KIMIKA 9: 65-72 (1993)
Printed in the Philippines

REVIEW PAPER

Chemical and microbial decolorization of molasses-derived melanoidin

ERNESTO J. DEL ROSARIO
Institute of Chemistry and National Institutes of Biotechnology and Applied Microbiology
University of the Philippines at Los Baños
College, Laguna

(Received October 20, 1992; accepted March 10, 1993)

Melanoidin, which results from the Maillard reaction between sugars and amines upon heating, has a complex polymeric structure whose molecular size is affected by pH and temperature. Chemical methods of decolorizing molasses- derived melanoidin include flocculation using either inorganic or organic compounds while microbial decolorization methods involve either enzymes or bio-flocculants. The present paper reviews relevant research work on the chemical and microbial methods of decolorizing molasses-derived melanoidin with emphasis on alcohol distillery effluent.

Key Words: decolorization, melanoidin, distillery effluent

COLOR POLLUTION OFFERS A SIMILAR, IF NOT MORE DIFFICULT, challenge to environmental scientists who are concerned with reduction of oxygen demands, either chemical (COD) or biochemical (BOD). Although somewhat intractable scientifically, it is more obvious to the layman than COD or BOD because of its visual nature. Thus, decolorization of liquid wastes deserves ample research and development efforts. The goal here is not only to solve an environmental problem but also to discover new knowledge about reactions of melanoidin, the chromophoric substance which is responsible for color pollution.

Molecular Studies on Melanoidin

Maillard reaction

The nonenzymatic reaction between reducing sugars and amino compounds was first reported by the French chemist Maillard in 1912 (1) who observed the formation of brown pigments or melanoidins after heating a solution of glucose and glycine. The reaction was subsequently called "Maillard reaction" and was expanded to include similar reactions between amines, amino acids or proteins on the one hand with sugars, aldehydes or ketones on the other hand.

The primary and secondary steps in the Maillard reaction for glucose and a primary amine are shown in Figure 1. The

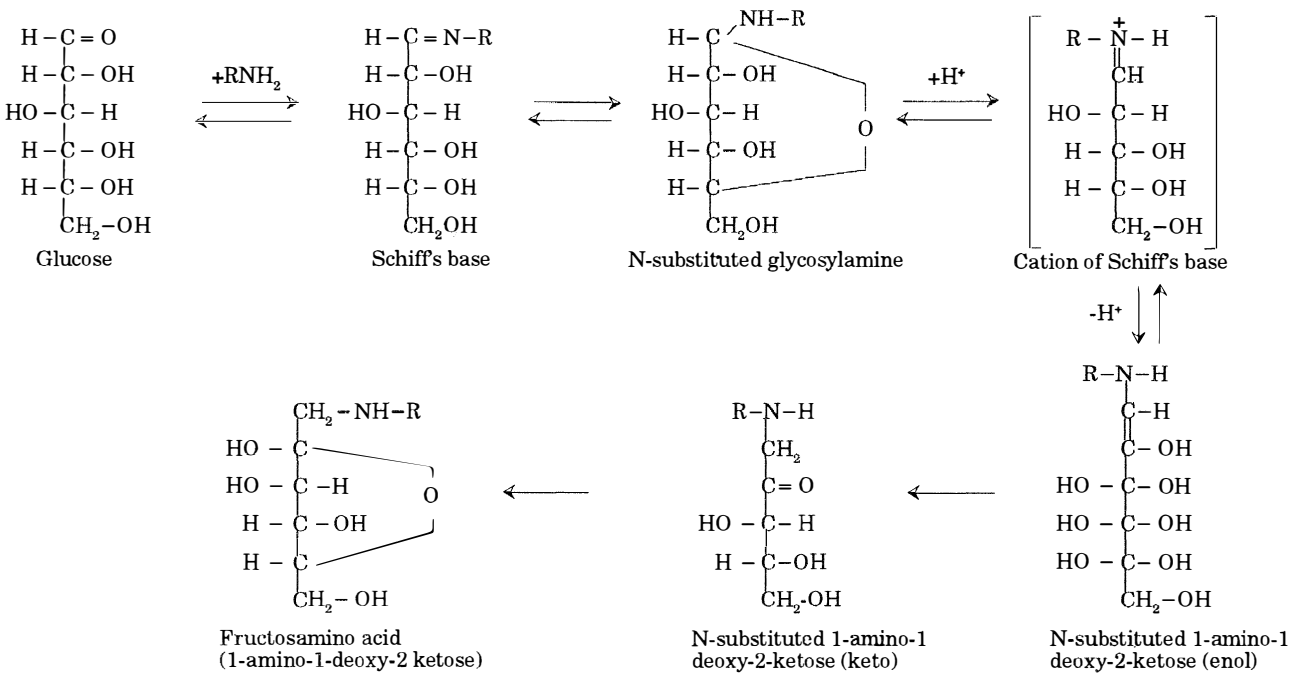


Figure 1. Initial steps in the Maillard reaction between glucose and a primary amine (31).

initial reactions involve formation of a Schiff's base and its subsequent cyclization to form an N-substituted glycosylamine followed by the Amadori rearrangement involving conversion of an aldose to a ketose sugar derivative. However, the reactions up to this point have all been shown to be reversible and the products are still colorless. The formation of melanoidin pigments results from the decomposition of Amadori compounds as shown in Figure 2.

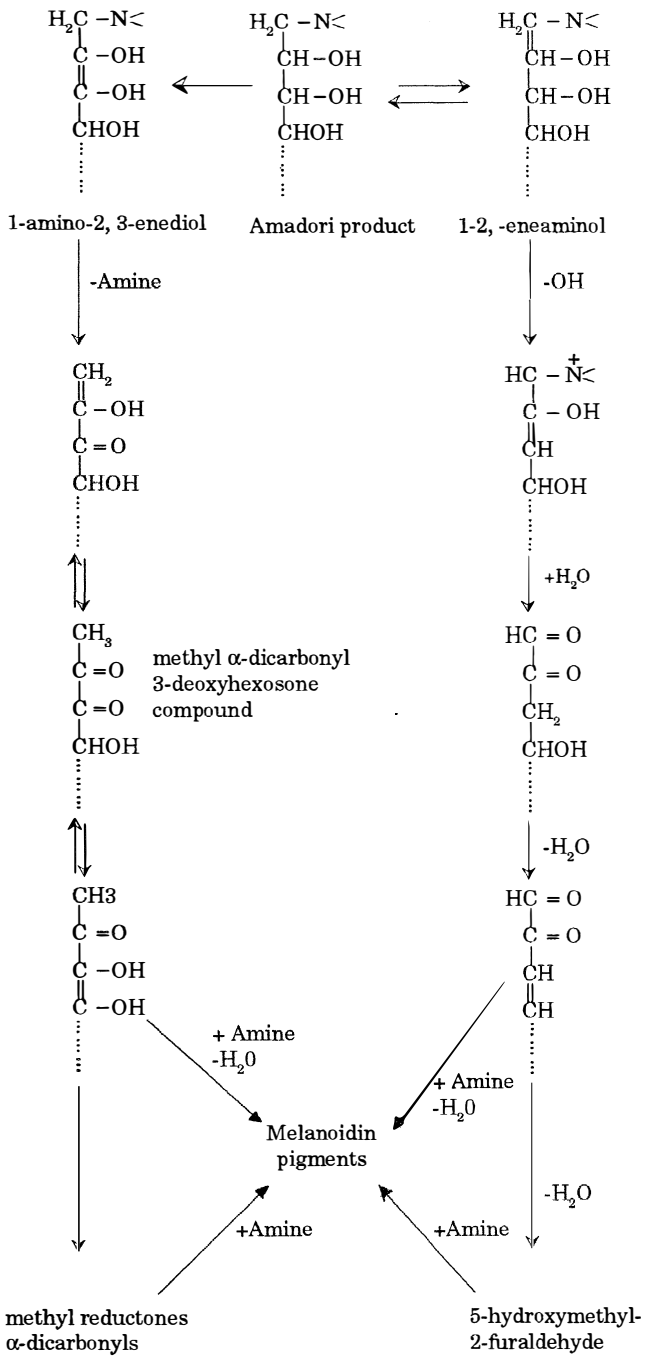


Figure 2. Decomposition reactions of Amadori compounds to produce melanoidin pigments (32).

Molecular structure of melanoidin

Kato and Tsuchida (2) evaluated molecular structures for melanoidin based on the results of pyrolysis of melanoidin which had been prepared from D-xylose or D-glucose and butylamine in methanolic solution. A possible structure which they claimed was supported by their experimental data is shown in Figure 3.

Preliminary studies have been conducted by the author (unpublished data, ref. 5) on the structural elucidation of molasses melanoidin in the biogas digester effluent from a local alcohol distillery. The crude melanoidin, which had been deionized by means of a mixed bed ion exchanger (Dowex 1 and Dowex 50W), was partially purified by column chromatography on octadecylsilane-(C_{18})-bonded silica (reversed-phase). The eluted highly-colored compounds were non-volatile, highly soluble in water or methanol and appeared to be a mixture of polymers when examined by thin- layer chromatography on C_{18}-bonded silica using suitable solvent systems. These compounds were examined by mass spectrometry, as well as infrared (IR), nuclear magnetic resonance (NMR) and ultraviolet (UV)- visible spectroscopic methods.

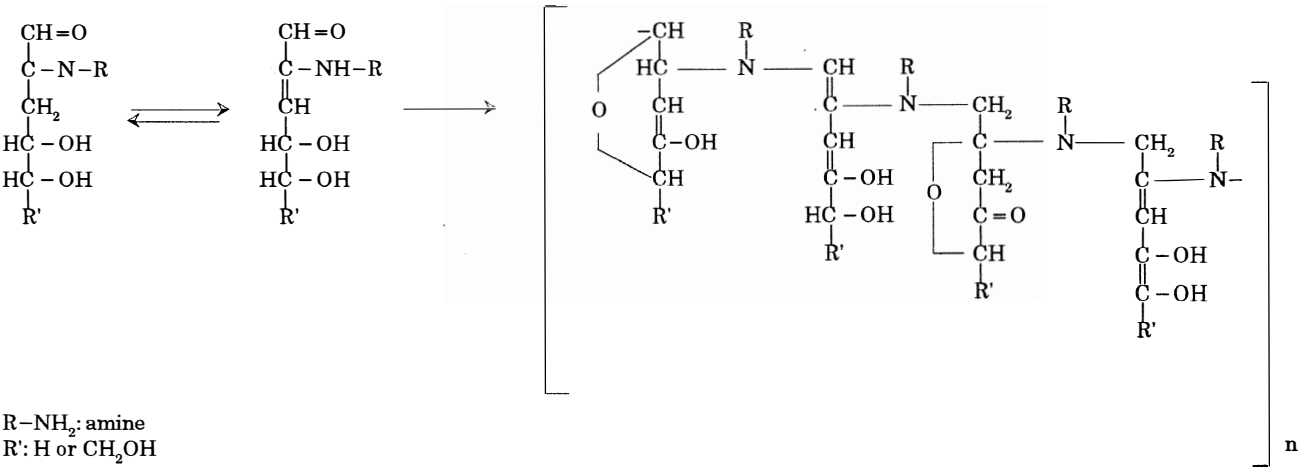
Mass spectrometry revealed the presence of some diagnostic fragments from melanoidin, notably CH_3O^+, $C_3H_7O_2^+$ and $C_4H_9O_2^+$ (Table 1). These fragments appear to be scission products from a polymeric structure such as that shown in Figure 3 (2). The infrared spectrum of partially purified melanoidin has the characteristic IR absorption bands showing the presence of chemical groups which are either supportive of, or not in gross conflict with, the suggested structure in Figure 3. However, preliminary results of NMR spectroscopy show significant inconsistencies with the said structure. This means that further research is needed in order to elucidate the detailed molecular structure of melanoidin.

The effect of pH and O_2 on the molecular size of non-dialyzable melanoidin was studied by Okada et al., who found the pK of the polymer to be 3.5 (3). The polymeric size of melanoidin increased under acidic conditions and decreased at high pH. At the latter conditions, the authors suggested that depolymerization was catalyzed by the hydroxide ion with the aid of molecular oxygen. The same authors in a subsequent paper (4) observed that during melanoidin preparation, the molecular size increased as the pH was reduced below 4.5. Similarly, repeated isoelectric precipitation at pH 2.5 caused an increase in molecular size. Acid treatment further increased the molecular weight.

Chemical Decolorization of Melanoidin

Decolorization by inorganic flocculants

The results of decolorization of molasses melanoidin in alcohol distillery biodigester effluent (ADBE) using ferric chloride ($FeCl_3$) or aluminum chloride ($AlCl_3$) are shown in Figure 4 (5). The left vertical axis (ordinate) represents the residual turbidity, while the right ordinate represents total organic carbon, of the supernatant solution after centrifugation of the flocculated melanoidin. Residual turbidity was calculated as the ratio of absorbances (at 475 nm) for the sample and control (water added without inorganic



$R-NH_2$: amine
R': H or CH_2OH

Figure 3. Possible molecular structure of molasses-derived melanoidin (2).

flocculant). As shown in Figure 4 the optimum concentration of either Fe^{3+} or Al^{3+} needed for decolorization was approximately 0.035 molar (M). At this concentration 93% decolorization of undiluted ADBE was obtained while reduction in total organic carbon in the supernatant solution was approximately 76%. An excess of either trivalent cation increased the residual turbidity and suspended organic carbon.

The corresponding data for the decolorization of undiluted ADBE using the commercial flocculant "Polytetsu" whose chemical formula is $[Fe_2(OH)_n(SO_4)_{3-n/2}]_m$ are presented 2 in Figure 5. Addition to ADBE of the optimal concentration of flocculant, i.e. 0.02 v/v corresponding to 0.039 M Fe^{3+} concentration in the final solution, resulted in more than 98% decolorization of ADBE and more than 90% reduction of the total organic carbon in the supernatant solution. These values are higher than the corresponding values using either $FeCl_3$ or $AlCl_3$ as presented in Figure 4. Similarly, addition of excess flocculant adversely affected the decolorization process.

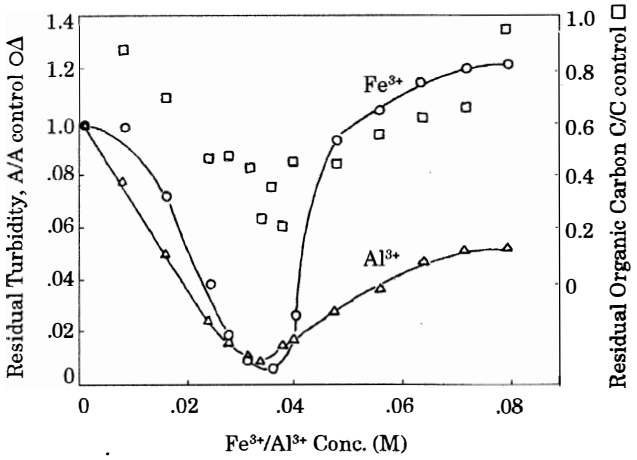


Figure 4. Decolorization of alcohol distillery biodigester effluent (ADBE) by $FeCl_3/AlCl_3$ (5).

Table 1. Diagnostic fragments from ADBE melanoidin obtained by mass spectrometry (5).

Mass/ Charge Ratio m/z	Probable Chemical Fragment	
	Formula	Structure
31	CH_3O^+	$+CH_2-OH$
75	$CH_3H_7O_2^+$	$+CH_2-CH(OH)-CH_2-OH$
89	$C_4H_9O_2^+$	$+CH_2-CH_2-CH(OH)-CH_2-OH$

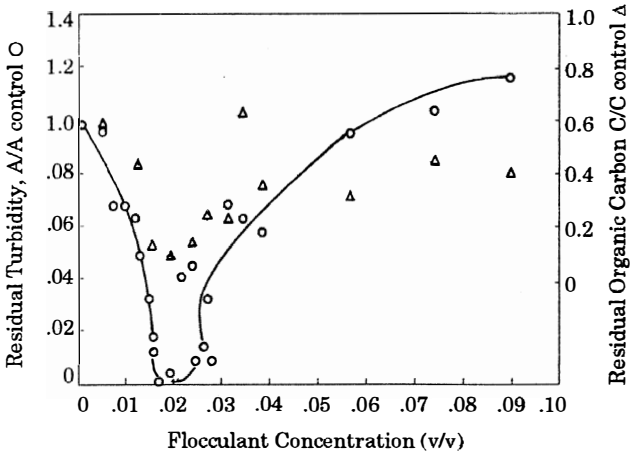


Figure 5. Decolorization of alcohol distillery biodigester effluent using polymeric flocculant with formula $[Fe_2(OH)_n(SO_4)_{(3-n/2)}]_m$ (5).

The concentration of the major ions of ADBE before and after addition of the commercial flocculant "polytetsu", as well as ion analysis of the latter, are given in Table 2. This table shows that the total ionic concentration of the supernatant solution was reduced to less than one-half of the initial value after flocculation. However, sulfate ions were added while the Fe^{3+} concentration was slightly increased after decolorization.

Recently, Migo et al. (6) have shown that addition of calcium ion (as $CaCO_3$) at the rate of 8 g/l improved decolorization of two distillery effluent samples with the commercial ferric flocculant "polytetsu" (0.04 v/v) to more than 94% calcium ions removed 95% of the dark color of dialyzed fresh slops in the presence of the commercial flocculant. Fluoride ions, as well as divalent ions were found to affect the extent of effluent decolorization.

It was observed that the trivalent anion PO_4^{3-} was much less efficient in flocculating the colloidal particles of melanoidin compared to the trivalent cations. Furthermore, the divalent cations, such as $Ca^{2+}$and Mg^{2+}, were less efficient flocculants than the trivalent cations in support of the Schulze-Hardy Rule (7,8,9). These observations strongly suggest that each melanoidin particle has a net negative charge and flocculation involves charge neutralization as shown in the mechanism of Figure 6. As depicted in this figure, the colloidal dispersion is stabilized by electrostatic repulsion between negatively- charged colloidal particles. Added cations neutralize the particle charges and allow the van der Waals attractive forces to operate, thereby causing flocculation of the particles. However, adsorption of excess cations cause deflocculation because of mutual repulsion between positively-charged particles. The electrical charge properties of colloidal particles may be explained on the basis of the concept of the electric double layer (7,9,10). Each colloidal particle, on account of its net electrical charge, is surrounded by ions whose distribution in solution may be described in terms of a double layer. This consists of an inner region which may include adsorbed ions and a diffused region in which ions are distributed according to the influence of electrical forces and random thermal motion. Various models have been proposed for the electric double layer, the most notable of which are the Stern and Gouy-Chapman models (7,10). As shown in Figure 7, the inner or Stern layer contains ions which are attached to the surface of the colloidal particle by electrostatic and/or van der Waals forces. The attachment is strong enough to overcome thermal agitation. Ions with centers located outside the Stern plane form the diffuse part of the double layer. The key features of the Stern model, such as the value of the Stern potential, the "thickness" ($1/\kappa$) of the double layer and the electrostatic or zeta (ζ) potential (7) are also indicated in Figure 7.

Table 2. Concentration of major ions in alcohol distillery biodigester effluent (ADBE) before and after addition of commercial flocculant "Polytetsu" (5).

Sample	Ion Concentration (ppm)					
	K^+	Na^+	Ca^{2+}	Fe^{3+}	Cl^-	SO_4^{2-}
ADBE before adding flocculant	3692	178	431	6.8	1340	0
ADBE after adding flocculant	440	79	56	8.5	107	625
"Polytetsu"	6398	5385	1224	7.8×10^4	0	56.8×10^4

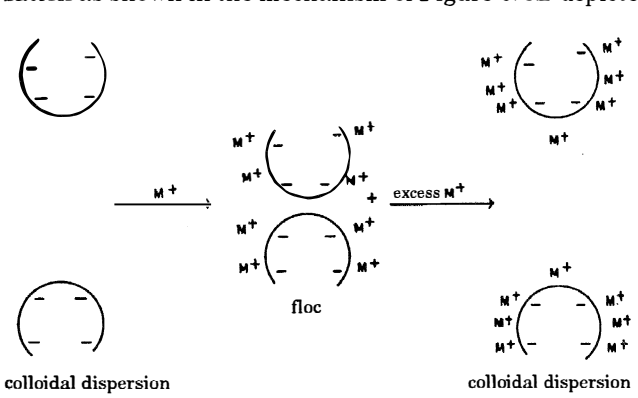


Figure 6. Possible mechanism for effect of cation M^+ on flocculation and deflocculation of colloidal melanoidin particles (5).

The above-mentioned mechanism (Fig. 6) for flocculation of melanoidin particles in the presence of trivalent (Fe^{3+} or Al^{3+}) ions is able to explain, to a large extent, the experimental results of Figure 4, wherein maximal flocculation/decolorization was observed at 0.035 M trivalent ion concentration. However, it is important, also, to take into account equilibrium reactions that determine the actual concentration of trivalent cations compared to other ionic forms (with net ionic charge less than 3+) which are less effective in charge neutralization and colloid coagulation. An example is shown in Figure 8 where the speciation of iron is plotted for ferric chloride solutions; the abscissa is expressed as the negative logarithm (base 10) of the total Fe^{3+} concentration. As shown in Figure 8 the maximum in $Fe(H_2O)_6^{3+}$ concentration is at a $pFe(III)_T$ value of about

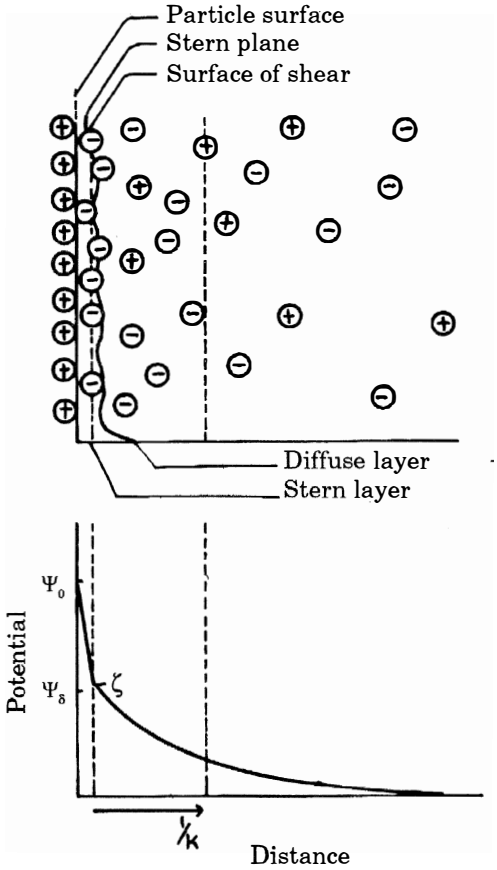


Figure 7. Schematic representation of the structure of the electric double layer according to Stern's theory (7).

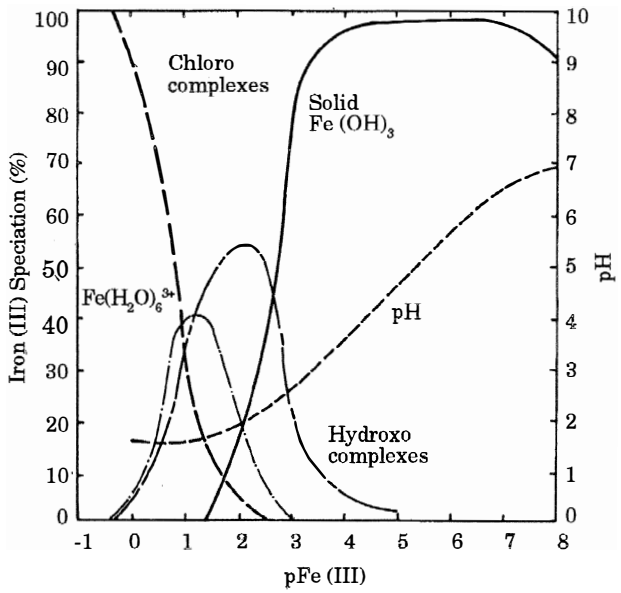


Figure 8. Species composition of ferric chloride solutions (33).

1.2 corresponding to a total ferric ion concentration $Fe(III)_T$ of 0.063 M. The discrepancy between this value and that experimentally observed (0.035 M) for the optimal concentration of Fe^{3+} needed for flocculation of ABDE (shown in Fig. 4) is presently unexplained.

Recent results obtained by V. P. Migo (unpublished data, ref. 6) have shown the following: a) melanoidin is affected by pH and precipitates at the isoelectric point of 2.5; at higher pH there is a change in the molecular size of the polymers, b) fluoride ions affect the color of melanoidin, c) low melanoidin colloid concentrations require low dosage of the polyferric sulfate flocculant, d) the resulting pH after addition of this flocculant to the melanoidin-containing effluent varied from 2.5 to 4.5; at this pH range, the forms of Fe(III) and the mechanisms of destabilization are sweep flocculation for $Fe(OH)_3$ and adsorption flocculation for $Fe(OH)^+_2$, $Fe(OH)^{2+}$ and $Fe_2(OH)_2^{4+}$ (11).

Decolorization by organic flocculants

Organic linear homopolymers have been found to be good flocculants of colloidal particles. Some synthetic polymeric flocculants are given in Table 3. These are classified as non-ionic, anionic and cationic.

A mechanism for flocculation brought about by organic polymers involves bridging between colloidal particles by adsorbed polymer chains. Detailed models for this bridging mechanism have been discussed in the literature (12).

Microbial Decolorization of Melanoidin

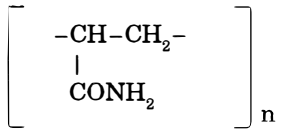
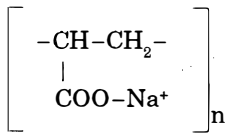
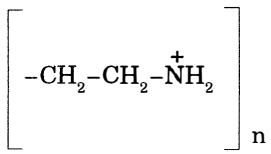
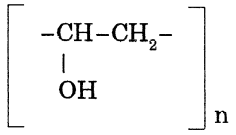
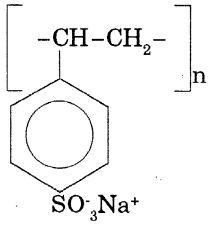
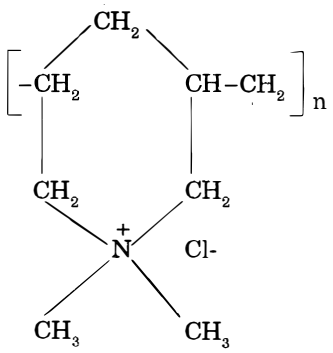
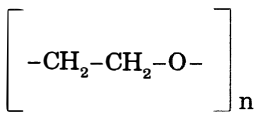
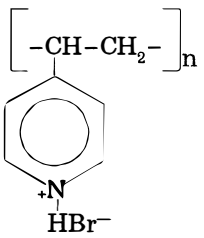
Decolorization by microbial enzymes

Various microorganisms have been found effective in decolorizing molasses wastewater. These include *Coriolus sp.* (13), *Coriolus versicolor* (14,15), *Aspergillus fumigatus* (16), *Aspergillus oryzae* (17), *Mycelia sterilia* (18,) other filamentous fungi (19), *Lactobacillus hilgardii* (20,21), agar bacteria (22) and *Bacillus sp.* (23,24). The decolorization of melanoidin exhibited by these microorganisms may be explained in terms of the action of the secreted microbial enzymes.

The melanoidin decolorizing enzyme of *Coriolus sp.* No. 20 was purified and characterized (13). The optimal pH and temperature of the enzyme were pH 4.5 and 35°C, respectively, and the molecular weight (MW) was about 200 kilodaltons (kD). The purified enzyme was identified as sorbose oxidase. Decolorization was observed in the presence of oxygen and a sugar such as glucose, sorbose, maltose, sucrose, lactose, galactose or xylose; glucose was converted into gluconic acid. It was suggested that active oxygen (O_2^-, H_2O_2) produced by the oxidase reaction was responsible for melanoidin decolorization.

Similar work on the enzymes of *Coriolus versicolor* Ps4a was reported by Ohmomo et al. (14). Two enzyme fractions were observed. The main fraction consisted of at least five enzymes, two of which were characterized. One enzyme (P-III) had a MW of about 49 kD, an optimal pH of 5.5, and optimal temperature of 30-35°C and required glucose and O_2 for activity. The other enzyme (P-IV) had a MW of about 44 kD, an optimal pH of 4.0-4.5, an optimal temperature of 30- 35°C and could decolorize melanoidin in the absence of

Table 3. Some synthetic polymer flocculants (12).

Nonionic	Anionic	Cationic
		
Polyacrylamide	Sodium polyacrylate	Polyethyleneimine
		
Polyvinyl alcohol	Sodium polystyrene sulfonate	Polydiallydimethylammonium chloride ("Cat-Floc")
		
Polyethylene oxide	Polyvinylpyridinium bromide	

glucose and O_2. This second enzyme (P-IV) was found to react directly with melanoidin in contrast with P-III enzyme which decolorized melanoidin indirectly as a side reaction of a sugar oxidase.

Results obtained in the author's laboratory on the decolorization of melanoidin in alcohol distillery biodigester effluent (ADBE) involved the use of two local isolates of *Bacillus subtilis* which had been immobilized in alginate gel beads (24). A maximal value of 75% relative color reduction (RCR) was observed after two days at ten-fold dilution of the effluent; lower relative color reduction was obtained for the undiluted effluent. Decolorization of the five-fold diluted effluent was approximately 54-58% after two days using either a 3.5-liter tower reactor or a 4-liter stirred tank reactor containing the immobilized bacteria. The uninoculated gel beads were found to contribute 30-40% of the RCR value observed for the immobilized bacteria.

Screening of 236 filamentous fungi resulted in 12 isolates which gave more than 40% RCR of ABDE at five-fold dilution. Two isolates (MD-1 and MD-2) which have been identified to belong to the genus *Aspergillus* using the slide culture technique were further tested for their ability to decolorize MPB at dilution factors of 10, 5 and 1 after 8, 11 and 14 days of incubation with shaking at room temperature (Table 4). These preliminary results indicate that these isolates are capable of decolorizing melanoidin at rates much higher than those observed for immobilized bacteria. Also, it has also been observed that these two isolates can decolorize pure melanoidin, something which has been observed in bacteria. Color reversal, however, has been observed in isolate MD-1 though the exact cause is yet unknown. It has also been observed that isolates MD-1 and MD-2 grow very rapidly in melanoidin pigment broth and a large amount of mycelia is produced.

Table 4. Relative color reduction of ADBE by fungal isolates MD-1 and MD-2 at different concentrations of melanoidin (24).

Isolate	Dilution Factor	Relative Color Removal (%)		
		8 days	11days	14 days
MD-1	10	67.8	78.6	74.7
MD-2		57.7	74.1	77.6
MD-1	5	67.4	77.8	76.2
MD-2		73.1	75.8	73.5
MD-1	1	47.7	20.1	3.6
MD-2		63.6	61.3	60.3

Decolorization by microbial flocculants

Some microorganisms secrete substances that are capable of flocculating and decolorizing molasses-derived melanoidin. These include *Aspergillus sojae* (25), *Paecilomyces sp.* (26), *Rhodococcus erythropolis* (27-29) and *Alcaligenes latus* (30).

Characterization of the flocculant produced by *Rhodococcus erythropolis* was recently done by Kurane (personal communication, 1991). A flocculant yield of 0.5 gram per liter of fermentation medium was obtained. The bio-flocculant was found to be a protein with a molecular weight of about one million daltons and with a large proportion (~35%) of hydrophobic amino acids. It is relatively heat stable and can withstand boiling for 10 minutes without loss of flocculant activity. It causes at least 80% decolorization of alcohol distillery effluent in less than 3 minutes, as well as pulp and paper mill wastes and other highly colored effluents.

Summary

Color pollution caused by fermentation industries using molasses as substrate is due to melanoidin which is formed by the Maillard reaction between amino- and carbonyl-containing compounds followed by chemical decomposition of Amadori compounds to form highly-colored pigments. Although its detailed molecular structure has not been determined, melanoidin probably consists of a distribution of polymers containing dihydroxyl and amino groups, as well as conjugated double bonds, as shown by spectroscopic techniques, in general conformity with the postulated structure of Kato and Tsuchida (2). The molecular size of melanoidin is affected by pH and temperature as shown by Okada et al. (3,4).

Chemical methods of decolorizing molasses-derived melanoidin include flocculation using either inorganic or organic compounds. Experimental results showed that optimal decolorization of alcohol distillery biodigester effluent (ADBE) by $FeCl_3$ or $AlCl_3$ was obtained at a trivalent cation concentration of approximately 0.035 M. At this concentration 93% decolorization of undiluted ADBE was obtained while reduction in total organic carbon in the supernatant solution was approximately 76%. Supportive data were obtained using a commercial flocculant whose formula is $[Fe_2(OH)_nSO_{4(3-n/2)}]_m$. The results may be explained in terms of the concept of the electric double layer wherein the negatively-charged colloidal particles of melanoidin are flocculated by cations through charge neutralization. However, an excess of cations caused deflocculation due to mutual repulsion between the positively-charged particles. Furthermore, the speciation of Fe^{3+} or Al^{3+} in solution is equally important.

Microbial methods of decolorization include enzymatic breakdown of melanoidin and flocculation caused by microbially-secreted substances. Several microorganisms have been reported in the literature which are capable of producing either melanoidin-decolorizing enzymes or flocculants. Such enzymes from *Coriolus* have been studied by Japanese researchers (13,15). The protein flocculant from *Rhodococcus erythropolis* has been characterized (30). Initial experiments done in the author's laboratory showed that ADBE melanoidin could be decolorized by *Bacillus sp.* immobilized in alginate gel beads. However, limited decolorization (~40%) has been observed so far on undiluted effluent and the decolorization activity progressively decreased with age of the bacteria-containing gel beads. Recent filamentous fungal isolates from local sources have shown promising results for decolorizing ADBE melanoidin (24).

Acknowledgments

Part of this paper served as the Filipinas Kao professorial chair lecture of the author who is grateful for the award of said chair. Some of the research described in this chair lecture was done at the University of Tsukuba during the author's stay there (April 27-May 20, 1991) under the Exchange Scientists Program funded by the Japan Society for the Promotion of Science (JSPS). Also included in this paper are initial results of an on-going research project on microbial decolorization of alcohol distillery effluent which is funded by the Philippine Council for Industry and Energy Research and Development (PCIERD). The author wishes to acknowledge the technical assistance of Mr. R. R. L. Gerona, Dr. S. Matsuyama and Ms. M. L. F. Paje, and the valuable help provided by Dr. M. Matsumura, Prof. H. Kataoka, Prof. M. Kuwahara, Dr. H. P. Hernandez, Dr. V. C. Sabularse, Dr. C. S. Abrigo, Jr., Dr. R. L. Calibo and Dr. I. F. Dalmacio. Ms. V. P. Migo was kind enough to provide advanced data of her research work.

References

1. Maillard, L. C. *Comp. Rend. Nat. Acad. Paris 154*, 66-68 (1912).
2. Kato, H. and Tsuchida, H. *Prog. Fd. Nutr. Sci. 5*, 147-156 (1981).
3. Okada, N., Ohta, T. and Ebine, H. *Nippon Nokiegaku Kaishi 55* (5), 407-414 (1981).
4. Okada, N., Ohta. T. and Ebine, H. *Nippon N. K. 56* (2), 93-100 (1982).
5. Del Rosario, E. J., Matsumura, M. and Kataoka, H. *Annual Reports of IC Biotech, Vol. 14* (Osaka Univ., Japan, 1991).
6. Migo, V. P., Matsumura, M., Del Rosario, E. J. and Kataoka, H. *Proc. 8th NRCT, NUS, DOST-JSPS Joint Seminar on Biotechnology* (Bangkok, Thailand, Oct. 29-31, 1992).
7. Shaw, D. J. *Introduction to Colloid and Surface Chemistry*, 2nd ed. (Butterworths, London, 1970).

8. Gregory, J. *In* Ives, I. J.(ed.) *The Scientific Basis of Flocculation*, pp. 89-99 (Sijthoff & Noordhoff, Alphen aan den Rijn, The Netherlands, 1978).
9. Adamson, A. W. *Physical Chemistry of Surfaces*, 4th ed. (J. Wiley & Sons, New York, 1982).
10. Hiemenz, P. C. *Principles of Colloid and Surface Chemistry*, 2nd ed. (Marcel Dekker, New York, 1986).
11. La Mer, V. K. *J. Colloid Science 19*, 291-293 (1964).
12. Gregory, J. *In* Ives, I. J.(ed.) *The Scientific Basis of Flocculation*, pp. 101-130 (Sijthoff & Noordhoff, Alphen aan den Rijn, The Netherlands, 1978).
13. Watanabe, Y., Sugi, R., Tanaka, Y. and Hayashida, S. *Agric. Biol. Chem. 46*, 1621-1630 (1982).
14. Ohmomo, S., Aoshima, I., Tozawa, Y., Sakurada, N. and Ueda, K. *Agric. Biol. Chem 49* (7), 2047- 2053 (1985).
15. Ohmomo, S., Itoh, N., Watanabe, Y., Kaneko, Y., Tozawa, Y. and Ueda, K. *Agric. Biol. Chem 49* (9), 2551-2555 (1985).
16. Ohmomo, S., Kaneko, Y., Sirianuntapiboon, S., Somchai, P., Atthasampunna, P. and Nakamura, I. *Agric. Biol. Chem 51* (12), 3339-3346 (1987).
17. Ohmomo, S., Kainuma, M., Kamimura, K., Sirianuntapiboon, S., Aoshima, I. and Atthasampunna, P. *Agric. Biol Chem. 52* (2), 381-386 (1988).
18. Sirianuntapiboon, S., Somchai, P., Ohmomo, S. and Atthasampunna, P. *Agric. Biol. Chem. 52* (2), 387-392 (1988).
19. Sirianuntapiboon, S., Somchai, P., Sihanonth, P., Attahasampunna, P. and Ohmomo, S. *Agric. Biol. Chem. 52* (2), 393-398 (1988).
20. Ohmomo, S., Daengsubha, W., Yoshikawa, H., Yui, M., Nozaki, K., Nakajima, T. and Nakamura, I. *Agric. Biol. Chem. 52* (10), 2429-2435 (1988).
21. Ohmomo, S., Yoshikawa, H., Nozaki, K., Nakajima, T., Daengsubha, W. and Nakamura, I. *Agric. Biol. Chem. 52* (10), 2437-2441 (1988).
22. Paje, M. L. F. and Ueda, K. *The Philipp. Agric. 72*, 231-235 (1989).
23. Paje, M. L. F., Matsumura, M. and Kataoka, H. *Annual Reports of IC BIOTECH.* Vol. 13 (Osaka Univ. Japan, 1990).
24. Magbanua, J. P. V., Bay, R. M., Hufemia, E. P., Gerona, R. R. L. and del Rosario, E. J. *Philipp. J. Biotech. 3* (2), (1992) in press.
25. Nakamura, J., Miyashiro, S. and Hirose, Y. *Agric. Biol. Chem. 40*, 377 (1976).
26. Takagi, H. and Kadowaki, K. *Agric. Biol. Chem. 49*, 3151 (1985).
27. Kurane, R., Takeda, K. and Suzuki, T. *Agric. Biol. Chem. 50* (9), 2301-2307 (1986).
28. Kurane, R., Toeda, K., Takeda, K. and Suzuki, T. *Agric. Biol. Chem. 50* (9), 2309-2313 (1986).
29. Kurane, R. *Biosc. & Biochem. 49*, 133-137 (1991).
30. Kurane, R. and Nohata, Y. *Agric. Biol. Chem. 55* (4), 127-29 (1991).
31. Eskin, N. A. M., Henderson, H. M. and Townsend, R. J. *Biochemistry of Foods* pp. 83-91 (Acad. Press, New York, 1971).
32. Whistler, R. L. and Daniel, J. R. *In* Fennema, D. R. (ed.) *Food Chemistry*, pp. 69-137 (Marcel Dekker, New York, 1985).
33. O'Melia, C. R. *In* Ives, K. J. (ed.) *The Scientific Basis of Flocculation*, pp. 219-268 (Sijthoff & Noordhoff, Alphen aan den Rijn, The Netherlands, 1978).