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A METHOD FOR THE PRODUCTION OF SUSPENSIONS OF ANAEROBIC MICRO-ORGANISMS

by

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In the study of the enzymatic capacities of micro-organisms it is essential to be able to produce large amounts of cells if the enzyme in question is to be extracted from the cells or from the substrate or if the biochemical activity of intact cells is to be studied. In order to create optimum conditions for cell multiplication in liquid media it is important that the substrate should be kept in motion throughout the cultivation period. Thus, the cultivation is usually performed in shaking machines. To a certain extent the shaking of the culture also prevents clumping and aggregating of the cells, a factor of importance when the activity is to be related to the number of living cells as determined from ability to colony formation on solid media.

The production of suspensions of living strictly anaerobic micro-organisms is sometimes extremely difficult, as good and rapid growth is obtained only at a very low oxygen tension. In fact, many of these organisms can be cultivated only in a hydrogen atmosphere almost or completely free from oxygen, since the oxidation-reduction potential is sufficiently low only under such circumstances.

The authors have worked out a method for the cultivation of anaerobic micro-organisms in liquid media under hydrogen-carbon dioxide atmosphere and under constant stirring (Fig. 1).

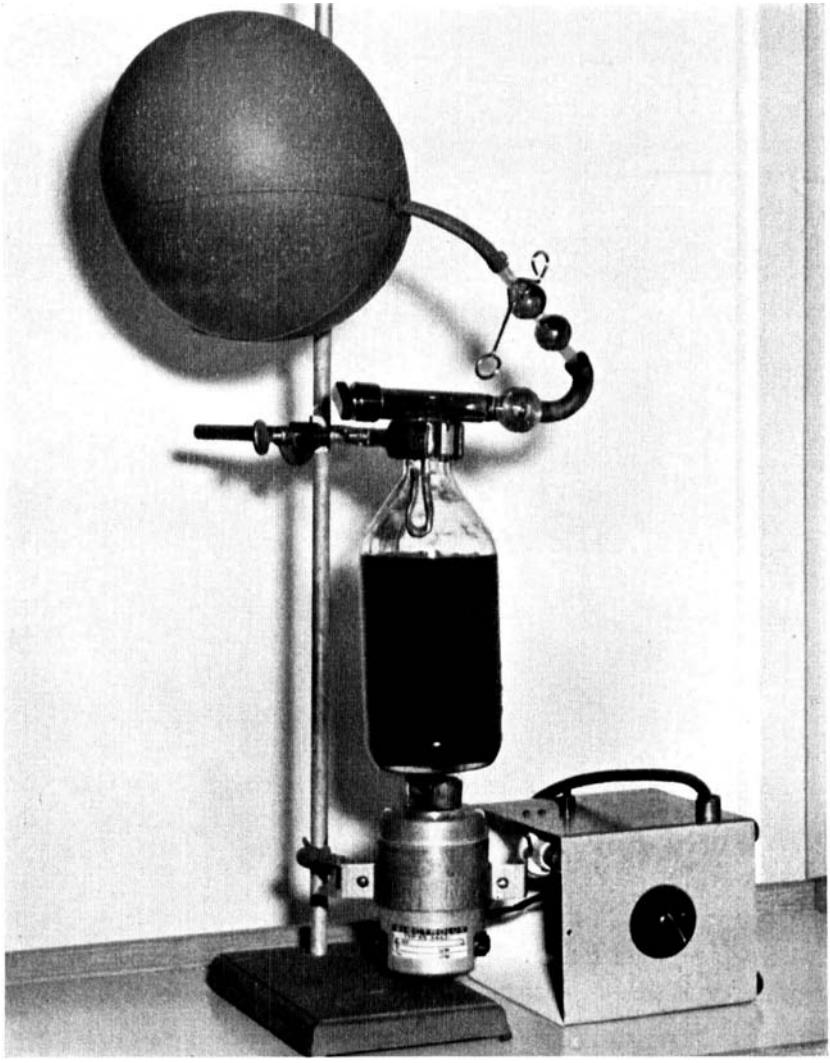


Fig. 1. Arrangement for anaerobic culture in liquid media.

METHOD

The substrate is sterilized in the usual way in a glass flask containing 1 litre of medium. The gas volume over the surface of the substrate was about 1 decilitre. The neck of the flask was thread-coiled and closed with a screw-cap during sterilization.

After the substrate was inoculated, a special Teflon-covered magnet stirrer* was transferred to the flask aseptically. The magnet was sterilized in the autoclave enclosed separately in paper.

Subsequently a special apparatus (Fig. 2) was applied to the flask. It is made of brass plated with chromium and consists of a chamber containing a palladium-asbestos catalyst enclosed in a brass net. At one end of the chamber there is a gas tap and at the other end an opening closed by a screw, which permits the inspection and exchange of the catalyst. To the gas tap a glass tube containing a cotton filter is connected by a rubber tube (Fig. 3).

The apparatus with the cotton filter may be sterilized separately in the autoclave enclosed in thick paper.

When the apparatus was applied to the flask the filter opening was connected to a vacuum pump and a manometer, and 80 per cent of the air was evacuated. A mixture of hydrogen and carbon dixoide (6 per cent, final concentration about 5 per cent) was taken from the pressure tube to a rubber balloon. This balloon was connected to the filter. The gas tap was opened so that the gas might enter the flask.

The flask was then placed over a rotating magnet, which caused the magnet inside the flask to rotate (Fig. 1). The motor had to be taken out from its cover, otherwise the medium was overheated. The balloon was connected to the apparatus during the cultivation period.

Great care must be taken when preparing the catalyst so that the gas mixture will not come into direct contact with the palladium and cause an explosion. When the authors were letting in the gas they had the apparatus covered in such a way that only the hand opening the gas tap was exposed. However, they have used hydrogen gas daily for three years without any acci-

* Kebo AB, Birger Jarlsgatan 120, Stockholm, Sweden.

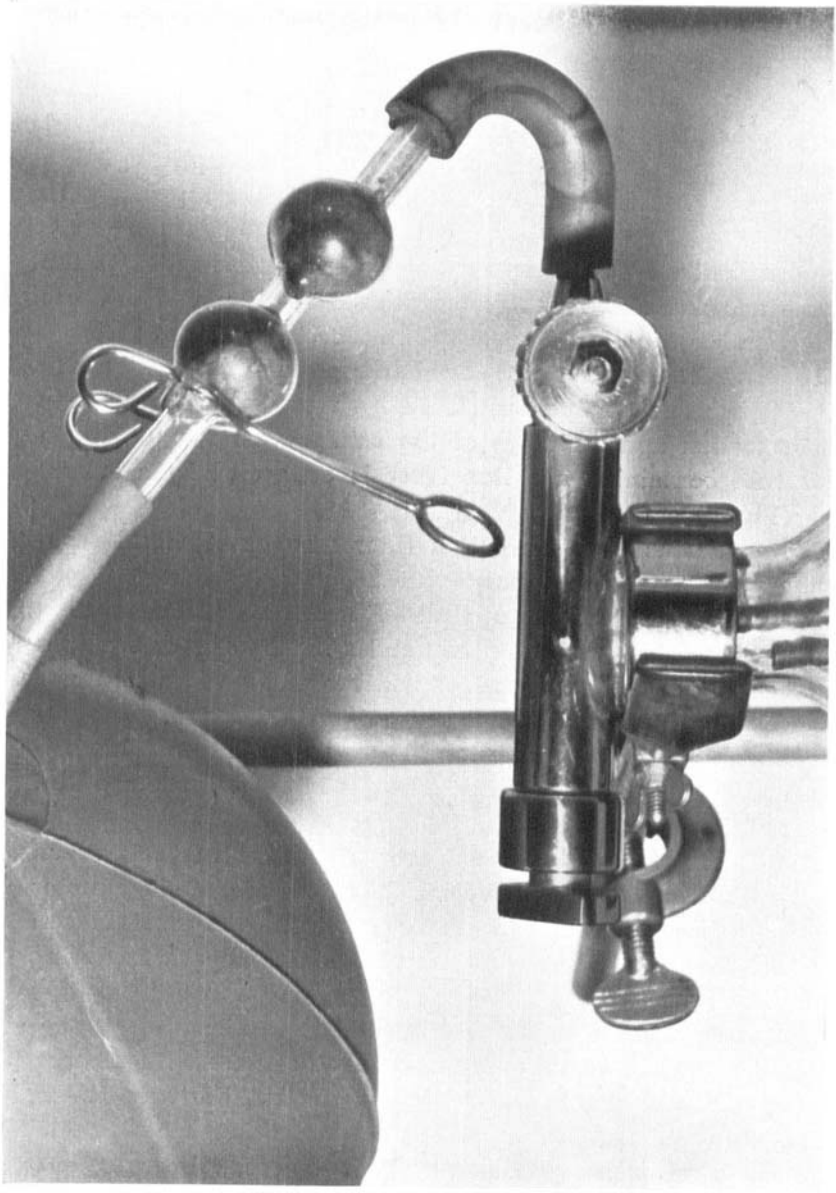
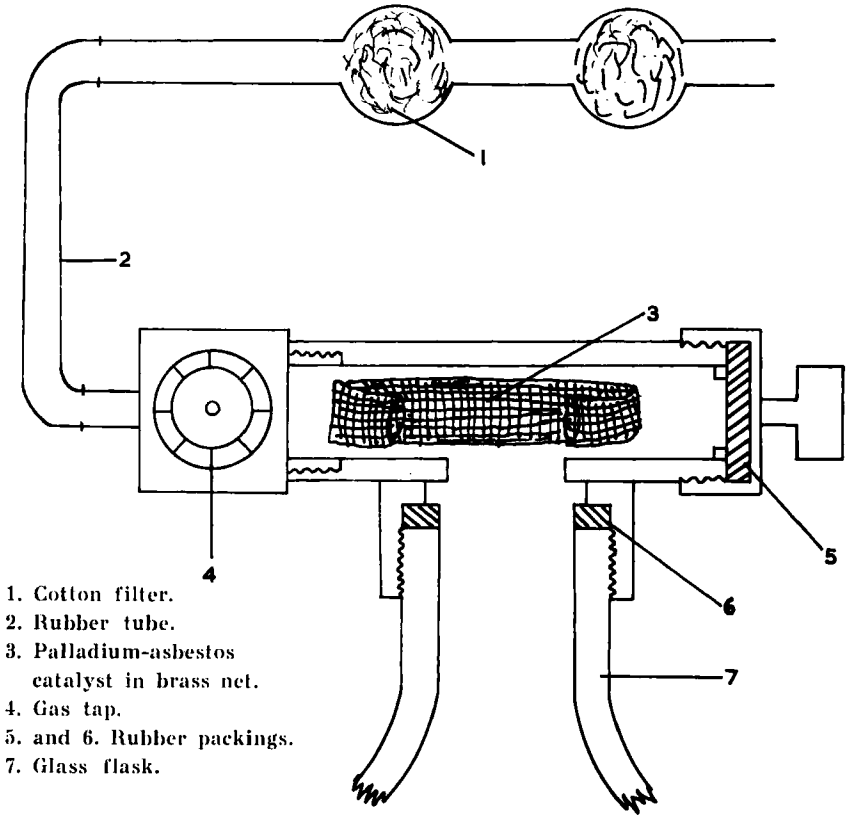


Fig. 2. Apparatus applied to a glass flask.



1. Cotton filter.
2. Rubber tube.
3. Palladium-asbestos catalyst in brass net.
4. Gas tap.
5. and 6. Rubber packings.
7. Glass flask.

Fig. 3. Section through the apparatus.

dents. The catalyst must be changed every six months, since the brass net will be weakened by the heat evolved during the reaction between hydrogen and oxygen.

EXPERIMENTS

Five experiments were performed with five different strictly anaerobic strains of Leptotrichia resembling those strains described by *Bøe* (1941) and *Hamilton & Zahler* (1957). The bacteriological study of these strains will be reported separately.

The strains were isolated from plates inoculated with material from between the teeth. The plates were incubated in an atmosphere of hydrogen with 5 per cent carbon dioxide, the oxygen

being consumed by hydrogen with palladium as a catalyst. In this atmosphere, the Leptotrichia strains will grow easily on ordinary horse-blood agar. After 6 days the colonies are often 20 mm in diameter.

Material was taken from a colony to a tube containing 10 ml of a medium consisting of Brain-Heart infusion (Difco) to which were added 0.3 per cent yeast extract and 0.03 per cent cysteine hydrochloride as suggested by *Möller* (1958). After incubation for 18—60 hours a heavy growth was visible in the tubes. The content of a tube was then transferred to a bottle of the type previously described, containing 1 litre of the same medium, the magnet was added and the apparatus was applied. After 80 per cent of the air was evacuated and hydrogen-carbon dioxide gas was let in, the flask was incubated until a heavy growth was visible. The incubation period varied between 18 and 60 hours, since the lag period was of different lengths in the different experiments. Once growth was visible, the substrate became turbid after a few hours.

When a moderate growth was visible, the flask was opened and the medium was centrifuged. A suspension was prepared as previously described (*Frostell*, 1957). In some flasks an opening was made in the wall 1 cm above the bottom. The hole was closed by a rubber stopper through which a glass tube was inserted. The glass tube was closed by a rubber stopper. Through this tube samples could be taken from the flask for turbidity determinations during cultivation.

Table I shows the results of these experiments. The wet weights and dry weights obtained from 1 litre of medium varied from 0.74 g to 2.26 g and from 0.08 g to 0.33 g respectively.

Table I.

Wet weights, dry weights and plate counts per mg weight obtained with 5 strains of Leptotrichia centrifuged and suspended in 10 mM/l phosphate buffer (pH 6.96).

	Strain No.				
	I	VIII	X	XV	XVII
Wet weight	1.87 g	2.26 g	0.74 g	1.03 g	2.21 g
Dry Weight	0.13 g	0.32 g	0.08 g	0.13 g	0.33 g
Plate count per mg w.w.	2.21×10^7	1.37×10^7	1.26×10^7	2.16×10^7	2.18×10^7

The plate counts determined immediately after dilution of the suspension to 10^{-7} with a buffered Ringer solution and inoculation on the surface of horse-blood agar plates incubated anaerobically, as previously described, for 2—3 days varied between 1.26×10^7 and 2.21×10^7 per mg wet weight.

In this series of experiments, no systematic efforts were made to achieve maximum amount of cell mass or living cell number per mg of weight.

It was found that the plate count of the suspensions rapidly decreased with time. Curves were obtained resembling those obtained with streptococci (*Frostell,* 1957). The results of these experiments and experiments performed in order to create stable suspensions of Leptotrichia will be reported separately.

SUMMARY

A method was elaborated for the anaerobic culture of organisms in liquid media in a hydrogen-carbon dioxide atmosphere and under constant stirring. The authors have devised a simple apparatus containing a palladium-asbestos catalyst which may be applied to a glass flask with screw neck. The stirring was performed with the aid of a motor-driven magnet. Suspensions were prepared by this method from five different strains of Leptotrichia, which were strictly anaerobic. The wet weights obtained from 1 litre of medium varied between 0.74 g and 2.26 g, and the plate counts varied between 1.26×10^7 and 2.21×10^7 per mg wet weight. The suspensions prepared were not stable, however, since a rapid decrease in viable cell counts was found in all experiments.

RESUME

UNE MÉTHODE POUR LA CULTURE D'ORGANISMES ANAÉROBIES

Une méthode a été élaborée pour la culture anaérobie d'organismes en milieu liquide, dans une atmosphère d'hydrogène-dioxyde de carbone, sous une agitation constante. Les auteurs ont construit un appareil simple contenant un catalyseur d'amiante de palladium qui peut être adapté à un flacon de verre à col fileté. L'agitation était obtenue à l'aide d'aimants mus par

un moteur. Les suspensions furent préparées par cette méthode à partir de cinq colonies différentes de Leptotrichia qui étaient strictement anaérobies. Les poids à l'état mouillé obtenus pour un litre de culture variaient entre 0,74 et 2,26 g et le nombre de cellules viables variait entre $1{,}26\times10^7$ et $2{,}21\times10^7$ par mg de poids à l'état mouillé. Les suspensions préparées n'étaient cependant pas stables, et toutes les expériences ont montré une rapide diminution du nombre de cellules viables.

ZUSAMMENFASSUNG

EINE METHODE ZUR HERSTELLUNG AKTIVER SUSPENSIONEN VON ANÄEROBEN MIKROORGANISMEN

Eine Methode für anäeroben Bebrütung von Organismen in Flüssigkeiten in einer Atmosphäre von Wasserstoff-karbondioxyd unter konstanter Bewegung ist ausgearbeitet worden. Die Autoren haben einen einfachen Apparat konstruiert, der einen Palladiumasbestkatalysator enthält, und der an einer Glasflasche mit Schraubenhals angebracht werden kann. Die Bewegung wurde mit Hilfe von Magneten erzeugt, die von einem Motor betrieben wurden. Durch diese Methode wurden aus fünf verschiedenen Stämmen von Leptotrichia, die anäerob waren, Suspensionen hergestellt. Das Feuchtgewicht, das aus einem Liter des Mediums gewonnen wurde, schwankte zwischen 0,74 g und 2,26 g, und die Keimzahl koloniebildender Zellen pro mg Feuchtgewicht schwankte zwischen $1{,}26\times10^7$ und $2{,}21\times10^7$. Die auf diese Weise hergestellten Suspensionen waren jedoch nicht stabil, da eine rapide Verminderung der Anzahl der lebensfähigen Zellen bei allen Experimenten beobachtet wurde.

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