ISSN 0026-2617, Microbiology, 2009, Vol. 78, No. 1, pp. 125–130. © Pleiades Publishing, Ltd., 2009.

EXPERIMENTAL ARTICLES

Improved Method for Direct Screening of True Lipase-Producing Microorganisms with Particular Emphasis on Alkaline Conditions[1]

D. Yu. Sorokin[a,2] and B. E. Jones[b]

[a] *Winogradsky Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia*

[b] *Danisco Genencor Division, Leiden, The Netherlands*

Received February 19, 2008

Abstract—A rapid and effective method for direct detection, selection and testing of microorganisms able to produce both cell-bound and extracellular true lipases is described. The method is based on formation of clearance zones on turbid solid media with emulsified olive oil around or under the colonies, cell fractions or culture supernatant of lipase-producing organisms. The method was successfully applied for the screening and isolation of microorganisms producing alkaline lipases.

Key words: lipase, lipolytic microorganisms, plate screening, alkaline lipase.

DOI: 10.1134/S0026261709010160

INTRODUCTION

Lipases are a group of enzymes hydrolyzing glycerol esters of long chain fatty acids. Microbial lipases, especially those stable and active at high pH values, are a subject of increasing interest in several branches of applied microbiology and biotechnology, such as medical microbiology (virulence factor in staphylococci and enterobacteria), organic synthesis (stereospecific ether synthesis in hydrophobic media), food (fat spoilage) and detergent industries [1–10]. During the last years of explosive molecular biology development, it becomes increasingly important to develop rapid direct methods to screen enzymatic activities in very large numbers of colonies, in for example, clone libraries [11, 12]. Furthermore, a test for lipase production is part of the routine procedure of identification of bacteria. Therefore, it is important to have a useful (rapid, simple and inexpensive) method for screening of lipase producing wild strains, to select active mutants and to test pure cultures.

The most widely used and recommended methods for detection of lipase activity cannot be assumed as satisfactory since they utilize artificial lipids as substrate (tributyrin, Tweens). Employment of such substrates can give false positive results because they can be hydrolyzed by esterases. The same is true for the most commonly used colorimetric assay for lipase activity in culture supernatants which use *p*-nitrophenyl esters of various fatty acids. The method is indeed rapid but it would give false-positive result in the case of the presence of an esterase in the mixture. Lipases do have esterase activity, but not vice versa. Other methods, employing natural fats or oils as substrates incorporated into solid medium, are either not sensitive enough and demand additional treatment with toxic reagents for detection of fatty acids or are difficult to perform in general [13].

In this paper we describe a simple and rapid method for detection of true lipase-producing microorganisms and its application for the selection of alkaline lipase-producing bacteria.

MATERIALS AND METHODS

Samples. To enrich and isolate alkaliphilic lipase producers, samples from highly alkaline habitats were used including dry and wet fluffy soda soils from Ararate valley (Armenia) and from soda lakes of south-eastern Siberia (Chita region, Russia). Their pH varied from 9.5 to 11, salinity—from 2 to 10% and carbonate alkalinity from 20 to 200 mM.

Principle of the method. The method we developed for detection of lipase-active microorganisms is based on the formation of the clearance zones around the colonies grown on solid turbid media supplemented with finely emulsified and stabilized olive oil. The quality of the oil emulsion and its stability during sterilization,

[1] The article is published in the original.

[2] Corresponding author; e-mail: soroc@inmi.host.ru

and preparation of the media are critical factors for successful lipase detection. It is a well known fact that lipases are active only at the surface of an oil-water interface. Therefore, a preparation of a fine and stable emulsion of oil in water is essential to provide suitable conditions for lipase action.

Preparation of oil emulsion. To prepare an emulsion of desirable quality various formulations that were utilized for measurement of lipase activity in enzyme preparation have been tried [14]. The best results were achieved using the following procedure: one part of olive oil of analytical quality (Serva or Sigma) was mixed with 4 parts of 5% solution of gum arabic (synonym is gum gellan, Sigma)—a polysaccharide serving as an emulsifier and stabilizer, placed onto an ice bath and sonicated for 3 min at 22 kHz and 50 W. This procedure allows preparation of an extremely fine and stable oil emulsion that can be sterilized at 120°C during 20 min and kept for several months without lost of quality.

Principles of the medium preparation. We have found that two main principles should be applied during preparation of a suitable test medium. First of all, the final concentration of oil in medium should be relatively low providing a high sensitivity of detection. The best results were obtained at oil concentrations 0.4–0.5% (vol %). Another important factor is the concentration of organic nutrients other than oil. A high concentration of the latter (in other words use of rich media) was found to decrease dramatically the amount of lipase excreted by the colonies of active organisms. The best results in our work with alkaliphilic microorganisms were obtained when the medium apart from oil was supplemented by a low concentration of organic nitrogen, in particular by 0.2 g/l of yeast extract and peptone. Yeast extract has the advantage of additional emulsifying properties. In this case growth of the colonies is limited whereas diameter of the clearance zone is maximal. The particular formulation of the mineral base for media will depend on the kind of samples used for enrichment cultures or properties of the pure cultures. In general, mineral base composition should be such as to provide optimal conditions for growth and lipase action. An example for preparation of the test media for alkaliphilic lipolytic microorganisms is presented below.

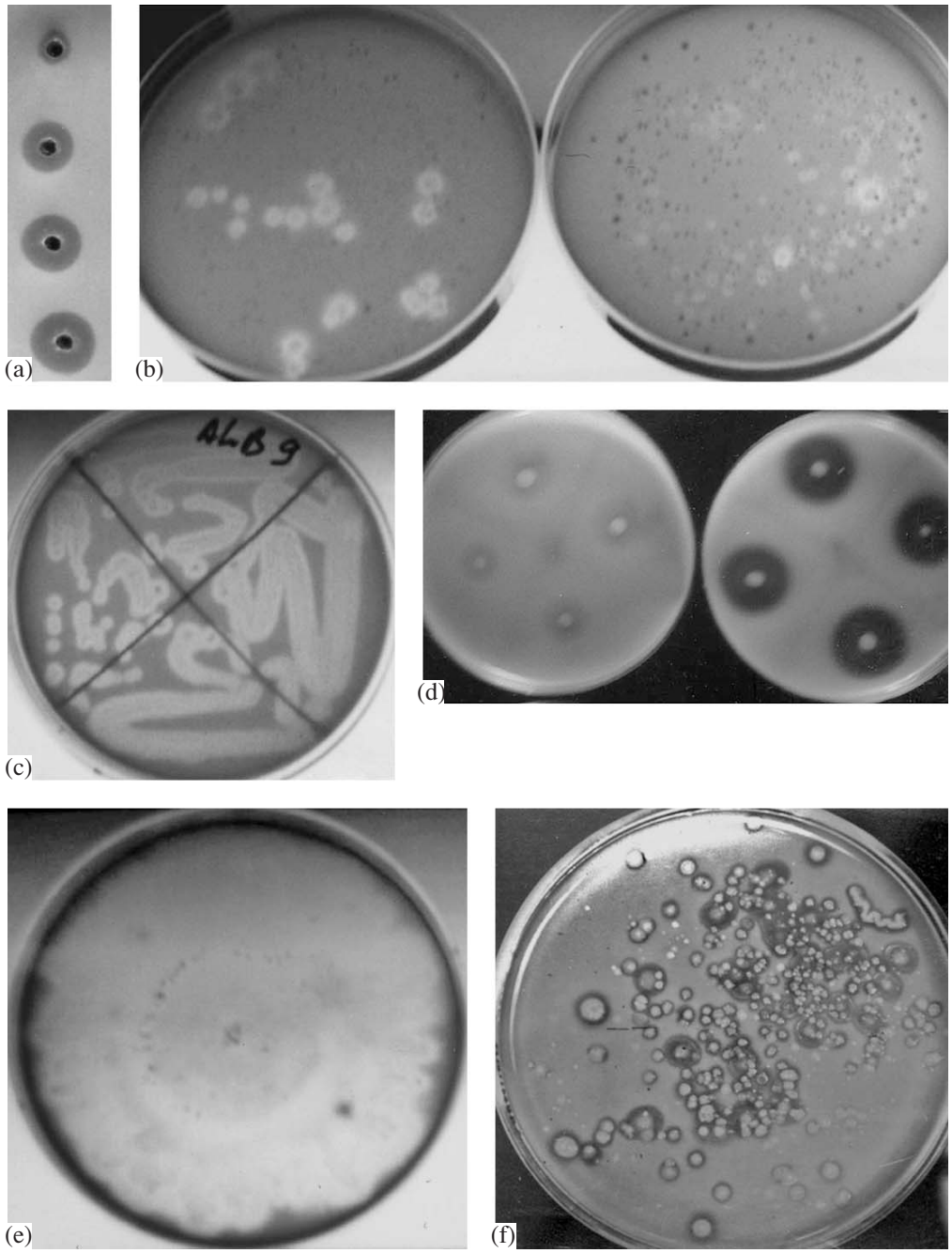
Preparation of agar medium for detection of alkaliphilic lipolytic microorganisms. Mineral base (MB) (0.6 M Na^+) included 23 g/1 Na_2CO_3; 7 g/l $NaHCO_3$; K_2HPO_4 and KNO_3 (both 0.5 g/1), final pH 10.05–10.1. After sterilization and supplementation with 1 ml/l of trace metal solution and 1 mM of $MgSO_4$ the MB was mixed with an equal volume of 3% wt % sterile agar at 60°C, followed by pre-warmed sterile 20% olive oil emulsion at final concentration 0.4% (vol %) and organic nitrogen. The final mixture was vigorously shaken and poured into the well-leveled Petri dishes to provide a uniform layer of about 2 mm. The resulting plates are homogenous milky turbid. Thicker layer may need much longer incubation for complete clearance of the emulsion. This medium was used to detect lipolytic alkaliphiles enriched from alkaline samples during 1 week on liquid medium of the same composition.

To test pH dependence of growth and lipase production by pure cultures of alkaliphilic microorganisms, different buffering agents at final concentration 0.6 M were utilized: HEPES-$NaHCO_3$ for pH range 7–8, $NaHCO_3$-Na_2CO_3 for pH 8.5–11. Tests for lipolytic activity in culture supernatants (filter-sterilized to prevent bacteria growth) and commercial lipase preparation were carried out on plates containing buffering agent, oil emulsion and agar. 1–10 μl portions were applied into the wells cut into agar.

RESULTS

Action of commercial lipase preparation. A commercial lipase preparation ("Lipolase", Novozymes, Denmark) was examined to test suitability of the proposed method for the detection of lipolytic colonies. Lipolase was chosen because it is claimed as an alkalistable enzyme [1, 15]. Different concentrations of the Lipolase diluted in 0.05 M Tris–HCl buffer (pH 8.0) were applied to wells cut in olive oil plates buffered at pH 7–11 and incubated at 36°C. Visible clearance zones appeared only on plates with pH 7–8; already after 1 hour incubation. The most significant difference between lowest and highest concentrations became evident after 3 hours incubation (figure, a). To check whether the lack of activity of the Lipolase on plates with pH 9–11 was determined by pH itself or by high soda concentration, the latter was replaced by organic buffers (0.1 M final): CHES was used for pH 9, CAPSO for pH 10 and CAPS for pH 11. In this case optimal pH was found at pH 8, where clearance zones were maximal and complete, but some activity with partially cleared zones was also detectable at pH 9–10.

Detection of lipase-producing colonies of haloalkaliphilic bacteria. Liquid medium with emulsified olive oil buffered at pH 10 was inoculated with samples from soda soils and soda lake sediments, and incubated with vigorous aeration during one week. In this time all of the oil substrate had been consumed in 9 out of 13 samples tested by microscopic examination and soap formation. Samples from positive enrichments were diluted and spread on plates with olive oil emulsion buffered at pH 10. Bacteria excreting lipase were easily detectable by appearance of clearance zones around colonies on a milky-turbid background (figure, b). These colonies were picked with a Pasteur pipette, sus-



Detection of lipase activity by plate assay, (a), detection of activity of commercial lipase Lipolase^{TM} at pH 8 (0.05 M Tris–HCl) with 2 times increasing loading steps from top to bottom, (b)–(f), haloalkaliphilic lipase-producing microorganisms at pH 10 (0.6 M Na^+): (b), detection of bacterial lipolytics from enrichment culture; (c), purified culture of lipolytic bacterium; (d), lipolytic bacilli on the medium with 1.5% (left plate) and 0.5% (right plate) agar; (e), colony of lipolytic micromycete; (f), detection of lipolytic actinomycetes from enrichment culture in presence of penicilline G.

pended in sterile mineral base and further purified by spreading on olive oil agar (figure, c). This procedure allows isolation of active bacterial strains producing alkaline lipase. It had, however, one complication. We found that some types of colonies (formed mainly by bacilli) with only slightly positive results on solid medium after prolonged incubation, rapidly consumed olive oil in liquid medium. It was found that in this case the agar concentration in oil plates is critical: bacilli strains produced large clearance zones only when the

agar concentration was below 0.6% (figure, d). Such bacteria appear to produce lipase complexes with high molecular mass that do not readily diffuse into concentrated agar. Therefore, to detect this type of lipolytic bacteria in a mixed population, we have tried to use direct mixing of the serial dilutions with oil agar at 45°C to obtain plates with a final agar concentration 0.5%. However, the results were in most cases not as clear as with hard agar plates, because the colonies of bacilli exhibited spreading growth inside the agar layer and rapidly mixed with each other. Hence, for investigating mixed bacterial populations, we recommend incubating plates comprising 2% agar for one month after the first isolation of readily detectable lipolytic colonies, then isolate weak lipolytic microbes and check their activity using plates with 0.5% agar.

To isolate lipase producing actinomycetes and micromycetes two approaches were employed. In one procedure, small aggregates of soils or sediments were directly placed on the surface of olive oil agar supplemented with filter-sterilized penicillin G (sodium salt, Sigma) at final concentration 1 g/l to suppress bacterial growth. After 1–2 weeks, mycelium of actinomycetes and micromycetes began to spread out around the aggregates. Those that produced oil clearance were picked by needle and purified using the same medium. In contrast to bacteria, clearance zones produced by actinomycetes and fungi did not usually spread far beyond the edge of the colonies, but were easy to detect under the spreading mycelium (figure, e). Another procedure was the same as for detection of lipolytic bacteria except that both enrichment medium and agar medium contained penicillin G (figure, f). Clearly, other antibiotics could be used in these procedures for selective advantage.

pH profile for growth and lipase activity. This important parameter can be rapidly measured using plates with olive oil emulsion strongly buffered at different pH. In most cases, we used plates with final agar concentration 2% except for bacilli when 0.5% agar was applied. All alkaliphilic bacteria isolated from soda habitats showed marked preference for alkaline, soda-containing media for growth and lipase activity with maximum at pH 10 and negligible growth and activity below pH 8. In contrast, fungal isolates from the same samples showed equal activity at pH range between 7 and 10.

DISCUSSION

Assay for true lipase activity (in contrast to esterase) is a complicated procedure demanding detection of free fatty acids liberated during oil/fat hydrolysis. Therefore, simplified procedures using agar media with incorporated oils or fats have long time been proposed for the purpose of rapid screening of lipolytic microorganisms [13]. The information on most of such approaches is summarized in the Table. They can be divided into 2 major groups. Indirect methods are based on the detection of free fatty acids produced during lipolysis by flooding of the plates with special reagents or with incorporated dyes. All these methods have their draw backs and cannot be recommended for high through-put screening. In direct assay methods, the detection of lipolysis is based on observation of changes in state of various lipids incorporated into agar media. The methods differ by the type of test substrate and manner of their preparation and distribution. The most useful method for screening of lipolytic colonies is based on the incorporation of emulsified and stabilized tributyrin because hydrolysis results in formation of water-soluble products and, therefore, complete clearance around the colonies producing "tributyrinase". However, correlation between the latter and true lipase activity is usually weak in our experience. Use of other artificial substrate, like Tweens, also may give false-positive results. The method proposed in this paper provides several advantages that make it useful for rapid and high through-put detection of lipolytic organisms and lipase in solution:

(1) Use of a natural substrate allows detection of the colonies producing true lipase.

(2) The manner of preparation and stabilization of the olive oil emulsion allows preparation of a turbid medium with a finely distributed substrate that is absolutely essential for lipase action (i.e. large surface area of the lipid phase). Such emulsions can be stored for extended periods and sterilized without lost of its properties.

(3) The method is very sensitive because complete clearance produced by lipolytic colonies is easy to detect on a homogenously turbid background.

(4) All reagents used for the medium preparation are inexpensive; no complex equipment is necessary.

We believe that this method would be especially useful for the screening of large numbers of clones with a lipase gene insertion. In addition, we have successfully extended this procedure to other natural oils and fats, e.g. soya oil, jojoba oil, palm oil and [French refined] tallow (melting point 50°C) and also applied it to the screening of thermophiles (e.g. *Thermococcus* sp., *Pyrococcus* sp., *Desulfurococcus* sp. and *Thermotoga* sp.) under strictly anaerobic conditions and temperatures 70–100°C using solidifying agents other than agar.

ACKNOWLEDGMENTS

This work was supported by the Genencor International (Palo Alto, USA) and by the Program on Molec-

Comparative data on plate methods for detection of lipolytic microorganisms

Method	Substrate	Preparation of the medium	Detection	Weak points	References
Indirect methods	Plant oil, cream	Direct incorporation, 1.5–2% agar	Detection of free FA by flooding with $CuSO_4$ solution or by special dyes	Low sensitivity high probability of false-positive reactions; problematic substrate dispersion; unsuitability for direct screening because of toxicity of the reagents	[13]
	Trioleine, Tristearate Tripalmitate	Emulgation of 0.5–1% substrate at 50–80°C with nutrient 1.7–2% agar before autoclaving	Flooding with $CuSO_4$		[16]
	Olive oil		Flooding with OsO_4		[17]
Direct methods	Tributyrine	Emulgation by sonication, final concentration is 0.25% in 1% agar	Clearance around the colonies	Low stability of emulsion; possibility of false-positive reaction (esterase instead of lipase activity)	[18]
		Direct additionat 1% to the melted 1.5% agar following mechanical emulgation with a stabilizer PVA and sterilization			[19]
	Trioleine, Tristearate Tripalmitate Trilaurine	Mechanical or ultrasonic homogenization at 0.1–1% concentration with melted 1–2% nutrient agar at 80°C following autoclaving	Formation of opalescence or precipitate around the colonies	Artificial substrates, problems with homogenous dispersion, low sensitivity for screening	[16, 18, 20]
	Olive oil	Spray of fine oil droplets (around 30 μm) on the agar surface	Change in appearance of oil droplets	Low sensitivity; unsuitable for screening	[21]
		Direct incorporation	Rhodamine B/UV detection	Poor distribution of substrate	[8]
	Linseed oil	Mechanical emulgation at 1% concentration with natural emulgants (saliva, rumen fluid) following sterilization with 2% nutrient agar	Clearance around the colonies	Low quality of the emulsion; difficulties with clearance zone detection	[22]
		Sonic emulgation without stabilizer			[20]
	Horse fat	Mechanical emulgation at 0.3% concentration with 0.2% melted agar	Clearance around the colonies	Low sensitivity for screening; no sterility; exotic substrate	[23]

ular and Cell Biology RAS. We gratefully acknowledge the excellent technical assistance of Daan Meijer.

REFERENCES

1. Arvidson, S.O., Extracellular Enzymes from *Staphylococcus aureus*, in: *Staphylococcal infections* Easmon, C.S.F. and Adlan, C., Eds., 1983, vol. 2, pp. 745–806.
2. Aaslyng, D., Gormsen, E., and Malmos, H., Mechanistic Studies of Proteases and Lipases for the Detergent Industry, *J. Chem. Technol. Biotechnol.*, 1991, vol. 50, pp. 321–330.
3. Vorderwurbecke, T., Kieslich, K., and Erdman, H., Comparision of Lipases by Different Assays, *Enzyme Microb. Technol.*, 1992, vol. 14, pp. 631–639.
4. Watanabe, N., Ota, Y., Minoda, Y., and Koichi, Y., Isolation and Identification of Alkaline Lipase Producing Microorganisms, Cultural Conditions and Some Properties of Crude Enzymes, *Agric. Biol. Chem.*, 1977, vol. 41, pp. 1353–1358.
5. Sellek, G.A. and Chaudhuri, J.B., Biocatalysis in Organic Media Using Enzymes from Extremophiles, *Enzyme Microb. Technol.*, 1999, vol. 25, pp. 471–482.
6. Reetz, M.T., Lipases as Practical Biocatalysts, *Curr. Opin. Chem. Biol.*, 2002, vol. 6, pp. 145–150.
7. Van den Burg, B., Extremophiles as a Source for Novel Enzymes, *Curr. Opin. Microbiol.*, 2003, vol. 6, pp. 213–218.
8. Vargas, V.A., Delgado O.D., Hatti-Kaul1, R., and Mattiasson, B., Lipase-Producing Microorganisms from a Kenyan Alkaline Soda Lake, *Biotechnol. Lett.*, 2004, vol. 26, pp. 81–86.
9. Leow, T.C., Zaliha, R.N., Abd Rahman, R., Basri, M., and Salleh, A.B., Thermoalkaliphilic Lipase of *Geobacillus* sp. T1, *Extremophiles*, 2007, vol. 11, pp. 527–535.
10. Salameh, M.A. and Wiegel, J., Purification and Characterization of two Highly Thermophilic Alkaline Lipases from *Thermosyntropha lipolytica*, *Appl. Environ. Microbiol.*, 2007, vol. 73, pp. 7725–7731.
11. Demirjian, D.C., Shah, P., and Moris-Varas, F., Screening for Novel Enzymes, *Top. Curr. Chemistry*, 1999, vol. 200, p. 1–29.
12. Demirjian, D.C., Moris-Varas, F., and Cassidy, C.S., Enzymes from Extremophiles, *Curr. Opin. Chem. Biol.*, 2000, vol. 15, pp. 144–151.
13. Shelley, A.W., Deeth, H.C., and Mc Rae, I.C., Review of Methods of Enumeration and Isoaltion of Lipolytic Microorganisms with Special Reference to Dairy Application. *J. Microbiol. Meth.*, 1987, vol. 6, pp. 123–137.
14. San Clemente, C.L. and Vadehra, D.V., Instrumental Assay of Microbial Lipase at Constant pH, *Appl. Microbiol.*, 1967, vol. 15, pp. 110–113.
15. Gormson, E. and Stenlenbjerg-Olson, B., Lipolase-TM, a Microbial Lipase for Detergents, *21st Detergent Symposium of the Japanese Oil Chemistry Society* Oct. 1989.
16. Willis, A.T., The Lipolytic Action of Some Clostridia, *J. Pathol. Bacteriol.*, 1960, vol. 80, pp. 379–390.
17. Kuimova, T.F., Shabanova, E.A., and Kazakov, G.A., Rapid Selection of Microorganisms Forming Extracellular Lipases, *Microbiology* (Moscow, Engl. Transl.), 1975, vol. 44, pp. 365–366.
18. Smeltzer, M.S., Hart, M.E., and Iandolo, J.J., Quantitative Spectrophotometric Assay for Staphylococcal Lipase, *Appl. Environ. Microbiol.*, 1992, vol. 58, pp. 2815–2819.
19. Mourey, A. and Kilbertus, G., Simple Media Containing Stabilized Tributyrin for Demonstration of Lipolytic Bacteria in Food and Soils, *J. Appl. Bacteriol.*, 1976, vol. 40, pp. 47–51.
20. Henderson, C., An Improved Method for Enumeration and Isolation of Lipolytic Rumen Bacteria, *J. Appl. Bacteriol.,* 1973, vol. 36, pp. 187–188.
21. Bulder, C.J.E.A., Some Observations on the Lipolytic Activity of Microorganisms and a New Method for Its Detection, *Antonie van Leeuwenhoek J. Microbiol. Serol.*, 1955, vol. 21, pp. 433–445.
22. Hobson, P.N. and Mann, S.O., The Isolation of Glycerole-Fermenting and Lipolytic Bacteria, *J. Gen. Microbiol.*, 1961, vol. 25, pp. 1227–1240.
23. Davies, M.E., A Study of the Diffusible Lipase Produced by Staphylococci and of Its Immunological Activity, *J. Gen. Microbiol.*, 1954, vol. 11, pp. 7–44.