concentration considerably as a result of atmospheric isolation. These may possibly result from some nonvolatile peroxide type precursor. It was noticed that terpinolene, the major carrot hydrocarbon, very readily underwent autoxidation when isolated in the pure state. This process probably also happens to some extent in the carrot. The peroxides formed by such oxidation might be expected to break down on heating, e.g., when carrots are cooked or during steam distillation at atmospheric pressure. Terpinolene has a methylene group between two double bonds (doubly allylic). Such methylene groups are well known to be very susceptible to autoxidation. It is fairly easy to see how α-terpineol and terpenin-4-ol could arise from terpinoline through oxidative processes in which the tetra-substituted double bond is attacked. When carrots are blended (which could increase enzymatic oxidation) these two alcohols increase in concentration relative to other components.

Peak 65 (ca. 0.2% of the whole oil), in our opinion, has a moderately intense raw carrotlike aroma. It could not be characterized with certainty even though mass, IR, NMR, and UV spectra were obtained. The mass spectrum measured on Time of Flight mass spectrometer was as follows (two most intense ions each 14 mass units above m/e 34, intensities in parentheses, molecular ion in boldface type): 39 (24), 41 (19); 53 (14), 55 (14); 65 (16), 67 (22); 79 (34), 82 (41); 91 (36), 93 (29); 105 (24), 107 (17); 117 (29), 121 (19); 135 (100), 136 (19); 149 (22), **150** (43). This mass spectrum was somewhat similar to that of carvacrol. CI mass spectrometry confirmed the molecular weight of 150. IR and NMR spectra, however, showed that there was no aromatic ring. IR absorption spectra indicated a probable alcohol with a band at 2.78 μm and a strong band at 9.6 μm. A ^{1}H NMR spectrum (90 MHz, $CDCl_3$) showed δ 1.5 (s, 1 H), 1.62 (s, 2 H), 2.08 (s, 2 H), 2.5 (m, 1 H), 4.5 (s, 2 H), 5.06 (s, 1 H), 5.55 (s, 1 H). A UV absorption spectrum in methanol showed an absorption maximum at 2600 Å with ϵ 2000 (ca.). Catalytic hydrogenation (Pd on charcoal, 10 psi H, 25 °C, 15 h) gave three isomeric alcohols with molecular weight 154 and some 4-isopropylmethylcyclohexane (two isomers). Unfortunately the authors could not come up with a structure that fitted all of these data. It is possible that the compound is changing during the handling.

The mass and infrared spectra of the components in Table I were compared directly with those of authentic samples. The mass spectra of most of the components are also available in the literature (e.g., Stenhagen et al., 1974) except for that of geranyl 2-methylbutyrate, which is listed below: 39 (5), 41 (49); 53 (5), 57 (69); 68 (53), 69 (100); 80 (17), 85 (21); 92 (6), 93 (32), 107 (3), 108 (1); 121 (13), 123 (2); 136 (11), 137 (2); 154 (1); 169 (1) (no molecular ion).

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On the Electrophoretical Differentiation and Classification of Proteins. 10. Comparative Investigation of Yeast Proteins of Various Genera by Means of Isoelectric Focusing in Polyacrylamide Gels

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The water-soluble proteins of eight different yeast genera (*Brettanomyces claussenii, Candida utilis, Cryptococcus laurentii, Debaryomyces hansenii, Kloeckera apiculata, Kluyveromyces lactis, Saccharomyces cerevisiae, Schizosaccharomyces pombe*) obtained by cell disruption with freeze-pressing were resolved by means of isoelectric focusing in cylindrical polyacrylamide gels. In the pH range of 3.5–10.0 carrier ampholytes, about 30 protein bands were found with isoelectric points of 4.5 to 9.7. By using a special one-rod electrode, the pH of the gel slices in minute quantities of distilled water were measured, and the trend of the pH gradient in the whole gel was determined. By means of densitometer tracing of the protein pattern in connection with the pH gradient, the isoelectric points of particular proteins were established. The protein patterns were dissimilar and reproducible under constant working conditions.

In the identification of microorganisms, increasing attention has been paid to physicochemical methods which are more rapid compared with morphological, physiological, and serological methods and which can ideally supplement them (Hedén and Illéni, 1975; Mitruka, 1975). Among these, electrophoresis of proteins has been proved to be a convenient method for diagnostic and taxonomic purposes.

In spite of the rapid increase in the use of electrophoretic methods in bacteriology, it has been used to a very limited extent with yeasts.

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Many workers have studied human pathogenic bacteria such as isolates of *Yersinia* (Hudson et al., 1973), *Mycoplasma* (Razin and Rottem, 1967; Rottem and Razin, 1967; Razin, 1968; Forshaw, 1972; Theodore et al., 1971), *Salmonella* (Ames, 1974), *Mycobacterium* (Haas et al., 1972; Affronti et al., 1972), *Streptomyces* (Gottlieb and Hepden, 1966), *Clostridium* (Dyer and Anderson, 1971), animal pathogens such as *Brucella* (Morris, 1973), *Haemophylus* (Neumann and Hinz, 1977), or phytopathogens such as *Pseudomonas* (Palmer and Cameron, 1971) and *Xanthomonas* (El-Sharkawy and Huisingh, 1971). Kersters and De Ley (1975a,b) identified and grouped *Zymomonas mobilis* and *Agrobacterium* by numerical analysis of their protein profiles and classified the genus *Zymomonas* (Swings et al., 1976; Swings and De Ley, 1977). Protein patterns were found useful also for comparative investigation of parasitic fungi such as *Septoria* (Durbin, 1966), *Phytophtora* (Hall et al., 1969), and *Verticilium* and *Fusarium* (Whitney et al., 1968).

The development of isoelectric focusing has provided a separation method of higher resolving power, caused by concentration of the proteins during their separation (Wadström and Smyth, 1973, 1975a,b). Electrofocusing has been used for the study of proteins of *Staphylococcus aureus* (Wadström et al., 1974), staphylococcal and clostridial toxins (Arbuthnott et al., 1975), and θ-hemolysin of *Clostridium* (Smyth, 1975). By means of zymogram methods L-asparaginase from *Serratia marcescens* and *Escherichia coli* (Stern et al., 1976), β-lactamases from various bacteria (Matthew et al., 1977) and proteases from *Pseudomonas aeruginosa* (Wretlind and Wadström, 1977) have been studied.

Malate dehydrogenases from *Schizosaccharomyces pombe* were studied by Flury et al. (1974) and from *Saccharomyces cerevisiae* by Hägele et al. (1978). Enzyme polymorphisms of *Saccharomyces* strains by starch gel electrophoresis were reported by Donhauser et al. (1976). We have chiefly compared the water-soluble proteins from *Saccharomyces cerevisiae*, syn. *carlsbergensis* (bottom-fermenting yeasts) and *Saccharomyces cerevisiae* (top-fermenting yeasts) with those of *Achromobacter alcaligenes* and *Pseudomonas ovalis* by thin-layer isoelectric focusing in granular gels (Drawert et al., 1973a) and in cylindrical polyacrylamide gels (Drawert et al., 1973b). The protein patterns of the microorganisms which have been systematically placed apart were dissimilar and reproducible with constant experiment conditions.

The protective and reproducible cell disruptions were carried out with freeze-pressing by means of Biox-X-technique, which is described elsewhere (Drawert and Bednář, 1974a). We have also compared the protein patterns from one run to another and we have done the comparative traces with very good reproducibility. The protein patterns from 2-year-old samples of *Cryptococcus laurentii* and *Schizosaccharomyces pombe* stored at 4 °C in darkness were identical with the fresh disrupted cells (Drawert and Bednář, 1978a). A comparative investigation of yeast proteins within the genus *Saccharomyces* and among various strains of the species *Saccharomyces cerevisiae* Hansen will be published (Drawert and Bednář, 1978b). The results from this also show very good reproducibility of protein patterns among several runs of the same sample.

MATERIAL AND METHODS

Organisms. The strains of yeasts used in this study were obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland, as follows: *Brettanomyces claussenii* Custers 1938, fr. beer, K. Andersen; *Candida utilis* (Henneberg) Lodder & van Rij 567, fr. distillery, R. Ciferri; *Cryptococcus laurentii* (Fuff.) Skinner 139, T, fr. palm wine, H. Kufferath; *Debaryomyces hansenii* (Zopf) Lodder & van Rij 768 (Deb. membranaefaciens), fr. air, K. Saito; *Kloeckera apiculata* (Reess emend. Klöcker) Janke 2590, fr. draught beer, B. Johnsson; *Kluyveromyces lactis* (Dombrowski) van der Walt 2359; *Saccharomyces cerevisiae* Hansen 381, H. Naganishi; *Schizosaccharomyces pombe* Lindner 5680 (Schiz. pombe v. acidodevoratus), fr. apple, H. H. Dittrich. All the strains have been described by Lodder (1971).

Culture Condition. The cells were transferred from the agar slants into 50-mL Erlenmeyer flasks containing 20 mL of the culture media. The succeeding dilutions of the initial inoculum were done in the cultivation flasks with fresh culture media (1:40). Difco YM Broth was used as the nutrient media. The incubation of the aerobic cultures was carried out on a rotating roller drum apparatus (Brunswick Scientific, N.J.) at 25 °C and 200 rpm. The cell mass needed for the Biox-X-Press cell disintegrator was obtained by harvesting the cultures at their exponential growth phase. The growth rate of the cultures were estimated by optical density measurement of the diluted (1:20, v/v) culture solutions (Barton, 1973).

Preparation of the Cell-Free Extracts. The yeast cells were centrifuged at 20 000*g* and washed in distilled water, and the process of centrifuging was repeated again. The cell mass was then transferred into a previously cooled disintegrator (Biox-X-Press LKB, Bromma, Sweden) and was deep frozen at −25 °C overnight. The cells were disintegrated as mentioned in our previous work (Drawert and Bednář, 1974a). The ruptured cell mass was centrifuged at 5000*g*, and the supernatant cell plasma was lyophilized in high vacuum. A total of 400–500 mg of dried extract was obtained at the end of this process.

Isoelectric Focusing in Polyacrylamide Gel. The separation of protein according to its isoelectric points (pI) was achieved in disc-electrophoresis apparatus (Desaga, Heidelberg, West Germany) coupled with a thermostat (Lauda, MGW, West Germany). As power supply we used the power unit Duostat, Model RD 2 (Beckman Instruments). The supply of current varied from an initial wattage of 2 mA/tube up to a maximum voltage of 500 V for about 5 h. Phosphoric acid solution (1% v/v) was used at the anode and ethylenediamine solution (2% v/v) at the cathode. The following separating gel was used: 7% (w/v) polyacrylamide gel with 5% (v/v) carrier ampholyte (Ampholine, LKB Bromma, Sweden) of pH 3.5–10.0.

Procedure. Glass tubes (5 × 150 mm) containing the polymerized gel (105 mm) were filled with 200 μL (w/v) of sample solution (1–2% w/v) of the lyophilizate in 25% (w/v) sucrose solution and overlayered with 20% (w/v) sucrose solution containing 2% (v/v) carrier ampholyte, avoiding the mixing of the two solutions. After focusing, the gel was taken out, and the protein was fixed in 12% (w/v) trichloroacetic acid and then dyed with 0.1% (w/v) of Coomassie Brillant Blue G 250 (Serva, Heidelberg, West Germany). The dye was prepared in a solvent mixture of methanol, acetic acid, and water (45:10:45). The excess dye adhering to the gel was washed out with the same solvent mixture lacking the coloring material, and the densitometric tracing of the gel was performed.

Densitometry. The gels were inserted in a Plexiglas sample holder and scanned with a Vitatron TLD 100 (Forshbach, West Germany), fitted with filter no. 580 and a shutter 0.25. The selected proportion of breadth and length of the densitograms was in the ratio 1:1 (densitometer advance, 0.5 cm/min in position 1 and the paper

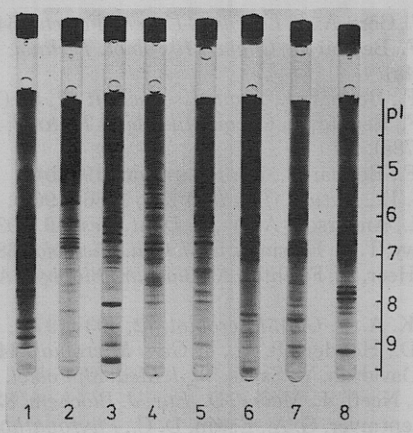

Figure 1.

advance, 0.5 cm/min in position 3).

pH Gradient Estimation. The estimation was done by cutting the gel into 5 mm long pieces, incubating in distilled water, and measuring the pH directly. It was possible to measure the pH of samples in minute quantities (100–200 μL) by using a special electrode (one-rod electrode, Ingold, West Germany).

RESULTS

The protein patterns in the eight selected strains of microorganisms belonging to various genera were dissimilar. In the pH range of 3.5–10.0 carrier ampholytes, 25–30 protein bands were found with isoelectric points of 4.5 to 9.7 (Figure 1).

Brettanomyces claussenii CBS 1938 indicated relatively uniform distribution of proteins in a pH range between 6–9.2. The four protein bands in the pH range of 8.5–9.2 have not as yet been reported in any other yeast varieties. The main bands were found at pH 7.5. In *Candida utilis* CBS 567, the majority of protein bands appeared in the acid range. The main bands occurred at pH 5.0 and between pH 5.5 and 6.8. Bands of diminished intensity were observed in the basic range up to pH 9.5. *Cryptococcus laurentii* CBS 139 showed strongly marked bands at pH 8.2–9.5. At pH 8.7 and 9.1, the two bands were relatively faint but sufficiently pronounced. The entire pattern, however, was otherwise distributed over a wide range beginning at pH 4.5. In *Debaryomyces hansenii* CBS 768, the protein bands were localized in pH ranges 5.1–6.0 and 6.6–8.2. *Kloeckera apiculata* CBS 2590 had two strongly marked protein bands at pH 5.8–5.9, more intensely stained than the other bands. The next relatively intense band occurred at pH 6.7. Only a few protein bands were detected in the alkaline range. The two main bands observed in *Kloeckera apiculata* CBS 2590 were also found in *Kluyveromyces lactis* CBS 2359. A conglomerate of protein bands of almost the same intensity was detected in the pH range of 6.3 to 7.7. In *Saccharomyces cerevisiae* CBS 381 predominant bands appeared in two zones, 5.3–6.2 and 6.6–7.2, as already reported (Drawert et al., 1973) for the brewing yeasts. In addition, the protein pattern has a distinctive band at pH 8.4. In *Schizosaccharomyces pombe* CBS 5680, three intense protein bands were noticed between pH 7.8 and 8.0 and a single band at pH 9.1. Double bands of approximately equal intensity were observed at pH 6.6. Numerous sharp bands were seen between pH 5.5 and 6.3.

Densitometric tracings of the protein profiles according to pH gradients are presented in Figures 2 and 3 for the different genera of yeasts.

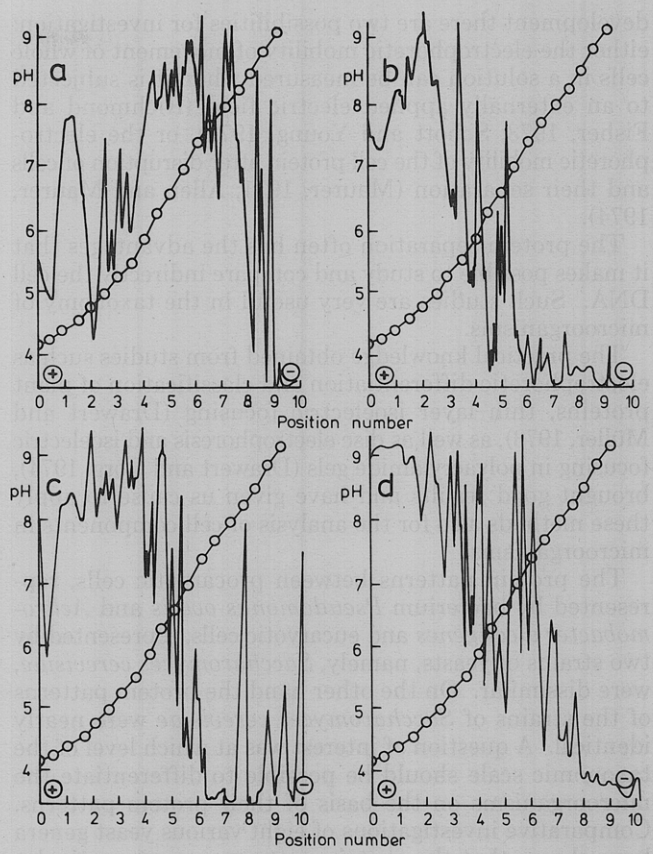

Figure 2.

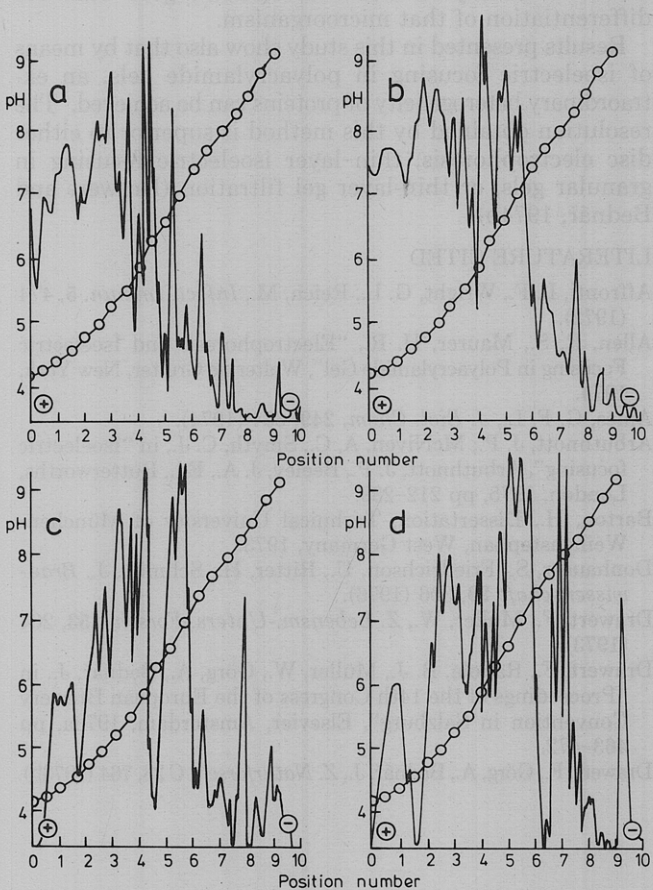

Figure 3.

DISCUSSION

Electrophoretic methods may be a very useful tool for chemotaxonomy of microorganisms. By way of methodical

development there are two possibilities for investigation: either the electrophoretic mobility of movement of whole cells in a solution can be measured when it is subjected to an externally applied electric field (Richmond and Fisher, 1973; Schott and Young, 1972), or the electrophoretic mobility of the cell protein after disruption of cells and their separation (Maurer, 1971; Allen and Maurer, 1974).

The protein separation often has the advantages that it makes possible to study and compare indirectly the cell DNA. Such studies are very useful in the taxonomy of microorganisms.

The practical knowledge obtained from studies such as electrophoretic differentiation and classification of plant proteins, thin-layer isoelectric focusing (Drawert and Müller, 1973), as well as disc electrophoresis and isoelectric focusing in polyacrylamide gels (Drawert and Görg, 1974), brought good results and have given us cause to apply these methods also for the analysis of cell components in microorganisms.

The protein patterns between procaryotic cells, represented by bacterium *Pseudomonas ovalis* and *Achromobacter alcaligenes* and eucaryotic cells, represented by two strains of yeasts, namely, *Saccharomyces cerevisiae*, were dissimilar. On the other hand the protein patterns of the strains of *Saccharomyces cerevisiae* were nearly identical. A question of interest was at which level of the taxonomic scale should be possible to differentiate the microorganisms on the basis of their protein patterns. Comparative investigations of eight various yeast genera have shown that the protein patterns among particular strains were very variable and formed a good basis for differentiation of that microorganism.

Results presented in this study show also that by means of isoelectric focusing in polyacrylamide gels, an extraordinary heterogeneity of proteins can be achieved. The resolution obtained by this method is superior to either disc electrophoresis, thin-layer isoelectric focusing in granular gels, or thin-layer gel filtration (Drawert and Bednář, 1974b).

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