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Culture Media for Large-scale Production of Micrococcin

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SUMMARY: A number of media have been developed for the production of micrococcin. A satisfactory medium consists of corn steep liquor to 1800 mg. N/l. with an aqueous extract of dried autolysed yeast to 1800 mg. N/l., which gives titres of the order of 40–60 μ g. micrococcin/ml. in 3 days. A soluble yeast extract such as Marmite may be used in place of the dried autolysed yeast. Moderate aeration is required for good titres but initial pH is relatively unimportant, any value in the range 6.0–7.0 being suitable. The main energy source in sugar-free media is probably the aerobic oxidative deamination of amino-acids. An inoculum that has been pre-incubated gives better results than an inoculum used immediately after it has been prepared. The addition of various inorganic ions to the medium was tried without any detectable influence on titre.

The production of micrococcin (Su, 1948) in quantity was undertaken to provide material with which other workers could examine extraction of the active substance and its behaviour both *in vitro* and *in vivo*. The principal object of the present experiments was to reduce the cost of the medium and to supply information necessary for fairly large-scale fermentation. Although a number of *ad hoc* experiments were carried out in shake flasks, stirred aerated flasks were used for most of the work. The correlation between the results obtained in stirred aerated flasks and those obtained in a 100 gal. stirred aerated fermenter was good.

METHODS

Stirred aerated flasks. The stirred aerated flask (Fig. 1), designed to study general fermentation problems, has been in use for more than 3 years. During this period a number of fungi have been examined. No difficulties, apart from control of foaming, were found in getting a sufficient supply of oxygen to the fungi examined, some of which had an extremely high oxygen demand.

The basis of the design is a bolt-head Chance Hysil flask of 5 l. capacity, normally containing 4 l. of medium. The flask is fitted with a black rubber bung having five moulded holes through which pass the aerators, stirrer, sampling arm and the air outlet filter. The rubber bung is retained in the flask by a suitably perforated metal plate clamped to a metal ring round the neck of the flask by butterfly nuts. The aerator arms, which have a bore of $\frac{1}{4}$ in., are not restricted at the nozzles. They carry baffle plates preventing vortices and increasing the agitation effect. A relatively small stirrer facilitates assembly. It operates above the aerator outlets and disperses the air in the medium. A range of stirring speeds from 100 to 1000 r.p.m. is available. Greater agitation can be obtained by the addition of links of stainless steel chain to the blades of the stirrer, but excessive speeds with such chains have

been found by Mrs Gill-Carey (private communication) to damage extensively a species of fungus. The stirrer shaft operates through a plain metal bearing which is filled with phenolated vaseline immediately after sterilization. A grease-cup, mounted on the shaft below the bearing, prevents the lubricant entering the medium.

Sterile air, pre-heated to the temperature required and automatically humidified, is passed into the flask via a Y-piece, and leaves by the outlet air filter, where its rate of flow is measured on a U-tube flowmeter. Units of four flasks, with an air filter and distributing manifold, are mounted on an aluminium frame which is sterilized with all connexions attached except the drive to the stirrer shafts and those to the air flowmeters. Four such frames are mounted, after sterilization, on a metal rack which carries the flowmeters and an electric motor with pulleys, gears and shafting for stirring. The stirrer shafts are connected by flexible metal cables to the vertical driving shafts. The method of transmitting the drive from the motor can be seen in the photograph of the rack (Pl. 1). The racks run on the monorail system devised by Green (1946) for the penicillin production unit, from which the racks were modified.

Shake flasks. Conical flasks (Pyrex, 200 ml.) containing 50 ml. medium were used on a horizontal shaker giving 84 oscillations/min., the length of throw being 7.5 cm.

Aeration characteristics of the apparatus employed. A number of determinations of the 'aeration constant' (Wise, 1951) for the stirred flasks under conditions normally used were carried out by Mr W. S. Wise. From the results and from experiments on the effect of stirring and aeration, it was clear that the aeration constant necessary to satisfy the peak oxygen demand of the micrococcus used was less than 1.0.

Organism. A strain of *Micrococcus* sp. isolated by Su (1948) was used. This strain is now available from the National Collection of Type Cultures (NCTC 7128).

Assay. The method used to assay fermentation samples was the solid medium dilution method of Su (1948), as modified by Heatley, Kelly & Smith (1952). It is a time-consuming technique, and the number of assay samples was kept to a minimum by pooling equal volumes of samples from replicate fermentations *before* assay. Arbitrary times for sampling were selected after preliminary observations on the course of the development of antibiotic activity.

Conditions used in comparison of media. Fermentations were carried out at 30° and the time of incubation was 4 days. Each flask was stirred at 300 r.p.m. and aerated at 3 l. air/min./4 l. medium. The inoculum was 15 ml. of a 17 hr. stirred aerated culture of the micrococcus grown in nutrient broth at 30°.

Foaming was controlled by the addition of a mixture of D.C. Antifoam 'A' (a Dow Corning silicone product) and tri-amyl citrate. Usual concentrations used were 40 p.p.m. of the former and 100 p.p.m. of the latter, but these were increased when necessary, e.g. with corn steep liquor (CSL), blood preparations

and casein. No toxicity or diminution in titre was demonstrated when using large amounts of these agents. The antifoam was added as a solution in chloroform before sterilization.

MEDIA

Su (1948) employed a medium comprising 1 % Lab-Lemco, 1 % Eupeptone no. 2 (Allen and Hanburys, Ltd.), and 0.5 % sodium chloride. It was desirable to find a cheaper and more easily available medium. To this end the materials included in Table 1 were chosen for examination as substitutes for the components of Su's medium. Control runs using Su's medium were always included as a standard of reference for efficiency in micrococcin production. In the first instance, substitutions were based on equal nitrogen contents, but promising combinations were further examined by varying the total nitrogen content and the relative proportions of the two constituents.

Table 1. *Source of supply and total nitrogen (TN) of materials tested in media*
(Total nitrogen determinations by micro-Kjeldahl.)

Material	Source of supply	Total nitrogen (%, w/w)
Constituents of Su's medium		
Lab-Lemco	Oxo Ltd.	6.98
Eupeptone no. 2	Allen and Hanburys Ltd.	15.0
Substitutes for constituents of Su's medium		
Corn Steep liquor (CSL)	Brown and Polson Ltd. (via The Distillers Co. Ltd.)	2.60
Dried autolysed yeast (DAY)*	The Distillers Co. Ltd.	7.16
Brewer's yeast	George's Brewery Ltd., Bristol	2.30
Baker's yeast	The Distillers Co. Ltd.	2.40
Guinness yeast extract	Arthur Guinness, Son & Co. Ltd.	5.60
Yeastrel	Brewers' Food Supply Co.	5.88
Marmite	The Marmite Food Extract Co.	6.04
Meat extract (Argentine)	Armour and Company Ltd.	8.45
Meat meal	British Glues and Chemicals Ltd.	9.15
Maggi's Bouillon powder	Marber and Co. (Food Products) Ltd.	4.80
Bacto-Peptone	Difco Laboratories Inc.	15.5
Fish meal	Hull Fish Meal and Oil Co.	13.2
Casein	Casein (Industries) Ltd.	12.7
Soya flour	Brodie and Co. Ltd.	7.3
Roths pot ale (concentrated)	The Distillers Co. Ltd.	15.0

* Dried autolysed yeast (DAY) extract was made by autoclaving an aqueous suspension (12 kg. DAY/100 l. water) for a short while and then clarifying with a Sharples centrifuge. Other partially soluble materials were acid-hydrolysed before use.

Finally selected medium based on statistical evaluation of the results of test runs. After numerous experiments in stirred or shaken flasks the best medium was found to be CSL to provide 1800 mg.N/l., DAY extract to provide 1800 mg.N/l., and 0.5 % sodium chloride. Marmite to the same nitrogen content could replace the DAY extract with only slight decrease of titre and had the advantage of eliminating the DAY extraction step in medium preparation. Some batches of CSL could be employed alone at 3600 mg. N/l. but others

appeared to contain something which was detrimental to micrococcin production at this concentration. All batches of CSL examined gave satisfactory results in combination with DAY extract or Marmite.

Larger scale production. The process resulting from the work here reported was successfully applied to the production of micrococcin in a 100 gal. stainless steel fermenter which was developed from a Kestner stirred and jacketed vessel. Using CSL/Marmite medium, titres of 40–50 μg . micrococcin/ml. were regularly produced in 66 hr.; under the same conditions in this apparatus Su's medium gave titres of 6–8 μg ./ml.

Comments on various factors. The experiments on media composition showed that any of the materials tried were satisfactory substitutes for the peptone in Su's medium, DAY extract and acid-hydrolysed dried blood in particular giving substantially better results. The best substitute for Lab-Lemco was CSL, but even this gave only slightly better results. CSL gave particularly good titres in combination with acid-hydrolysed dried blood, but this was not pursued further as it proved difficult to recover micrococcin from broths which included blood.

The fermentation is relatively insensitive to variations in conditions. The initial pH was varied over the range 6.0–7.4 without detectable effect. It was found to be advantageous to increase the rate of stirring to 600 r.p.m. with an aeration rate of 3 l. air/min., but even under these conditions the aeration constant (Wise, 1951) of the apparatus employed is still less than 1.0.

It was possible to use as inoculum a suspension obtained by washing off 20 oz. slants directly, or after only 7 hr. incubation, with only a slight drop in yield. Increases in concentration of sodium or chloride ion were made without effect on titre, as were additions of potassium, magnesium, phosphate, and sulphate ion. There was some evidence of a slight drop in micrococcin production with very long sterilization times at 15 lb./sq.in. but no difference was observed up to 1 hr.

BIOCHEMICAL OBSERVATIONS

During fermentation in a 100 gal. vessel with CSL/Marmite medium, lactate was rapidly destroyed, acetic acid and ammonia accumulated and the pH value rose (Fig. 2). The ammonia found was equivalent to about 25 % of the available nitrogen.

By the use of cell suspensions of the micrococcus it was observed that α -alanine, glutamic acid and arginine were deaminated with the production of ammonia; histidine, lysine, valine and phenylalanine were not. The deamination of alanine occurred only in presence of oxygen, and the rate of deamination was dependent on the rate of aeration (Fig. 3). This reaction proceeded fastest at about pH 6–6.5, decreasing rapidly on either side of this range. No pyruvate was demonstrated after cell suspensions had been aerated in the presence of alanine even when toluene was present (Stephenson, 1949) but, whether toluene was present or not, acetic acid equivalent to about 15 % of the alanine deaminated was formed. Neither pyruvate, lactate, citrate nor glucose influenced the rate of deamination of alanine by washed cell suspen-

sions. Experiments using gelatine or corn steep protein as substrate indicated that the micrococcus had little proteolytic activity.

Statistical analysis of fermentation experiments in shake flasks showed that alanine could, with advantage, replace part of the CSL nitrogen in CSL/

Fig. 1

Fig. 1. Diagram of aerated stirred flask. *A*, inoculator; *B*, stirring shaft; *C*, sampler; *D*, aerator; *E*, stirrer; *F*, outlet air filter.

Fig. 2

Fig. 3

Fig. 2. Chemical changes in a 70 gal. micrococcin fermentation. Medium: CSL to 1800 mg. N/l.; Marmite to 1800 mg. N/l.; sodium chloride, 0.5 %. Micrococcin, —●—; pH, —△—; ammonia, —○—; acetic acid, —⊗—; lactic acid, —□—.

Fig. 3. Form of curve relating aeration rate to rate of deamination of α -alanine by the micrococcus.

Marmite media, while alanine/Marmite medium was considerably better than peptone/Marmite. Lactate also could replace part of the CSL nitrogen but pyruvate could not (Table 2). Glucose was added in experimental fermenta-

Table 2. *The influence of alanine, pyruvate, and lactate on micrococcin production*

(Basal medium: CSL to provide 1.8 g. N/l.; Marmite to provide 1.8 g. N/l.; Sodium chloride 0.5 %; Sodium sulphate 0.05 %; pH value = 6.5.)

Medium	Micrococcin $\mu\text{g./ml. (mean)}$
Undiluted	56
Diluted 1/2	28
Diluted 1/2 + alanine to restore to 3.6 g. N/l.	74
Diluted 1/2 + pyruvate to same molarity as alanine above	28
Diluted 1/2 + lactate to same molarity as alanine above	45

tions as an alternative energy source, but it was observed that the low pH which resulted inhibited micrococccin production. The use of calcium carbonate in such fermentations, was not successful as it did not maintain the pH high enough (above pH 6) for maximum production.

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EXPLANATION OF PLATE

PLATE 1

Fermentation rack showing two frames mounted on one side with stirrers connected up.

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