrochloride	protease (aminopeptidase)	Tris/ HCl (pH 7.4) [1]
4-Methylumbelliferyl-phosphate	phosphatase	Tris/ HCl (pH 8.3) [1]
4-Methylumbelliferyl butyrate heptanoate palmitate 3)	Lipase (esterase)	Tris, HCl (pH. 7.4) [1]

Table 1 : Substrates and buffer systems used in this study

1 Tris 0.1 Ml^{-1}/HCL 0.1 N
2 0.2 Ml^{-1} Disodiumphosphate + 0.1 Ml^{-1} Citrid acid, toluene 20 ml/ l buffer as a preservative
3 This substrate was used for the lipase activity measurement of bacteria pure cultures in liquid medium.

Detection of specific extracellular enzymatic properties of bacteria colonies

Chromatography filter papers of the same size as the Petri dishes used were soaked with MUF-substrate working solutions. The soaked filter papers were then carefully layed on bacteria colonies grown on the standard agar plate (c.f. bacteria inoculation). One of the standard agar plates parallels was used for one MUF-substrate only. Care must be taken to avoid air bubble formation between the agar layer and the soaked chromatography filter paper because entrapped air bubbles result in decreased sensitivity. After *ca.* 3 minutes at room temperature the filters were removed from the agar surface and placed on a glass plate or a clean and empty Petri dish. The filter papers were then observed under an HBO fluorescence microscope lamp (365 nm) and the resultant fluorescent spots were counted. A conventional UV lamp could not be used for this purpose.

Because MUF has its maximal fluorescence at alkaline pH values (optimal pH 10.3) the filter papers were exposed to concentrated NH_3 vapors before observation to enhance fluorescence intensity (Fink and Kochler, 1970, Guilbault 1973). Exposure to concentrated NH_3 in a desiccator lasted about 1 minute. NH_3 treatment was especially important when MUF-glucosaminide was applied because the working solution for this substrate has a pH of 4.95 which permits only a very low fluorescence emission. With other MUF-substrates the working solution pH is already near the pH of maximal fluorescence and only minor improvement of fluorescence intensity was observed.

Substrate analogue tests

To test whether or not MUF-substrates are analogues for polymeric substrates commonly used for the detection of biochemical properties of bacteria (e.g. gelatin, starch, chitin and Tween-80 (sorbitan monooleate)) we examined the response of pure cultures towards the two types of substrates. The pure cultures were isolated from the Baltic Sea and from the Atlantic Ocean (The Azores, Portugal) and cultivated in ZoBell 2216 E liquid medium with a salinity of 0.8 % and 2.4 %, respectively. For this purpose the cells were harvested and washed three times with autoclaved brackish water (sea water/distilled water mixture 1 : 3) via moderate centrifugation. The washed bacteria pure culture were suspended in autoclaved brackish water and separated into four 20 ml portions. One of the substrates was then added aseptically to one of the subsamples. The final concentration of MUF-substrates was 2 $\mu M l^{-1}$. Incubation was performed at 20°C in the dark. Relative fluorescence intensity of samples was measured three times, i.e. immediatly after substrate addition, after 4 hours and after 24 hours, with a spectrofluorometer (Jasco model FP-1050, excitation wavelenth 364 nm, emission wavelength 445 nm). Growth patterns of the same bacteria cultures were comparatively studied by the use of ZoBell agar medium supplemented with one of the following substrates: 0.4 % gelatin, 1 % soluble starch, precipitated chitin (precipitated chitin was added until the agar medium became milky in appearance) or 1 % Tween-80. The supplemented media were autoclaved at 125°C for 20 minutes and poured into sterile Petri dishes. The liquid pure cultures were streaked on the prepared agar plates with a loop. Inoculated agar plates were incubated at 20°C for 2 weeks. Chitinase and lipase activity of each colony on the chitin-and Tween-80-containing media was observed directly, as indicated by a distinct clear zone and halo zone around active colonies, respectively. Protease was detected by applying a layer of mercuric chloride-hydrochloric acid coagulant solution to the plate resulting in a halo around active colonies. Amylase was similarly determined using dilute Lugol's solution leading to a starch-iodine reaction indicating zones of hydrolysis.

Results and Discussion

Some examples from our work on extracellular enzymatic activity of marine bacteria are provided here to demonstrate the applicability of the new technique for ecological as well as physiological field studies. It should be premised that photographic documentation of the results is not fully satisfactory due to the effects of long-wave UV radiation and the dark blue background emission of the filter paper disks treated with MUF-substrate. Furthermore, light emission from the HBO microscope lamp used here was not evenly distributed over the total area of the filter paper. Light scattering did not fully prevent this effect because it is always associated with a certain degree of light extinction. All these implications are, however, not inherent problems for the visual observations of enzymatic reactions on the filter paper disks. For the human eye the background (filter paper) appears nearly black and the reactive spots glow intensively light blue or are simply bright. Colonies lacking extracellular enzymatic activity do not provoke any MUF fluorescence on the filter paper. Moreover, the somewhat irregular excitation irradiance from the UV-lamp is not visible to the unaided eye.

Extracellular enzymatic activities of standard ZoBell agar plates

Inoculated with water from the Kiel Fjord, a ZoBell agar plate, as it is used to obtain standard colony counts, is presented in Fig. 2a. Fig. 2b shows the filter paper which was soaked with MUF-leucine and exposed to the agar plate colonies for 3 mn. Spots of enzymatic reaction become visible with long-wave UV irradiation. Even those spots caused by very small colonies are clearly recognizable. Two relatively large colonies did not exhibit protease activity in the sense of MUF-leucine breakdown.

Extracellular enzymatic activity from selected colonies

7 agar plates were inoculated with 10 randomly selected colonies from standard ZoBell agar plates. One example is given in Fig. 3a. Some enzymatic activities (protease, phosphatase, lipase) are documented with photographs (Fig. 3b-d). The complete results are shown in Tab. 2. It becomes obvious that esterase as well as phosphatase and protease activity was nearly a universal feature of these colonies, some of which lacked abilities for enzymatic α-glucoside, β-glucoside and N-acetyl-β-glucosaminide decomposition. It was

No. of colony[1]	α-glucosidase	β-glucosidase	N-acetyl-β-glucosaminidase	protease	phosphatase	Esterase[2]	
						but.	hep
1	+	+	—	+	+	+	+
2	+	+	+	+	+	+	+
3	—	+	+	+	+	+	+
4	+	+	+	+	+	+	+
5	—	+	—	—	+	+	+
6	+	—	+	+	+	+	+
7	—	+	—	+	+	+	+
8	—	+	—	+	-	+	+
9	+	+	+	+	+	+	+
10	+	—	+	+	+	+	+

Table 2 : Comparison of enzymatic activities which were obtained from the 10 bacteria isolated from standard agar plates (Kiel Fjord).

+ positiv, —, negativ 1 see Fig 3a 2 see Tab. 1

interesting to see that at least one glucose compound could be split by each of the bacteria colonies. Analysis of this type could be used to characterize extracellular enzymatic activities of bacteria isolated from such ecological niches as macroalgal surfaces, outer and intestinal surfaces of zooplankton, interfaces, etc... There is some indication that enrichment of polymers or continuous excretion of certain compounds leads to a stabilization of a corresponding microflora in these environments. For activities and properties of bacteria living in association with benthic macroalgal mats this has been demonstrated by Mow-Robinson (1983).

The question still open is whether enzymatic response of bacteria towards conventional 'selective' media and to MUF-substrates is the same. Results from corresponding experiments conducted with pure culture bacteria from the Baltic Sea and from the Atlantic (Azores) are listed in Tab. 3. It is clearly evident that in most cases growth and extracellular enzymatic activity occurred with MUF-substrates when the bacteria strain in question also grew on the natural polymeric substrate analogue (selective medium). There were a few exceptions from this general pattern of observation, this may account for bacterial growth on supplementary substrates other than the selective agent or it may demonstrate that in a few cases MUF-substrates, more similar to dimeric molecules, are not suitable substrate analogues for the polymers in question. However, it must be emphasized that in the majority of the cases MUF-substrates were, indeed, good analogues for naturally occurring substrates and growth as well as qualitative enzymatic activity can be tested with them. The main problem involved in this kind of study is, of course, the quality of substrate analogism of MUF-substrate and whether only extracellular enzymes are considered. In the literature it has been shown that hydrolysis of MUF-α-glucoside and MUF-β-glucoside is competitively inhibited by maltose and cellobiose, respectively (Hoppe, 1983).

No. of bacterial pure culture	MUF—leu	Gelatin	MUF—α—glu	Starch	MUF—glucosa	Chitin	MUF—pal	Tween—80
1763	++	+	+	+	++	+	++	+
1766	++	+	+	+	++	+	+	+
1777	++	+	+	+	++	+	+	+
1782	+	+	+	+	+++	+	+++	+
1787	+	+	++	—	—	—	++	+
1788	++	+	+	+	++	+	++	+
1789	+	+	+	+	+++	+	+++	+
1796	++	+	+	+	++	+	++	+
1808	+	+	—	—	—	—	—	+
1810	+	+	—	—	—	—	—	+
1851	++	+	+	+	++	+	++	+
1863	+++	+	+++	+	—	—	+++	+
1865	+	+	—	—	—		++	+
1873	++	+	++	+	—	—	—	—
1901	++	+	—	—	+++	+	++	+
1927	+++	+	—	—	+++	+	+	+
1930	++	+	—	—	+++	+	—	+
1956	—	+	++	—	—	—	—	+
1957	+++	+	+		—	—	+++	+
1966	—	+	++	-	+	—	—	+
1967	+++	+	—	—	—	—	++	+
1989	++	+	—	—	—	—	+++	+

Table 3 : Comparison of methodological approaches for the detection of bacterial enzymatic activities Growth of bacteria pure cultures in liquid MUF-substrate media is compared to growth on selective agar plates which were supplemented with polymeric substrates.

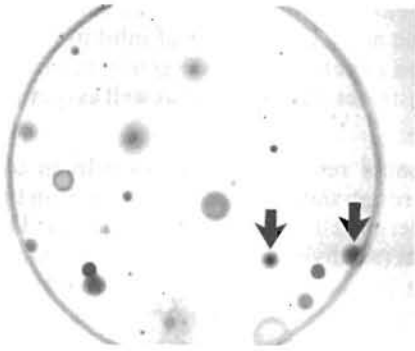

Figure 2a : Bacterial colonies from sea water developed on ZoBell agar plate photographed under visible light illumination. Arrows indicate colonies which lack protease activity, see Fig. 2b.

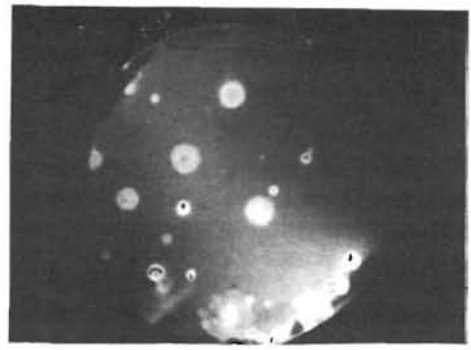

Figure 2b : The corresponding chromatography filter paper soaked with 1.0×10^{-4} M MUF-leucine in a Petri dish protographed under 365 nm UV light illumination (c.f. Fig 2a).

Fig. 3. Enzymatic activities of bacteria pure cultures investigated by means of MUF-substrates Bright spots indicate enzymatic decomposition of the substrate in question.

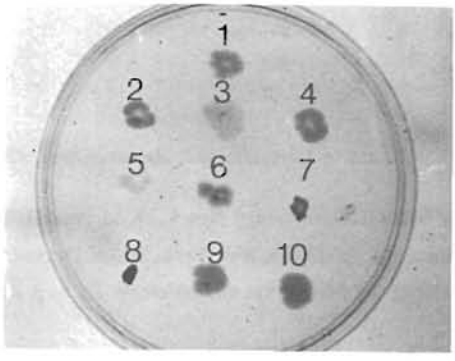

Figure 3a : ZoBell agar plate with 10 bacteria pure culture originating from standard agar plates for the enumeration of population.

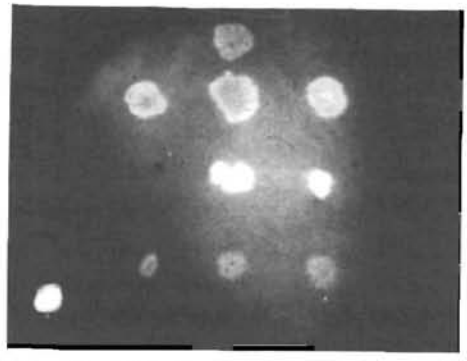

Figure 3b : Colonies capable for the decomposition of MUF-leucine (protease activity).

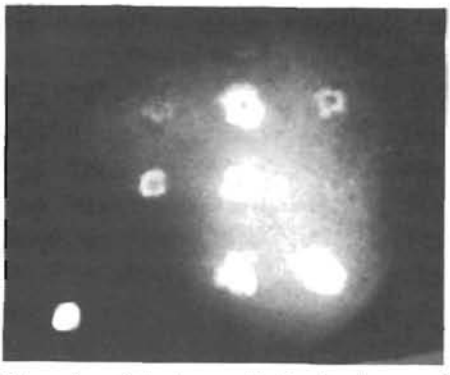

Figure 3c : Colonies capable for the decomposition of MUF-phosphate (phosphatase activity)

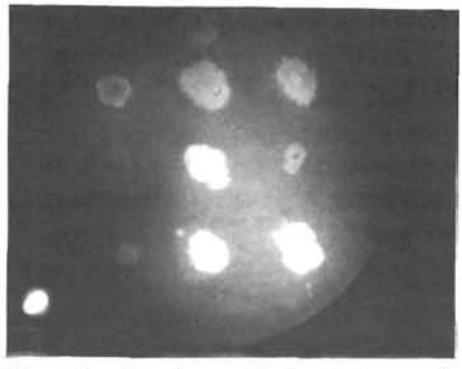

Figure 3d : Colonies capable for the decomposition of MUF-butylate (esterase activity).

Enzymatic decomposition of MUF-leucine is weakly inhibited by serum albumin and MUF-phosphate is inhibited by phosphogluconic acid. The manner of inhibition is not clear in the latter two cases. Further competition experiments are urgently required to more precisely define the usefulness of MUF-substrates in qualitative as well as quantitative studies of the enzymatic activity of bacteria.

Liberation of intracellular bacterial enzymes could result from lysis of cells in older bacteria colonies. This cannot be fully avoided through shortening of the incubation time, because some colonies may grow faster and undergo cell lysis earlier than others. There may also be some criticism concerning the solvent (methylcellosolve) used to prepare the stock solution of MUF-substrates. However, it has been shown that the effect of the solvent at its working solution concentration is negligible. The usage of water as a solvent for those MUF-substrates sufficiently water soluble (MUF-β-D-glucoside, MUF-N-acetyl-β-D-glucosaminide, MUF-phosphate) is, nevertheless, recommended.
The number of colony-forming bacteria (saprophytes) plays an important role as an easily assessable indicator of pollution and of autochthonous organic matter accumulation. In this regard the identification of "physiological groups" of saprophytes could lead to a better understanding of organic matter cycles in ecosystems. Despite the uncertainties still involved, the described method can be used as a rapid and sensitive measure of extracellular enzymatic properties of saprophytes and could also be a promising tool in the research of mutants in physiological studies.

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