MARINE YEASTS OFF THE INDIAN COAST

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In contrast to the attention that has been lavished on terrestrial yeasts since early times, the yeasts associated with marine environments have not formed a popular subject for investigation. Even granting that marine microbiology is of recent development, it is difficult to understand why most investigators have persistently cold-shouldered this interesting group of organisms whilst the bacteria from similar environments have received perhaps more than their fair share of attention. The extent of our ignorance on marine yeasts is best summed up by quoting ZoBell (1946) who admits, "it is problematical whether certain of these organisms are indigenous to the sea because they occur so commonly in the air"! That a 'study of marine yeasts, using modern taxonomic methods, might yield interesting ecological data' has also been expressed by Mrak and Phaff in their recent review on yeasts (1948).

No doubt the occurrence of yeasts in the sea has often been reported, though usually as incidental to other micro-organisms. Fisher (1894 *a*), Fisher and Brebeck (1894), Issatchenko (1914), Nadson and Burgwitz (1931), and ZoBell and Feltham (1934) are some investigators who have reported the occurrence of yeasts along with moulds and bacteria in the sea. Our present knowledge on the role of yeasts in the sea will tend to be largely speculative, but they probably have an important part in modifying and preserving the marine environment.

This investigation, which to the best of our knowledge, represents one of the first attempts at understanding this neglected group of organisms, was undertaken to determine whether the sea has an autochthonous yeast flora, and if so, to characterise and establish the identity of the species usually encountered. The knowledge gained was also expected to have a utilitarian application in the control of marine foods vulnerable to yeast spoilage.

Methods and Media

It has been stated (Reuszer, 1933; Fred *et al.*, 1924) that the effect of the proximity of the land does not extend beyond about a mile from the

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open shore. Throughout these investigations therefore samples of sea water ensured to be free from terrigenous contamination were collected at a distance ranging from 2 to 6 miles off the coast. Sterile glass samplers were used for the purpose and when the samples could not be immediately worked upon they were stored at 0 to 5° C., but in no case was a sample worked upon which had been stored for more than 48 hours.

The isolation of yeasts from the sea water was best achieved by aerobic and anaerobic enrichment culture methodology. The following two media were routinely employed and were found to be very satisfactory: Both were made in a mineral base of pH 7 containing K_2HPO_4, 1 g.; NaCl, 20 g.; $MgSO_4 \cdot 7H_2O$, 0·1 g.; $FeSO_4 \cdot 7H_2O$, 0·02 g.; $CaSO_4 \cdot 2H_2O$ (saturated solution), 10 ml. and yeast extract (for vitamin requirements) 1 g. per 1000 ml. of distilled water. Whereas medium No. 1 was incorporated with 2% (w/v) glucose and $NaNO_3$, 0·05% respectively as the carbon and the nitrogen sources, the medium No. 2 contained 10% commericial sucrose and 0·05% $(NH_4)_2SO_4$ for the same purposes. The latter medium in conjunction with the use of anaerobic glass-stoppered bottles has been consistently observed to be well suited for the isolation of yeasts in general (Lobo *et al.*, 1953). The third serial enrichment when streaked over the same medium solidified with 3% agar yielded colonies of yeasts which were purified by restreaking several times or until colonies were as homogeneous in appearance as space relationship would permit. Lodder and Kreger-van Rij (1952) state that plating methods give practically the same guarantee of purity as making single cell or single spore cultures provided all colonies on the plate are uniform in type. Incubation in all cases was at room temperature (27 to 29° C.), this being compatible with the optimum for yeasts and the surface temperature of the ocean, which in tropical waters is usually reported to range from 28 to 30° C.

The media and methods employed for the characterisation of isolates were those currently favoured in the study of terrestrial yeasts, but the media were fortified with 2% NaCl to simulate the marine environment. The taxonomic procedures adopted for the identification was by resort to the recent system of classification suggested by Lodder and Kreger-van Rij (1952).

Morphological and growth characteristics.—The morphological features of yeasts have been used as important criteria in their classification and are undoubtedly of basic taxonomic importance. The general cell morphology and mode of reproduction were studied in purified and clarified malt extract (15 ° Balling) containing 2% NaCl. Observations on the nature of growth, pellicle and sediment formation, and the color, form, consistency and margin

of growth on malt extract agar were of differential value. The formation of arthrospores, pseudomycelia or true mycelia were studied on Rivalier and Seydel's medium (1932) which consists of peptone, 2 g.; glucose, 2 g.; NaCl, 2 g. and distilled water, 100 ml. This medium was found to be superior to the potato-glucose medium used for this purpose by some workers. Sporulation and the formation of ascospores have long been recognized to be of value in taxonomy and several media have been recommended for the purpose. The extensive work of Phaff and Mrak (1948, 1949) on sporulation has indicated carrot wedges to be well suited for this study and we found this medium to be satisfactory. Young, active, well-nourished cultures were inoculated on freshly autoclaved carrot wedges containing a small amount of NaCl. A vegetable juice agar (Wickerham, 1951) was also used with success for this purpose.

Physiological characteristics.—The morphological characteristics of yeasts in general are subject to the limitations of variation which are not infrequent in culture. Physiological characteristics as a rule are less variable and have been extensively used in yeast systematics. Fermentation characteristics, the assimilation of sugar, ethanol and nitrate, acid production and changes in litmus milk are some of the important physiological criteria exploited in species differentiation. The fermentation of glucose, maltose, sucrose, lactose, galactose, raffinose, melibiose and starch were studied in Durham tubes containing a basal inorganic solution containing also 0·1% yeast extract and 2% NaCl to which the separately sterilized carbohydrate solution was added to give a final 2% concentration. Results were recorded daily upto a period of 10 days. Assimilation tests were carried out in clean glass-ware and observing all precautions. The basal medium employed was the one suggested by Lodder and Kreger-van Rij (1952) and which has ammonium sulfate for the nitrogen source and a mixture of vitamins of the B complex family for the growth requirements. The various carbon sources were separately sterilized and added to this medium on the basis of 10 mg. of carbon per 10 ml. of the medium.

For assimilation of nitrate, 1% glucose was used as the carbon source and 0·078% of potassium nitrate as the nitrogen source. Results were recorded daily and after 1, 2, and 3 weeks for sugar assimilation, after 1 month for ethanol assimilation and after 1 week for nitrate assimilation. For the last mentioned test, a second set of tubes was also inoculated with growth from the first to minimize errors due to the presence of soluble nitrogen excreted by cells from the inoculum. This test was also supplemented by the routine biochemical tests for the reduction of nitrate recommended by the Society of American Bacteriologists (1944). The production of

starch was demonstrated by the formation of a blue color on the addition of a few drops of 0·02 N iodine to cultures grown in a medium recommended by Wickerham (1951). The browning of arbutin agar made in a nitrogen base synthetic medium containing 0·1% glucose denoted the splitting of this glucoside. Fat hydrolysis was studied by the appearance of clear zones around the growth after 10 days of incubation in Gorodkowa's tributyrin agar. The various changes brought about in litmus milk (modified by the addition of separately sterilized NaCl to give a final 2% w/v concentration) were also studied and found to be of some value. The inocula for all physiological tests were obtained from homogeneous sterile water suspensions of the organisms grown on yeast extract agar.

RESULTS

Altogether over 80 isolates of marine yeasts were obtained from 17 samples of sea water examined during the course of this investigation, of which 74 have been identified so far. Though none of these corresponded exactly to the species described in Lodder and Kreger-van Rij's (1952) treatise, the differences observed were only of a minor nature and related to the morphology and cultural characteristics which are known to be subject to variation.

Both the families *Endomycetaceæ* (ascosporogenous) and *Cryptococcaceæ* (non-ascosporogenous) were represented in our collection, but the majority of isolates were placed in the latter family. Thus only 18 isolates represented sporogenous yeasts, 10 being placed in the genus *Saccharomyces* and 8 in the genus *Debaryomyces* whilst the remaining 56 isolates belong to the genera *Candida* (30 isolates), *Torulopsis* (16 isolates), *Rhodotorula* (6 isolates), *Cryptococcus* (2 isolates) and *Trichosporon* (2 isolates), all representing the asporogenous yeasts. The general characteristics of these strains are outlined below.

Morphological and cultural characteristics.—The general morphological form consisted of round to oval cells occurring singly, in pairs, or frequently in clumps. The size varied from 1·4 to 8·4/9·8 microns. Vacuoles and refractile granules were invariably present. All isolates showed vegetative reproduction by budding. Most species grew moderately in solid and liquid malt extract media producing floccular or granular sediment in the latter medium. Uniform good turbidity with a pellicle typified growth in sucrose yeast extract broth and on the corresponding agar medium the colonies were usually round, smooth, convex, opaque, and moist or dry with an entire margin. Size of the colonies ranged from 0·5 to 3 mm. and colour from porcelain white to off-white except for the *Rhodotorula* which

produced pink, orange or red pigments. Colony characteristics in general cannot be said to be of much differential value.

Physiological Characteristics.—All the 74 identified isolates were non-acid producing and nonlipolytic, and were unable to utilise nitrate as the sole nitrogen source. Starch was synthesized by 1 isolate only and there was no marked reaction in litmus milk but for the production of alkalinity and reduction of litmus. The majority of the isolates gave luxuriant growth including a pellicle with ethanol as the sole source of carbon. The fermentation as well as assimilation of lactose and starch was in general poor. Glucose was assimilated by all isolates, the majority giving a luxuriant growth with a creeping pellicle.

The isolates made from the sea water and identified by the methods alluded to were the following species or near variants of them:

Saccharomyces :—S. steineri, S. fructuum, S. rosei.

Debaryomyces :—D. hansenii, D. nicotinæ, D. subglobosus, D. klœckeri.

Candida :—C. tropicalis (26 strains), *C. guilliermondi, C. melibiosi.*

Torulopsis :—T. glabrata, T. candida, T. famata.

Trichosporon :—unidentified strains.

In addition to these, a few unidentifiable strains representative of almost all the above genera have been isolated.

Discussion

The presence of several species of yeasts in association with a marine environment suggests that the sea has an autochthonous yeast flora. One of the most striking features of this marine flora is the relative predominance of asporogenous yeasts, an observation which is in agreement with that of the earlier workers (Fisher, 1894 *a*; Nadson and Burgwitz, 1931) inasmuch as those workers referred to them as Torula. If our collection may be regarded in any measure as representative of marine yeasts occurring off the Indian coast, the genus *Candida* may be singled out for special mention. Further, the regularity with which *Candida tropicalis* was encountered in the sea shows that it probably is one of the most common species of marine yeasts.

Yeasts have also been recovered from marine air (Fisher, 1894 *b*; McLean, 1918), fish nets (Freitas, 1953), and several varieties of marine foods (Hunter, 1920, 1922; Hanzawa and Takeda, 1931; MaCormack, 1950; Phaff *et al.*, 1952). The majority of informations incriminating yeasts as spoilage agents of marine foods point to the *Torula* which from all

available evidence appears to represent the most dominant of marine yeasts. Studies on other aspects of the physiology of marine yeasts with particular reference to their nutritional and salinity requirements (unpublished data) seem to suggest that the species described here have their origin in the sea. Furthermore, if one were to scrutinize the sources from which the species named above were originally isolated, one would be surprised to observe that most of them referred to have been derived from brined cucumber, salted beans, dates, grape must, fermented tobacco, palm wine, animal products, butter and human sources (Lodder and Kreger-van Rij, 1952) and this leads one to the speculation that in all probability the sea is the natural habitat of these yeasts.

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SUMMARY

Microbiological analysis of samples of sea water collected off the coast of Bombay resulted in the isolation of over 80 yeasts by the enrichment culture methodology. A study of 74 strains indicated the preponderance of asporogenous over the sporogenous yeasts in the sea. The species *Candida tropicalis* probably occurs in the sea at all times and the other species belonging to the genera *Saccharomyces*, *Debaryomyces*, *Torulopsis*, *Cryptococcus*, *Rhodotorula* and *Trichosporon* have also been suggested as of marine origin.

REFERENCES

Custers, M. Th. J. .. "Onderzockingen over het gisgeslacht Brettanomyces," *Diss.*, 1940, Delft.

Fisher, B. .. "Die Bakterien des Meers nach den Untersuchungen der Plankton Expedition unter gleichzeitiger Berücksichtigung einiger älterer und neuerer Untersuchungen," (*Ergebnisse der Plankton-Expedition der Humboldt-Stiftung*), 1894 *a*, **4**, 1–83.

——— .. "Ergebnisse einiger auf der Planktonexpedition ausgeführten bakteriologischen Untersuchungen der Luft über dem Meere," *Zeitschr. f. Hyg.*, 1894 *b*, **17**, 185–94.

——— and Brebeck, C. .. *Zur Morphologie, Biologie und Systematik der Kahmpilze, der* Monilia candida *Hansen und des Soorerregers, G. Fischer*, Jena, 1894, 55 pp.

Fred, E. B., Wilson, F. C. and Davenport, A. "The distribution and significance of bacteria in Lake Mendota," *Ecology*, 1924, **5**, 322–39.

Freitas, Y. M. .. "Studies on the role of micro-organisms in the deterioration of fish nets and cordage," *Diss.*, Bombay University, 1953.

Hanzawa, J. and Takeda, S. "On the reddening of boned codfish," *Arch. f. Mikrobiol.*, 1931, **2**, 1–22.

Hunter, A. C. .. "A pink yeast causing spoilage in oysters," *U.S. Dept. Agric. Bull.*, 1920, No. 819, 1–24.

———— .. "The sources and characteristics of bacteria in decomposing salmon," *J. Bact.*, 1922, 7, 85–109.

Issatchenko, B. L. .. *Investigations on the Bacteria of the Glacial Arctic Ocean*, Monograph (Russian), Petrograd, 1914, 300 pp.

Lobo, F. X., Pathak, S. M., Kachwalla, N. and Bhat, J. V. "A suitable medium for the enrichment of yeasts," *Curr. Sci.*, 1953, **22**, 174–75.

Lodder, J. and Kreger-van Rij, N. J. W. *The Yeasts, a Taxonomic Study*. North Holland Publishing Company, Amserdam, 1952.

McCormack, G. .. *Pink yeast isolated from oysters grown at temperatures below freezing*, Fish and Wildlife Service, U.S. Dept. Interior, 1950, **12**, 11 A.

Mclean, A. L. .. "Bacteria of ice and snow in Antartics," *Nature*, 1918, **102**, 35–9.

Mrak, E. M. and Phaff, H. J. "Yeasts", *Ann. Rev. Microbiol.*, 1948, **2**, 1–46.

Nadson, G. and Burgwitz, G. "Hefen des Nördlichen Eismeeres," *Compt. rend. Acad. Sci., URSS.*, 1931, 103–10.

Phaff, H. J. and Mrak, E. M. *Sporulation in Yeasts*, Parts I and II. Wallerstein Labs. Commun., 1948–49, **11**, 261–79 and **12**, 29–44.

—, — and Williams, O. B. "Yeasts isolated from Shrimp," *Mycologia*, 1952, **64**, 431–51.

Reuszer, H. W. .. "Marine bacteria and their role in the cycle of life in the sea, III. The distribution of bacteria in the ocean waters and muds about Cape Cod," *Biol. Bull.*, 1933, **65**, 480–97.

Rivalier, E. and Seydel, S. *Nouvean procede de culture sur lames gelosees applique a l' etude microscopique Humanie et Compar.*, 1932, **10**, 442–52.

Smith, B. W. .. *The World under the Sea*. D. Appleton Century Company, N.Y., 1940.

Society of American Bacteriologists *Manual of Methods for Pure Culture Study of Bacteria*, Leaflet V 1944, 9–10.

Wickerham, L. J. .. "Taxonomy of yeasts," *U. S. Dept. Agric. Tech. Bull.*, 1951, 1029.

ZoBell, C. E. and Feltham, C. B. "Preliminary studies on the distribution and characteristics of marine bacteria," *Bull. Scripps. Inst. Oceanograph., Tech. Ser.*, 1934, **3**, 279–96.

ZoBell, C. E. .. *Marine Microbiology*. Chronica Botanica Company, Waltham, Mass, 1946.