THE PRODUCTION AND ACTION OF A TARTRATE
DECOMPOSING ENZYME

DISSERTATION

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for the Degree Doctor of Philosophy in the Graduate
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By

RICHARD RUSSELL BARTON, B.S., M.S.

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INTRODUCTION

One of the most important operations in the production of grape products is the elimination of argols (crude potassium bitartrate crystals). Because of the low solubility of this salt, precipitation starts within a few days after the extraction of juice or pulp from the grapes. As these argols detract from the quality of grape products, it is necessary to remove them before offering these products for sale.

The principal methods of eliminating argols from grape products is to speed up their formation and to remove them by filtration. Pederson and Tressler (50) have reviewed the principal methods used to remove argols from grape juice. These methods involve freezing and thawing the juice, storage at temperatures just above the freezing point, addition of calcium salts and storage at low temperature, or the removal of protective colloids which will hasten the deposition of argols. Recently a patent was issued to Meschter (42) for the prevention of grape product crystallization through the use of ion-exchange methods. While these methods will eliminate argols in grape products, they are expensive. Precipitation methods are expensive because of the extra handling and storage facilities needed. Ion-exchange methods are expensive because of the materials used. In addition there is the possibility of impairing the sensitive color and flavor of grape juice by use of ion-exchange resins.

Despite the fact that many micro-organisms are characterized by their ability to utilize tartrates, there has been no effort to use them

to prevent argol formation. The object of this study was to find a micro-organism that was capable of synthesizing a tartrase enzyme. This involved the testing of fungi to ascertain their abilities to utilize tartaric acid or potassium bitartrate as their source of carbon. Once such an organism was found, the next step was to ascertain the ability of the various strains of this organism to produce an active tartrate decomposing enzyme. The optimum methods of producing and preparing the enzyme extract and the action of this preparation were also determined.

REVIEW OF LITERATURE

Ability of Fungi to Produce Enzymes

The fungi are characterized by their ability to produce a large number of enzymes, and this physiological attribute accounts in part for their ability to grow on many materials and in the presence of very little organic material. This characteristic of fungi has attracted interest for many years. In 1915, Crabill and Reed (9) worked out qualitative tests for demonstrating the production and activity of microbial enzymes. These tests consisted of culturing the organisms on a stock medium containing specific carbon substrates. Endocellular enzymes were demonstrated by growth of the organism and exocellular enzymes by the production of a halo on the medium or by a change in color of an indicator incorporated into the medium. In a series of studies on the physiology of fungi, Schmitz and Zeller (69) studied the enzyme action of Armillaria mellea, Daedalea confragosa, and Polyporus lucidus. These studies indicated that the fungi produced twelve to thirteen different enzymes. In addition, when fibrin was used as a substrate, an additional enzyme, erepsin, was formed by all three of these fungi. In a later paper, Schmitz (68) gave the procedures for testing for the presence of exocellular and endocellular enzymes in the wood destroying fungus Fomes pinicola. These procedures indicated that eight exocellular enzymes and six endocellular enzymes were formed.

Garren (22) has also studied the enzyme-producing ability of the coniferous sap-wood destroying fungus Polyporus abietinus. This fungus was shown to form fifteen of the more common hydrolytic enzymes. Garren states

that the variety of the enzymes produced by this fungus indicates that the material stored in the wood parenchyma is of considerable importance in the nutrition of the organism. In this work a comparison of in vitro and in vivo methods indicated that the former more accurately demonstrated the total number of enzymes that a given organism is capable of producing.

While the above papers indicate that fungi are capable of producing a large number of enzymes they do not indicate all of the enzymes capable of being produced. In a recent paper, Shimazono (70) has reported that a wood white rot fungus is capable of producing an enzyme which decomposes oxalic acid. In this enzyme catalyzed reaction, oxalic acid is broken down to formic acid and carbon dioxide. The optimum conditions for the reaction are temperatures of 35° to 40° C. and a pH of 2.5 to 3.0.

Tartaric acid has been investigated as a growth substance by many workers. Fulmer and Werkman (21) have listed the results of some of these investigations. Wehmer (1891) and Raistrick and Clark (1919), reported that Aspergillus niger decomposed tartaric acid to oxalic acid. In 1912, Kestytshew and Afanassajewa reported that Aspergillus niger produced ethyl alcohol from this acid. Karczag isolated a yeast that produced butyric, lactic, propionic and succinic acids from tartaric acid. Several bacteria have also been isolated which were capable of utilizing tartaric acid. Koniz (1881) stated that Bacterium termo produced acetic, propionic and succinic acids; Grey (1923) reported that Bacterium coli communis produced acetic and propionic acids and ethyl alcohol, and Nagai (1923) wrote that Bacterium coli and Bacterium lactis aerogenes produced acetaldehyde from tartaric acid.

During World War II, crude potassium bitartrate was an important by-product of winery operations. It was found that unless the tartrates were separated quickly the yields were very low. Stadtman, Vaughn, and Marsh (74) investigated the situation and found that several microorganisms were decomposing the tartrates before they could be extracted. Penicillium digitatum, Aspergillus niger, and Aspergillus oryzae were found to be the fungi that could most efficiently utilize tartrates as a source of carbon.

Methodology of Fungus Metabolism

In order to study the biochemical and physiological properties of fungi, it is necessary to take into consideration the methods used for growth. The type of media and cultural conditions play an important part in the activity of micro-organisms. In addition there is evidence that the enzymatic make-up of fungus cell material depends on the cultivation conditions during the growth phase.

Foster (17) has discussed the three general types of growth techniques used in biochemical and physiological studies of fungi. Agar colony cultures are useful in the production and detection of mutants or variants and in other types of cultural work. Because fungi are aerobic organisms, the enormous surface area of loose solid materials offers certain advantages. Media such as wheat brans are useful in promoting rapid growth and sporulation because they supply a large surface area as well as nutrients for the fungus. This type of culture has also been used for the commercial production of mold products.

Because of the ease in which a large number of experimental treatments can be handled, cultivating molds on the surfaces of liquid media has been the favorite cultural method. A great deal of valuable information has been obtained by comparative studies of different organisms or from the effect of different treatments on one organism grown in stationary liquid media. As it is necessary for the nutrients in liquid culture to reach the surface growing molds by a process of diffusion, this type of culture media is not as rapid or efficient as submerged culture techniques.

With the development of penicillin, impetus has been given to perfecting methods of submerged culture. With the constant agitation given to such a type of culture, the mold is in constant contact with the nutrients. Because of this, growth is rapid and there is a more efficient utilization of the nutrients available. Another advantage is that the mold mycelium can be obtained in a pure form free from particles of media. One disadvantage is that some molds cannot be grown using submerged culture techniques.

In addition such factors as moisture, temperature, and acidity play an important part in the growth and biochemistry of molds. Monteith (43) studied the relation of soil temperature and soil moisture to infection by Plasmodiophora brassicae. He found that infection developed over the temperature range of 9° to 30° C. The soil moisture was a more important factor in the development of disease. When the moisture content of the soil was 60 per cent of the water holding capacity or higher, infection took place. At a soil moisture of 45 per cent of the water holding

capacity, the disease did not develop. Monteith postulated that at the lower moisture contents, there was insufficient moisture to form a film around the spores to enable germination to take place.

Gray and Martin (25) made some improvements in the soil burial method for testing the resistance of fabrics to decay by micro-organisms. They found that at moisture contents of 21 to 25 per cent decay of fabric was more rapid than at 35 per cent moisture content. This indicated that the cellulose-destroying organisms in the soil were able to grow better at the lower soil moisture content.

Edson and Shapovalov (13) found that there was a certain degree of correlation between the temperatures and some potato rot fungi. This correlation was particularly striking with the wilt producing fungi Fusarium oxysporum. A temperature of about 4° C. would hold Fusarium tuber rots in check in storage, but at higher temperatures infection would occur.

In studying the fruiting of Physarum polycephalum, Gray (24) found that the factors of pH and temperature were related and interdependent. At a constant pH, the higher the temperature the longer the time required for fruiting; the higher the temperature the smaller was the percentage of fruiting. At a constant temperature, the higher percentage of fruiting occurred at pH 3.0. The maximum temperature at which fruiting occurred was between 32.5 and 35° C.

Karrer (32) has shown that the hydrogen-ion concentration affects amylase production by fungi. Fusarium sp. produced the greatest concentration of the enzyme in a solution with an initial pH of 4.5 and final pH of 7.8. An initial pH of 7.0 and final pH of 7.9 was the optimum for

amylase production by Colletotrichium gossypii. In the case of Penicillium italicum, culture solutions with an initial pH of 3.0 or 4.5 were equally favorable.

Wolpert (84) has reported on the growth of certain wood-destroying fungi in relation to the hydrogen-ion concentrations of the media. Though the pH range in which these fungi will grow and the amount of mycelium growth will depend on the individual organism, the major portions of the growth curves for all the organisms tested were on the acid side of neutrality. In some cases the entire curve was on the acid side.

An interesting example of the effect of pH on the biochemical activity of fungi can be found in the formation of itaconic acid by Aspergillus terreus. Prescott and Dunn (55) report that in order to get the maximum yield of this acid, the pH of the culture media must be low initially and maintained at a low level. The optimum pH for itaconic acid production is in the pH range 1.8-2.0. At a higher pH level, the acid probably is not formed. This is an example of shunt metabolism.

Fungus Nutrition

While fungi are capable of growing on a wide variety of organic materials, most of them grow better on some materials than others. The type of organic material used as a substrate will have considerable influence on the amount and type of fungal growth. By the proper balance of nutrients, high conversion of carbohydrate material to metabolic products other than cell material is possible. In addition to organic materials, certain inorganic substances have been shown to be necessary for the growth and activity of fungi. While some fungi are capable of synthesizing the

vitamins necessary for growth, other organisms are unable to do so. For this reason certain vitamins have to be supplied from an outside source or these fungi do not grow.

Carbon and Nitrogen

Because of the great importance that carbon and nitrogen have on the metabolism of fungi, a great many papers have considered the influence of both of these materials on fungi. In several of these papers it has been shown that concentration of carbon and nitrogen as well as the sources are important in determining the amount and type of fungal growth.

Brown (5) made an analysis of the factors which determined the growth forms of strains of Fusarium sp. He found that up to a certain point, an increase in the concentration of the medium increased sporulation of these organisms. When starch was used as the carbohydrate, an increase in concentration produced ~~greater~~ sporulation and a diminution of aerial mycelium. Glucose had the opposite effect. Color formation in the medium depended on a high carbon: nitrogen ratio in the nutrient.

In a study of the nutrition of Ophioliobus graminis, Fellows (14) found that this organism was specific in its nitrogen requirements. He found that egg albumen, casein, peptone, and nucleic acids were the only nitrogen containing compounds utilized by this fungus. This specificity was not affected by the hydrogen-ion concentration of the media, by the source of carbon, or by the presence of inorganic or organic growth promoting substances.

La Fuze (33) determined some of the common nutritional characteristics of the wood destroying fungi Polyporus betulinus, Fomes pinicola and

Polystictus versicolor. He reported that of the carbon sources tested, polysaccharides supported the best growth of mycelium. The pentoses were not as effective, and there was no growth on lactose. The type of nitrogen was of considerable importance. Amino and ammonium nitrogen were better sources than nitrite and nitrate nitrogen. The best growth was obtained on proteins containing large amounts of glutamic acid and tryptophane. Amino acids containing phenyl and disulfide radicals retarded growth.

Lockwood, Ward, and May (39) studied the factors influencing the production of d-lactic and fumaric acids by Rhizopus oryzae. A temperature of 40° C. was more favorable for growth and glucose utilization than 30° C., but the higher temperature was less favorable for d-lactic acid production. The glucose concentration of the medium most favorable for d-lactic acid production was 15 percent. Fumaric acid production was suppressed in cultures containing more than 6 grams of ammonium nitrate per liter. Ammonium nitrate in a concentration range of 1.6 to 6 grams per liter was favorable for d-lactic acid production

Robbins (60) has divided plants into four groups according to their ability to assimilate different types of nitrogen. The first group are those that can utilize gaseous nitrogen, nitrates, ammonium salts, and organic nitrogen. Included here are Azotobacter, Rhizobium, and Clostridium. The next group can assimilate nitrates, ammonium salts and organic nitrogen. Contained here are many bacteria, actinomyces, some yeasts, algae, and the higher green plants. The third group can assimilate ammonium salts and organic nitrogen. Most yeasts are grouped here as there is conflicting evidence as to their ability to assimilate organic

nitrates. The last group contains those organisms that can assimilate organic nitrogen. Here are found some bacteria, some filamentous fungi, and some algae.

Work on factors influencing the growth of some thiamine requiring fungi was done by Leonian and Lilly (35). The growth of these fungi was greatly increased when a 0.02 molar concentration of acetic, lactic succinic, glutaric, fumaric or tartaric acid supplemented a 2.5 per cent glucose solution. Aspartic acid was found to be a better source of nitrogen than asparagine. Ammonium nitrate increased the effectiveness of aspartic acid.

Blank and Talley (3) found that Phymatotrichum omnivorum could utilize glucose, fructose, and mannose or compounds from which the fungus could obtain these simple sugars as carbon sources. They also stated that the utilization of polysaccharides was correlated with the ability of this organism to hydrolyze them and the rates at which the polysaccharides were hydrolyzed.

Leben and Keitt (34) studied the influence of carbon and nitrogen sources on the in vitro growth of Venturia inaequalis. They found that cellulose, dextrin, fructose, glucose, maltose, mannitol, mannose, melibiose, raffinose, and sucrose were adequate carbon sources for this organism. Of the organic nitrogen compounds, arginine, glutamic acid, histidine, proline, and urea could be utilized by this fungus. Ammonium nitrate, ammonium sulfate, and potassium nitrate were the inorganic nitrogen compounds that could be used.

Steinberg (77) has written a review paper in which the carbon and nitrogen requirements of fungi were discussed. One outstanding statement

in this paper is that Aspergillus niger was capable of using amino acids synthesized from sugars and a nitrogen source almost as efficiently as a hexose.

Mineral Nutrition

Just as carbon and nitrogen are essential elements for fungi, certain metallic substances used are so small in comparison to other materials, they are sometimes referred to as trace elements. At the present time, the optimum concentrations of some of these substances for growth have not been accurately determined. The difficulty has been that the minute amounts found in glassware and as contaminants of chemicals are often sufficient to permit abundant growth of the fungus under test.

In his book, Foster (18) has reported the classical work of Raulin on the nutrition of Aspergillus niger. He supplied the ash of Aspergillus niger mycelium as a complete mineral supplement. Both zinc and iron were found to be present in this ash. Raulin also developed a synthetic medium that contained a large number of metallic salts that he considered essential for growth of fungi.

In his study on the production of citric acid by Aspergillus niger, Porges (54) found that certain metallic salts were necessary. The presence of both iron and zinc were essential for rapid growth of the fungus and for an increased yield of citric acid. He further stated that zinc favored citric acid production by this organism. In addition, when zinc was present in the medium, the spores of A. niger were dark colored.

The paper by Lewis (37) shows that the requirement of metallic substances can vary with the desired end point. This work was concerned

with the relationship of iron nutrition to the synthesis of vitamins by Torulopsis utilis. Lewis found that more thiamine was produced with an iron deficiency, with 0 and 3 p.p.m. of iron more riboflavin was produced than when 0.1 p.p.m. of iron was present. The greatest amount of nicotinic acid was produced with an iron deficiency. More biotin was produced when there was 3 p.p.m. of iron present in the media. In the case of pyridoxine, there was a greater amount produced when there was a deficiency of iron in the media.

Steinberg (76) succeeded in developing a procedure for removing traces of metals from the chemicals used for synthetic media. Using this technique, it was possible for him to determine that two to six p.p.m. of calcium were necessary for maximum yields of Aspergillus niger, Rhizoctonia solani, Sclerotium rolfsii, Cercospora nicotinae, Fusarium Oxysporum, Rhizium irregulare, and Thielamopsis basicola.

Perlman (51) was discussed the effects of minor elements on the physiology of fungi. He agreed with the views of others that many ions are necessary for growth of fungi, but raised questions as to the method in which these ions act. He states that since it appears that some metallic ions can replace others, there is the possibility that the enzymatic systems in fungi are catalyzed by several different ions or that possibly there are several alternative mechanisms that exist for the production of these enzymes. The other possible explanation for the above is that growth is possible because of the presence of contaminants in the chemicals used in the media.

Nason (45) studied the effect of zinc deficiency on the synthesis of tryptophane by Neurospora sp. extracts. When cell free extracts of

Neurospora sp. grown on zinc deficient media were analyzed, it was found that considerably less tryptophane was produced from indole and serine than in control extracts. The addition of zinc did not improve the synthesis in 53 hours. In 0.002 and 0.0002 molar concentration, zinc caused inhibition of this organism.

As can be seen from the above, the work with trace elements, with few exceptions, is inconclusive. Foster (18) has reviewed the entire subject and has stated that iron, zinc, copper, cobalt, molybendum, and gallium are necessary for the nutrition of fungi. Further work will have to be done to definitely establish the essentiality of the other metallic ions.

Vitamin Nutrition

Considerable work has been done to determine the vitamin requirements of fungi. It has been clearly demonstrated that vitamins, particularly of the B group, are essential for the nutrition of many organisms.

Leonian and Lilly (36) determined the necessity of thiamine in twenty-five different fungi. When a synthetic media consisting of inorganic salts and dextrose was used these organisms failed to grow. On the addition of 0.5 p.p.m. of thiamine to this media, ten of the fungi grew well. Two intermediates of thiamine, 4-methyl-5- β -hydroxythiazole and 2-methylethoxymethyl-6-amino-pyrimidine, were inactive when used singly. However, when these intermediates were used together, eleven of the test organisms grew. Further work with thiamine derivatives indicated that an amino group in position 6 is necessary for activity of pyrimidine and a hydrogen ion in the 2 position and a β -hydroxyethyl group in position 5 is necessary on the thiazole derivative.

Working with Fusarium avenaceum, Robbins and Ma (64) reported that biotin is necessary for growth. They found that much of the biotin necessary could be supplied in the agar used in the culture media. As little as 0.001 microgram of this vitamin increased the growth of the test organism.

Considerable work has been done by Robbins and Kavanaugh (61) (62) (63) on vitamin nutrition of fungi. In a review (61) of vitamin deficiencies of fungi, it was found that the ability of fungi to produce vitamins varied widely. Aspergillus niger was able to produce thiamine from sugar, minerals and nitrogen, while Phytophthora cinnamomi was unable to synthesize this vitamin. These workers indicated that the need of vitamins is specific as they act as co-enzymes. In another paper (62) a study was made of the specificity of pyridoxine for Ceratostomella ulmi. The effect of twelve analogs of pyridoxine on the growth of this fungus also determined that the diacetate and triacetate analogs of vitamin B₂ were as beneficial as pyridoxine. Four analogs had 5 per cent or less of beneficial activity of pyridoxine, two were inactive, and four inhibited growth. Further work (63) was done on the effect of hypoxanthine as a growth regulator for Phycomyces sp. This compound, obtained as the silver salt from potato tubers, was more effective than xanthine as a growth substance. It is pointed out in this work that it is necessary for the amino groups and oxygen to be in the proper position on the pyrimidine ring in order for the substance to be effective as a growth substance.

In a later paper Robbins and Ma (65) extended their studies on vitamin nutrition of fungi. The deficiencies of twelve fungi for pyridoxine, thiamine and biotin were reported. Five fungi had a complete or nearly

complete thiamine deficiency, four were partially deficient in thiamine requirements, and three showed no deficiency for thiamine. Six of these organisms were slightly or partially deficient in pyridoxine, and five were partially deficient in biotin.

Wooster and Cheldelin (85) studied the growth requirements of Penicillium digitatum. They found that thiamine in a concentration of 0.01 to 3 gammas per 25 milliliters of culture solution were necessary for growth of this organism.

In a study of the nutritional requirements of the yeast Schizosaccharomyces pombe, Clark, Bannister and Mitchell (8) found that several vitamins were essential. In 100 grams of media, 25 gammas of thiamine, 0.1 gamma of biotin, 100 gammas of nicotinic acid; 100 gammas of calcium pantothenate, and 1000 gammas of inositol were required for the growth of this yeast.

In studying the nutrition of Sclerotium delphinii, Perlman (52) found that thiamine was necessary for growth. Seventy micrograms of thiamine per liter of culture solution produced maximum growth; 50 micrograms gave one half maximum growth. When the concentration of thiamine was reduced to one microgram, there was no growth of S. delphinii.

Adaptive formation of Enzymes; Variation of enzyme producing ability of different strains of organisms; and production of mutations in fungi.

In a preceding section it was pointed out that fungi are capable of elaborating a great number of enzymes. Most of these enzymes are constitutive in that they are formed independent of the type of nutrition supplied. However, other enzymes are adaptive in that they are produced only in the presence of a particular substrate.

Menon (41) made a comparison of the enzyme extracts of various parasitic fungi. He reported that the nutrient supplied the organisms influences the enzyme extract of a particular fungus. In order for Botrytus cinerea and Monilia fructiginea to produce pectinase, it was necessary to include pectin in the media.

A patent has been issued to Bucherer (6) for a method of producing microbial enzymes for decomposing specific metabolic poisons. The principal factor in producing such enzymes was the incorporation of the specific poison to be decomposed into the nutrient media.

Hassed and Dondoroff (presented in Nord) (47) have discussed the enzymatic synthesis of disaccharides. The enzyme, sucrose phosphorylase, used is produced by Pseudomonas saccharophila only when it is cultured with sucrose or raffinose as a substrate.

Virtanen (82) has discussed the possible formation of adaptive enzymes by micro-organisms. The protein content of young cells is largely enzyme protein. In the enzyme-nucleoprotein complex enzyme formation occurs autocatalytically. The formation of a specific adaptive enzyme is caused by the presence of a specific substrate which is loosely bound to the enzyme-nucleoprotein complex from which the enzyme arises. He states that the transformation of adaptive enzymes to more permanent ones can be brought about by the presence of a particular substrate for hundreds or thousands of generations.

Another factor of considerable importance in the production of microbial enzymes is the inherent ability of different strains of a given organism to elaborate the specific enzyme in adequate concentrations.

Harter and Weimer (26) made a comparison of the pectinase produced by different species of Rhizopus. They found that parasitic organisms "secrete" small amounts of pectinase, while the non-parasitic types "secrete" larger amounts of this enzyme into the medium.

Perhaps the most striking example of strain differences is shown by Raper, Alexander, and Coghill (59) in the production of penicillin by organisms of the Penicillium notatum - chrysogenum group. In the early work they found that the production of penicillin by naturally-occurring strains varied from 0 to over 100 units of this drug per milliliter of culture solution.

Foster (19) reviewed the work of Proskuriakov and Ossipov on the production of pectinase by Aspergillus oryzae. Here it was also demonstrated that there was a considerable strain specificity. One strain of A. oryzae was four times as active as others with respect to this enzyme.

The difference between strains producing a given enzyme is due to spontaneous or induced mutation. While it is important to take into consideration this difference between strains, it is sometimes difficult to obtain strains which show considerable differences. For this reason, the artificial production of mutants by chemical or physical methods has been resorted to. In this manner a large number of mutants having different physiological and metabolic characteristics can readily be obtained.

Dickson (11) has reported on the effect of X-rays, ultra violet light in producing saltants of Chaetomium cochloides. It was found that saltants were produced more frequently in the older portions of the cultures. Saltants produced by both X-rays and irradiation remained true to type. While X-rays had little effect on the germination of spores, ultra violet light caused some inhibition of the growth of spores.

Oster (56) studied the effect of ultraviolet light on Saccharomyces sp. He found that there were several actions involved in cell changes resulting from irradiation. One of the actions was that inhibition of normal sized colonies occurs at a low energy level. The reproductive mechanism was so damaged in some cases that only a few budded cells were produced. This resulted in the production of giant cells.

At higher incident energy levels, the metabolic functions were damaged as indicated by lowered oxygen consumption.

Steinberg and Thom (78) worked on the production of mutations and reversions in the reproducibility of Aspergillus niger with nitrites, colchicine and d-lysine. Nitrous acid readily produced mutants in A.niger and other Aspergilli. Positive results were obtained with colchicine if an excess of calcium carbonate was present in the media. Because many of these mutants could be reverted to the original strain by the addition of d-lysine, they concluded that mutations were caused by the destruction of certain amino acids in the hereditary mechanism.

Fries (20) found that a somewhat higher rate of mutation could be obtained from Ophiostoma multiannulatum by impregnating the spores with uranium. No qualitative changes in the composition of the mutants could be observed. Ophiostoma mutants were characterized by the production of numerous parathiotropic strains. Fries found that these mutants could be separated from the physiologically normal spores by filtration through a suitable filter as the germinated mutants produced shorter mycelium.

Bonner (4) has shown that it is possible to produce biochemical mutants in Penicillium sp. After irradiation of P. chrysogenum, mutants with altered

nutritional characteristics were observed. Investigation showed that in the different mutant strains, enzyme blocks were established which prevented the breakdown of glutamic acid to ornithine to citrulline and finally to arginine, which can be utilized by the fungus. Since this is similar to the changes in *Neurospora*, whose genetic modifications are shown, Bonner concluded that there is genetic control of the biochemical reactions of Penicillium sp..

Extraction and Preparation of Enzyme Extracts

In order to use an enzyme, it is generally desirable to separate it from the organism which elaborated it and the media on which the organism was cultured. Since all of the enzymes studied have proved to be proteins, methods for the separation and precipitation of proteins have been used for the preparation of enzyme extracts. Because most enzymes are readily denatured by some of the chemicals used in their separation, it is often necessary to make the separations in the cold or under conditions that will minimize this denaturation.

Northrop (49) has given the procedure for the preparation of a crystalline pepsin. After preliminary solution in an alkaline solution, the acidity of the solution is adjusted to the isoelectric point and the pepsin is salted out of solution with magnesium or ammonium sulfate. With repeated solution with alkali and precipitation with acid, a pure crystalline enzyme can be obtained. The composition, optical activity and proteolytic activity of this enzyme remained constant throughout the crystallization procedure.

An active amylase preparation was obtained by Shukla (71) from the root of the herb *Kasern*. The aqueous extract was precipitated with ammonium

sulfate. After dialysis the purified enzyme was precipitated with ethyl alcohol.

Tilden, Adams and Hudson (80) have given the method for the preparation of amylase produced by fungi. The enzyme was dissolved and filtered through a Seitz filter to remove mold myelia and spores. The enzyme was then precipitated from solution with acetone. Purification was accomplished by absorption of the enzyme on aluminum hydroxide and elution with an alkaline phosphate solution.

One of the simplest procedures for the production of the enzyme preparation is given by Tauber (79). To obtain amylase from Aspergillus oryzae, the mold was inoculated on moist bran and incubated at about 30° C. for thirty to sixty hours. Dry soluble mold enzyme was obtained by percolating water through the moldy bran and by adding alcohol to the aqueous extract to an alcohol concentration of 70 per cent. The alcohol precipitate is dried in vacuo or in a blast of warm air.

The procedure of Schmitter and Balls (67) for the isolation of crystalline alpha amylase from germinated barley also involves the use of alcohol to precipitate the crude enzyme from solution. The alcohol precipitate is purified by adsorption on wheat starch granules. After elution from the starch the purified enzyme is salted out of solution with ammonium sulfate.

Wallerstein (83) developed a method of precipitation and recovery of mold proteases using lignin and tannin. The enzyme is precipitated from solution in the form of a lignin or tannin complex. The full activity of the enzyme is restored by redissolving the complex at a slightly alkaline pH.

Jeffries (29) has described the commercial production of mold enzymes by means of a continuous tray method. The substrate was sterilized, spread evenly on sterile trays, cooled and inoculated. The inoculated trays were placed on trucks and held in tunnels where temperature and humidity can be carefully controlled. After incubation, the moldy substrate was removed from the trays and dried. The dry substrate was then ground and sold as a crude enzyme extract.

In a study of the specificity of leucine amino peptidase, Smith and Polglase (73) developed a method for the extraction and purification of this enzyme from hog mucosa. After maceration of the mucosa a water extract was obtained and dried by lyophilization. The dry material was suspended in 10 volumes of acetone, filtered and air dried. The acetone precipitate was dissolved in water and fractionally precipitated by the addition of solid ammonium sulfate.

The method of Crewther and Lennox (10) for the preparation of crystals containing protease from Aspergillus oryzae, is carried out at temperatures of 2° to -25° C. to minimize the denaturing effects of the alcohol used as a precipitating agent. The purification of the crude alcohol precipitate involved fractional precipitation with ethyl alcohol. The first precipitate contained catalase and sucrase and the next fraction contained the protease. This fraction was crystallized by the addition of ammonium sulfate and holding the solution at 2° C. until crystallization took place. It is reported that this is the first preparation of crystals containing mold enzymes.

Fisher, Meyer, Noelting, and Piquet (15) have developed a method of purification and crystallization of malt beta amylase. A mixture of alpha

and beta amylases and 30 per cent sugar are held at pH 3.6 and filtered to remove the alpha amylase. Beta amylase is precipitated when the solution is made 0.6 saturated to ammonium sulfate at pH 5.0. The precipitate is dissolved in water and fractionally precipitated twice with acetone. The second fraction was dissolved and brought to pH 7.8, on precipitation with acetone the precipitate contained 51 to 56 per cent beta amylase.

A temperature of 70° C. was used by Stark and Tetrault (75) for the isolation of starch saccharifying enzymes produced by bacteria. The bacterial cells were removed from the culture medium, which was then dialyzed against running tap water. Precipitation of the enzymes was brought about by ethyl alcohol. This alcohol precipitate was dried over calcium chloride.

Within the past few years there have been two developments which have facilitated the preparation of enzymes. One of these is the Waring blender. This piece of equipment has a set of blades that revolve at 10 to 20 thousand revolutions per minute and is very useful in breaking up mycelia and solid media for the complete extraction of enzymes. The other development is lyophilization whereby liquids can be dried from the frozen state. Because it avoids heat, this procedure has been widely used for drying heat-labile enzyme preparations. However, it has been discovered that both of these developments have harmful effects on certain enzymes and proteins.

Dounce and Schwalenberg (12) have reported that the lyophilization of crystalline beef liver catalase reduces the activity of this enzyme to one half its original activity. They state that the iron atom in the protein part of the molecule is loosened and is rendered reducible.

Naimark and Mosher (44) state that the activity of pepsin is lowered or completely lost by use of a Waring blender. They claim that the loss of activity is due to surface denaturation caused by heat generated by the shearing action of the apparatus, by oxidation, and by copper contamination.

Activation of Enzymes

It has been shown that many materials, notably metallic ions, can increase the activity of enzyme preparations. They differ from co-enzymes in that they are not necessary for the activation of enzymes. Since many enzyme preparations have been obtained only in the crude state, it is difficult to differentiate between co-enzymes and activators in some instances. Gortner (23) has listed the following theories concerning the role played by enzyme activators. (1) An activator may act as an emulsifier thereby increasing the surface of contact, (2) salt solutions may peptize the enzyme bringing additional functional groups into action; (3) an activator may make the substrate more reactive to the enzyme, (4) activators are reagents which stabilize or protect the sulphydryl or other groups of the action of enzyme poisons, and (5) activators may inhibit the action of enzyme poisons.

Linderstrom-Lang (38) has shown that the zinc ion works in two directions on the activity of peptidase. In concentrations of less than 0.003 normal, zinc is necessary for activity with the maximum effect in concentrations 0.004 to 0.0008 normal. At a concentration of 0.016 normal, it causes 90 per cent inactivation of peptidase.

Johnson, Johnson, and Peterson (30) found that magnesium chloride in a concentration of 0.010 molar was necessary for the activation of leucyl

peptidase of animal protein. They state that the magnesium ion forms a reversibly dissociable compound with the enzyme substrate complex.

Baumann and Heumuller (2) studied the effect of the addition of metallic ions on phosphatase activity. Manganese, magnesium and iron ions increased activity, nickel ions had slight effect, and cobalt ions caused inactivation of phosphatase.

Smith and Bergmann (72) also found that manganese could act as an enzyme activator. When used in 0.01 molar concentration, manganese sulfate caused activation of intestinal peptidases. They found that this enzyme is a protein-metal complex as evidenced by inhibition caused by hydrogen cyanide and hydrogen sulfide.

While most of the enzyme activators studied have been metals, there are a few references that certain vitamins act as activators.

Jacobsohn and Cruz (28) studied the influence of folic acid and vitamins B₁ and B₂ on several enzymes. They found that vitamins B₁ and B₂ were without effect. Folic acid had no effect on aconitases but increased the rate of fumaric acid hydration.

Ajl, Hart, and Werkman (1) determined the effect of biotin in succinic acid oxidation. During dialysis the activity of the enzyme was decreased. On the addition of 0.025 to 0.1 micrograms of biotin, the activity of the enzyme was restored.

Factors Affecting the Activity of Enzymes

Although the activity of an enzyme preparation is due to the inherent characteristics of the preparation, there are several external factors which play an important role in influencing activity. Some of these factors

are pH, concentration of the substrate, concentration of the enzyme, pH, temperature and length of time the reaction is allowed to proceed.

Harter and Weimer (27) studied the amylase of Rhizopus tritici with consideration to its production and action. Hydrolysis proceeded at a maximum rate of 45° C, at 60° C. inactivation of the enzyme took place. There was an increase in the formation of sugar with an increase in the concentration of the substrate up to a certain point. Beyond this there was a decrease in sugar formation.

Roughton and Booth (66) determined the effect of external factors upon the activity of carbonic anhydrase. There was a linear relationship between the concentration of the enzyme and its activity. In the pH range from 5.0 to 10, the activity increased with the increase in pH. Enzymatic activity increased with an increase in the substrate concentration.

In a study of a new enzyme, dextranase, Nordstrom and Hultin (48) found that the optimum conditions for activity were a temperature of 30° C. and a pH of 5.9 to 6.1. Above pH 6.1 irreversible inactivation of the enzyme sets in rapidly.

Calesnuck, Hills, and Willaman (7) determined the properties of a commercial fungal pectase preparation. The first 40 per cent of de-esterification took place in the first hour, and it was completed in six hours. Up to a certain level, increase in pectin concentration gave increased enzyme activity and then leveled off. Maximum activity took place of pH 5.0 and temperatures from 30° to 40° C. Calcium chloride in concentrations of 0.01 to 0.1 molar caused activation.

Happold (edited by Nord) (46) has studied the tryptophanase-tryptophan reaction. He found that at low enzyme concentrations, there was a

linear relationship between the concentration of the enzyme and the velocity of the reaction. At pH 7.5 there was maximum activity, and it decreased as the acidity increased or decreased from this level.

In a review paper, Joslyn (31) has discussed the action of enzymes in concentrated solutions and in the dried state. It is estimated that for several enzyme-substrate systems the initial rate of reaction increases with concentration of the substrate. Maximum rate of reaction then is reached and then levels off within a wide range. Finally the reaction decreases with a further increase in concentration of the substrate. Joslyn further points out that oxidation-reduction enzymes reach maximum activity at lower substrate concentration than do any of the hydrolytic enzymes.

METHODS AND MATERIALS

The Ability of Fungi to Utilize Tartrates

Several fungi that were able to utilize tartrates as their sole source of carbon were isolated from grape pomace piles and wine lees by Powers (53). The fungi were separated by their morphological differences and cultured on Sabourand Dextrose Agar or Wort Agar.

To ascertain the ability of these fungi to utilize tartrates the following synthetic media containing either potassium tartrate or potassium acid tartrate were inoculated with spores of the fungi and incubated at 30° C. for 17 to 33 days.

<u>Medium A</u>	<u>Percentage</u>
Ammonium nitrate	1.0
Ferrous sulfate	0.2
Potassium chloride	0.1
Sodium chloride	0.1
Magnesium sulfate	0.1
Dicalcium phosphate	0.4
Potassium acid tartrate	1.75
 <u>Medium B</u>	
Ammonium nitrate	0.2
Dipotassium phosphate	0.1
Magnesium sulfate	0.05
Potassium chloride	0.05
Ferrous sulfate	0.001
Potassium tartrate	1.00

The ability of these fungi to utilize tartrates was ascertained by determining the tartrates utilized. The sodium metavanadate colorimetric determination of tartrates of Underhill, Peterman and Kraus (81) was used.

Growing Fungi for Production of Tartrase

Using the fungus isolated by Powers (53) which was most effective in

utilizing tartrates as a test organism, a study was made of liquid and solid media and submerged culture technique to ascertain which method of culture was most suitable for tartrase production. Liquid media was unsuitable as growth of the fungus was slow, and tartrase activity was low. Submerged culture technique was unsuitable as the organism was unable to grow under this type of culture. The best type of medium was found to be a solid medium. From the standpoint of availability and ability to support heavy and rapid fungus growth, a grape pomace medium was found to be the best solid medium tested. The grape pomace was removed from the press cloths and thoroughly mixed before it was canned and sterilized at 15 pounds pressure for one hour. Before use, the pomace was ground in a food chopper and 1 per cent potassium bitartrate added. Fifty-gram portions of this medium were spread out in the bottom of 500 ml. wide-mouth Erlenmeyer flasks and sterilized at 15 pounds pressure for 20 minutes.

In order to obtain rapid growth, the fungus spores were first inoculated in about 8 ml. of a sterile liquid medium and incubated at 30° C. for 24 hours to permit germination to take place. This liquid medium contained the following materials:

Yeast extract	6.7%
KH_2PO_4	0.5%
Dextrose	5.0%
Distilled water	93.8

The germinated spores and liquid medium were used to inoculate fifty grams of growth medium. This medium was incubated at 30° C. for three to five days or until a heavy growth of the organism had developed but before heavy sporulation had occurred.

Extraction and Preparation of a Tartrase Preparation:

After the fungus under test had produced sufficient growth, 500 ml. of cold water (8° C.) was added to each 50 grams of moldy medium. This mixture was then macerated in a Waring Blendor for five minutes. Particles of media and mycelium were separated from the extract by vacuum filtration using about 200 grams of Dicalite filter aid to speed up the process and to obtain a clearer filtrate. When it was found that some tartrase activity was being lost due to maceration in a Waring blendor, this procedure was modified. The mixture of moldy medium and water was placed in a three-liter beaker and stirred at 8° C. with a mechanical mixer for two hours.

Separation of the enzyme from the liquid extract was accomplished by adding two volumes of cold (8° C) 95% ethyl alcohol to each volume of enzyme extract. When necessary the pH was adjusted with sulfuric acid until the iso-electric point was reached. The alcohol insoluble material was removed from solution by centrifuging at 2000 RPM for five minutes. This moist enzyme preparation was used to determine the activity of tartrase activity. This moist preparation would lose its activity in 18 to 24 hours.

To determine the relationship between tartrase activity and such factors as time, temperature and pH, it was desirable to use a dried stable enzyme preparation. It was found that the best method for obtaining such a preparation was freeze-drying or lyophilization. Equipment similar to that developed by Flosdorf (16) for drying drugs was used. After the moist alcohol insoluble material was extracted from solution, it was redissolved in a minimum of water. This solution was frozen on the

sides of the short necked Kjeldahl flask by rotating it in a mixture of solid carbon dioxide and 75 percent alcohol.

When frozen solidly, the flask was attached to the apparatus and a vacuum of about 50 millimicrons of Hg was created by means of a high vacuum pump. Drying was allowed to take place overnight or until the preparation was thoroughly dried. This dried, slightly hygroscopic preparation, was stored at 8° C until used.

Separation and Culture of Single Spore Isolates:

In order to ascertain if there was any difference in the activity of tartrase produced by different strains of a fungus, single spore isolates were obtained by means of a micromanipulator. A drop of 2 percent agar was placed on a sterile microscope slide cover. Before the agar had completely solidified, a loopful of spores from an agar slant culture was placed on it. The prepared slide cover was placed on an inclosed box on the stage of a microscope. Using medium power, the separated spores could be observed. By means of a glass rod that had been drawn into an extremely fine point, the individual spores were "picked" off the agar drop. Each spore was transferred to a drop of solidified 2 per cent agar on a microscope cover slide. Using aseptic techniques, these agar drops were transferred to a petri dish containing a solid media consisting of dextrose, yeast extract, potassium acid phosphate and agar. The position of each spore was marked so that more than one spore could be placed in each petri dish. After the spores had germinated and growth was apparent, these isolates were aseptically transferred to agar slants. These slants were incubated at 30° C until good growth resulted then they were stored at 8° C to

Figure 1. Lyophilizer Used for the Production of Dried Tartrase

reduce the rate of physiological changes. About 50 per cent of the single spores isolated germinated and grew so that of approximately 200 single spores isolated, 91 single spore isolate cultures were obtained.

Testing the Activity of Tartrase Produced by Different Strains of *Aspergillus Versicolor*:

In an effort to obtain the most active tartrase preparation, it was necessary to test all the strains of *Aspergillus versicolor* obtained from other laboratories and those obtained from single spore isolate cultures. Spores from each culture were inoculated into about 8 ml. of sterile liquid germinating media and incubated for 24 hours at 30° C. The germinated spores were used to inoculate 50 grams of solid media. After incubation at 30° C. for three to five days or until heavy growth of the fungus developed, the enzyme was extracted according to the procedure given above.

In order to use a uniform amount of material from each culture, the enzyme from 50 ml. of aqueous extract was used. This material was precipitated with cold (8° C) ethyl alcohol and removed by centrifuging. The moist preparation was added to 100 ml. of 1% tartaric acid or potassium bitartrate solution. After incubation at 30° C for 20 hours, the tartrate ion concentration of the substrate was determined. From this the activity of the enzyme, expressed as per cent bitartrate decomposed could be calculated.

It was found that the tartrase activity of these single spore isolates decreased after the cultures had been stored at 8° C and after several sub-culturings. In order to maintain the physiological constancy of the fungi, it was necessary to use a spore production medium that contained potassium bitartrate as a major source of carbon. Because potassium bitartrate has a

calcium sequestering action, it was not possible to use agar (the calcium salt of alginic acid) as a firming agent. Gelatin was used in its place.

The medium was composed of the following materials:

	gms.
Potassium acid phosphate	2.0
Yeast extract	2.8
Potassium bitartrate	4.0
Gelatin	60.0
Dextrose	2.0
Distilled water	400.0 ml.

Approximately 5.0 ml. of this medium were poured into test tubes and autoclaved at 15 lbs. pressure for 15 minutes. The tubes were slanted at 8° C. and the mediums allowed to solidify. Because the gelatin would liquify at room temperature, it was necessary to slant the tubes when the fungi were growing and sporulating. After sporulation had taken place, the slants were stored at 8° C.

Relationship Between Constituents of Media and Tartrase Activity:

This work was divided into four sections. The first section was concerned with the use of the common fungus nutrients yeast extract, and potassium acid phosphate. These constituents were added in concentrations of 0.7 per cent and 0.5 per cent to the basic medium of ground grape pomace containing an added 1 per cent potassium bitartrate.

Section two was a determination of the relationship of pH of the medium and tartrase activity. The pH of the medium was adjusted to 2.0, 3.0, 4.0, 5.0, and 6.0, with sulfuric acid or sodium hydroxide.

The relationship between added metallic ions and tartrase activity was the object of the third section. The following is the metallic salts and their concentrations that were used Zinc sulfate - 0.1 per cent, ferrous chloride - 0.1 per cent, cuprous chloride - 0.0005, ammonium

molybdate - 0.01 per cent, magnesium sulfate - 0.25 per cent, and manganese sulfate - 0.02 per cent.

The relationship between added vitamins and tartrase activity was the last part of this study. The following are the vitamins and their concentrations that were used: Thiamin - $12.5\mu\text{g}/50\text{ gm}$, calcium pantothenate - $50\mu\text{g}/50\text{ gm}$, pyridoxine - $50\mu\text{g}/50\text{ gm}$; nicotinic acid - $50\mu\text{g}/50\text{ gm}$, biotin - $0.05\mu\text{g}/50\text{ gm}$, inositol - $500\mu\text{g}/50\text{ gm}$, and folic acid - $50\mu\text{g}/50\text{ gm}$.

In all these studies the constituents were added to the basic media before sterilization. Duplicate samples of each and control samples were run with each determination. To maintain a uniform amount of enzyme, the precipitate from 50 ml. of aqueous extract was used. The substrate in each case was 100 ml. of approximately 1% potassium bitartrate. In the study of vitamins, two aliquots from each treatment were used. One was added to a substrate containing 0.1 per cent zinc sulfate and ferrous chloride and the other to a pure potassium bitartrate substance.

Relationship Between Metallic Ions Added to the Substrate and Tartrase Activity:

Using the same metallic salts and concentrations as described in the preceding section, a study was made of their action when added to the potassium bitartrate substrate. The enzyme from 50 ml. of aqueous extract was added to 100 ml. of the substrate containing the added metallic salts. After 20 hours incubation at 30°C , the concentration of the tartrate ion was determined. Further work was done using a combination of ferrous chloride and the other salts under test.

The last study of this nature was to determine the action of various concentrations of ferrous chloride and a combination of ferrous chloride and zinc sulfate. The ferrous chloride was used in concentrations ranging from 0.04 to 0.2 per cent in .02 per cent increments. The concentrations of ferrous chloride and zinc sulfate were used in concentrations ranging from 0.04 to 0.20 per cent. In each case the total salt content was maintained at a concentration of 0.24 per cent.

In all these studies, the 1 per cent potassium bitartrate substance was incubated at 30° C. for twenty hours. Three replicates and control samples were used for each treatment. Enzyme activity was calculated as per cent potassium bitartrate decomposed.

Relationship Between External Factors and Tartrase Activity:

Time:

To determine the relation between time and tartrase activity, 10 mg. of a dried tartrase preparation was used with 10 ml. of 1 per cent potassium bitartrate solution containing 8 mg. of ferrous chloride. After incubation at 2, 4, 6, 8 and 26 hours at 40° C., the per cent bitartrate decomposition was determined. Using the equation for a first order reaction, the velocity constants of the reaction were calculated. Three replicates were used in the determination.

Temperature:

For a study of the relationship between temperature, the same concentrations of dried enzyme and ferrous chloride were added to 10 ml. aliquots of 1 per cent potassium bitartrate solutions. The samples were incubated for two hours at temperatures of 20° C., 30° C., 40° C., 50° C., and 60° C. Enzyme activity was calculated as per cent tartrate decomposition. Velocity constants were calculated using the equation for a first order reaction.

pH:

Ten ml. portions of 1 per cent potassium bitartrate substrate were adjusted to pH 2.0 and 3.0 with sulfuric acid. Sodium hydroxide was used to obtain a pH of 4.0, 5.0, and 6.0. The pH measurements were made with a McBeth line operated pH meter. To these substrates, 10 mg. dried tartrase and 8 mg of ferrous chloride were added. Two replications of each pH were incubated at 30° C. for four hours. Enzyme activity was calculated as per cent tartrate decomposition.

Concentration of Substrate:

To determine the relationship between tartrase activity and substrate concentration, 0.25 per cent, 0.50 per cent, 1.0 per cent and 2.0 per cent potassium bitartrate solutions were used. To each 10 ml. aliquot of substrate, 10 mg. of dried enzyme and 8 mg. of ferrous chloride were added. Two replicates of each concentration were incubated for four hours at 30° C. Enzyme activity was calculated as per cent bitartrate decomposition and mg. of tartrate ion per ml. decomposed.

Concentration of Enzyme:

The dried enzyme was used in concentrations of 0.005, 0.01, 0.05, 0.10, 0.15, 0.20, 0.50, and 1.00 per cent. Due to the difficulty of accurately weighing very small amounts, the lowest concentration of enzyme was obtained by adding 5 mg. of dried tartrase to 100 ml. of substrate. The next four concentrations were obtained by adding 5, 25, 50, and 75 mg. of the enzyme to 50 ml. of substrate. The two highest concentrations were obtained by adding 50 and 100 mg. of tartrase to 10 ml. of substrate. Three replicates of each concentration were incubated at 30° C. for 20 hours. The enzyme activity was expressed as per cent tartrate decomposition.

Determination of Enzymatic Breakdown Products of Tartaric Acid:

The first step in determining the breakdown products of tartaric acid was to make pH and total acid measurements of the substrate before and after the enzyme reaction had taken place. The pH measurements were made with a McBeth line operated pH meter. The total acid determinations were made by titrating 10 ml. aliquots of the substrate with N/10 NaOH using phenolphthalein as an indicator.

Identification of the organic acids present were made using an adaptation of the filter paper chromatogram procedure of Luggs and Overell (40). The immobile aqueous phase and the mobile solvent phase were allowed to come to equilibrium in a Chromatocab manufactured by the Chromatography Division of the University Apparatus Company, Berkeley, California. Concentrations of not more than 20 μ g. of the known acids and substrate containing the unknown acids were placed about 4" from the end of a 10"x 18" Whatman #1 filter paper. The initial location of each acid was marked on the paper. When the acids had dried, the paper was clipped to a glass rod and placed in the Chromatocab for two to three hours to allow it to come to equilibrium with the mobile and immobile phases in the apparatus. Then the end of the paper closest to the acids was dipped into a mixture of 75 per cent chloroform and 25 per cent butanol containing one mole of formic acid per liter. The solvent was allowed to move down the paper for 16 hours. After marking the distance that the solvent had moved, the paper was air dried for 48 hours to allow the solvent to completely volatilize. To locate the position of the acids the paper was sprayed with brom cresol green indicator. When the indicator had dried, the acid spots were marked. The distance that the acids and

the solvent had moved from the same spot was measured. The Rf value of each acid was calculated by dividing the distance the acid moved by the distance that the solvent moved. As these Rf values are constant for each organic acid, the identity of the unknown acids in the substrate could be determined.

Action of Tartrase in Concord Grape Juice:

To 100 ml. of Concord grape juice 50 mg. of the dried tartrase preparation were added. In order to inhibit growth of micro-organisms, 0.2 ml. of toluene were also added to each sample. After incubation at 40° C. for twenty hours, the tartrate content of the juice was measured. From these determinations, tartrase activity was calculated as per cent tartrate decomposition.

The juice used in this work was hot pressed Concord grape juice made in 1948 and 1951. After pasteurization this juice was held at 8° C. until used. The tartrate content was brought up to the concentration present after pressing by either adding C.P. potassium bitartrate or heating the juice to redissolve the crystals that had precipitated out of solution.

RESULTS

Ability of Fungi to Utilize Tartrates

Of the thirty-three fungi isolated by Powers (53), six were able to utilize significant amounts of tartrates as a carbon source. These were identified by Raper (57) as Penicillium Jensenii Zalesici, A Trichoderma sp., Aspergillus versicolor (Vuill.) Tiraboschi, Penicillium fellutanum, Biourge, Penicillium expansum Link, and Trichoderma glaucum Abbot. These fungi were incubated for 17 days on a medium containing potassium bitartrate and for thirty-three days on a medium containing potassium tartrate. As can be seen in Table 1, Aspergillus versicolor was the most effective in that it utilized 87.7 per cent of the potassium bitartrate and 87.0 per cent of the potassium tartrate in solution. Penicillium Jensenii was the next most effective organism in that it utilized 85.8 per cent of the potassium bitartrate in solution. Because the other fungi utilized considerably smaller amounts of potassium bitartrate they were not used for the production of tartrase.

In the research reported herein cultures of these fungi were held on Sabour and Dextrose Agar or Wort Agar slants for approximately one year and then transferred to new slants. At this time A. versicolor was again tested and found to utilize only 77.5 per cent of the tartrate ion (supplied as potassium bitartrate) as compared with 87.7 per cent a year earlier. Despite the fact that some of its ability to utilize tartrates had been lost, this organism was able to use a sufficient amount of this ion to justify its use for the production of tartrase.

Selection of Media for Producing Tartrase

Preliminary work indicated that tartrase was an endocellular enzyme. The alcohol insoluble material obtained from an extract of fungal mycelium decomposed 41.3 per cent of the tartaric acid in solution while an extract of the liquid medium on which the fungus grew failed to decompose any tartaric acid. A heavy production of mold mycelium was necessary to obtain an appreciable amount of tartrase. In order to obtain rapid production of mycelium, liquid and solid media, and submerged culture techniques were investigated to ascertain the optimum type of culture for *A. versicolor*.

Table 1:

ABILITY OF SIX FUNGI TO UTILIZE POTASSIUM BITARTRATE AND
POTASSIUM TARTRATE AS CARBON SOURCES (Powers 53)

ORGANISM	PER CENT UTILIZATION OF TARTRATE ION	
	Potassium bitartrate medium	Potassium tartrate medium
<u>Aspergillus versicolor</u>	87.7	87.0
<u>Penicillium expansum</u>	--	65.0
<u>Penicillium fellutanum</u>	79.5	14.4
<u>Penicillium Jenseni</u>	85.8	19.6
<u>Trichoderma glaucum</u>	20.0	58.0
<u>Trichoderma sp.</u>	11.9	7.6

The liquid medium was a modification of Sabourand Dextrose medium. In addition to 1.0 per cent peptone and 4.0 per cent dextrose, 1.0 per cent potassium bitartrate was incorporated into the medium. This

solution was autoclaved in 200 ml. aliquots in quart bottles at 15 lbs. pressure for fifteen minutes. After cooling to room temperature, it was inoculated with spores of A. versicolor and incubated at 30° C. until fungal growth covered the surface of the liquid. During the five weeks necessary to produce sufficient mycelial growth, the older portions of the mycelia sporulated heavily. When the tartrase was extracted and tested, it was found that after 48 hours incubation, it had decomposed only 24.3 per cent of the tartaric acid originally in solution. Due to the long growth period and this relatively low tartrase activity, the use of this medium was discontinued.

Submerged Culture Technique

The liquid medium was inoculated with spores of six strains of A. versicolor and agitated by a steady stream of sterile air for 96 hours during which only one strain showed any growth. The mycelium separated from the medium by filtration was subjected to aqueous extraction, the extract filtered to remove remaining particles of mycelium and the enzyme precipitated with alcohol. After this separation the alcohol insoluble material was added to a 1 per cent tartaric acid substrate and incubated at 30° C. for 48 hours. A determination of the tartrate content of the substrate showed that there was only 7.2 per cent tartrate decomposition. As the tartrase activity of the extracts produced under submerged culture technique was low, this method of culture was discarded.

Solid Media

Since these fungi are aerobic in nature, a large surface area will facilitate heavy and rapid growth. To supply this condition, ground

grape pomace containing an added 1% potassium bitartrate was investigated. In order to obtain rapid growth it was necessary to germinate fungus spores in a liquid medium before inoculating the grape pomace medium. When this solid medium was so inoculated a satisfactorily heavy growth of the organism resulted in three to seven days depending on the strain used. This basic medium of ground pomace and potassium bitartrate was used to determine the activity of strains of A. versicolor.

As can be seen in Table 2, samples of medium containing 0.5 per cent KH_2PO_4 and others containing 0.7 per cent yeast extract were compared with the basic medium. The pH of all these samples ranged between pH 3.2-3.5. The results indicated that the combination of KH_2PO_4 and yeast extract had little or no effect in increasing tartrase activity. Because of this the basic medium of ground grape pomace with 1% added potassium bitartrate was used to produce tartrase.

In another study, the pH of the basic medium was adjusted to 1.8, 2.5 and 3.0 with 5 per cent sulfuric acid and to pH 3.5, 3.9 and 4.5 with 10 per cent sodium hydroxide before it was sterilized. The same strain of A. versicolor was used as in the study of the constituents of the medium on tartrase activity. The results of the study are presented in Table 3. There was no significant difference in tartrase activity from fungi grown on media with pH 2.5-3.5, but at pH 1.8, 3.9, or 4.5 enzyme activity was significantly lower than at pH 2.5 or 3.0. Because of this, the pH of the medium was adjusted to pH 3.0 for the further production of tartrase.

The activity of tartrase was low during the studies on the effect of added constituents to the medium due to physiological degeneration of the strain of A. versicolor being used. When the effects of pH of the medium

TABLE 2

EFFECT OF CONSTITUENTS OF MEDIA ON PRODUCTION OF
 TARTRASE BY ASPERGILLUS VERSICOLOR
 STRAIN #76

Medium	Constituents of Medium	Percent Potassium Bitartrate Decomposition*
1	Grape Pomace 1% $\text{KHC}_4\text{H}_4\text{O}_6$	21.6
2	Grape Pomace 1% $\text{KHC}_4\text{H}_4\text{O}_6$ 0.5% KH_2PO_4	19.8
3	Grape Pomace 1% $\text{KHC}_4\text{H}_4\text{O}_6$ 0.7% Yeast Extract	21.7

*Average of two replications

No significant difference between treatments

TABLE 3

RELATION BETWEEN pH OF GRAPE POMACE MEDIA
AND TARTRASE PRODUCTION BY ASPERGILLUS VERSICOLOR
STRAIN #76

MEDIUM	pH	Per Cent Potassium Bitartrate Decomposition*
1	1.8	34.0
2	2.5	40.3
3	3.0	39.5
4	3.5	38.5
5	3.9	34.8
6	4.5	35.3

*Average of two replications

LSD 3.44 and 5.40 at 5% and 1% Levels

Figure 2. Colonies of the Eight Original Strains of Aspergillus versicolor Tested for Tartrase Production

on tartrate activity were determined, the activity of the culture had been restored.

Ability of Strains of *Aspergillus versicolor* to Produce Tartrate

Investigation showed that 11 to 30 per cent of the potassium bitartrate present would precipitate out as crude argols from grape juice held at 40° F. (8° C.). Thus to be satisfactory, a tartrate preparation would have to decompose a minimum of 30 per cent of the tartrates present in grape juice. In an effort to find one suitably active, eight strains of *Aspergillus versicolor* were obtained, one being that isolated by Powers, another obtained from Dr. W. D. Gray of the Botany Department, The Ohio State University, and six strains obtained from Dr. K. B. Raper of the Northern Regional Research Laboratory (see Fig. 2). These fungi were grown on basic grape pomace medium and the enzymes separated by water extraction and precipitated with alcohol. The activity of these tartrate preparations was tested on 1% tartaric acid solutions, with the results shown in Table 4. It will be noted that the tartrate activity ranged from 36.1 to 49.1 per cent tartrate decomposition and that while the difference between strains NRRL 225, NRRL 235, and Gray (OSU) 67 were insignificant, this last strain produced the most active enzyme.

By use of a micromanipulator, 91 single spore isolates were obtained from this strain in an effort to obtain a still more active enzyme. Tartrate preparations from 30 of these cultures were tested

on tartaric acid substrate. As will be seen in Table 5, the activity of these preparations varied over a wide range, the most active preparation decomposing 64.5 per cent of the tartrates and the least active preparation only 30.6 per cent. When analyzed it was found that the mean decomposition was 46.6 per cent and the standard deviation was 9.26 per cent.

Because the argols in grape products consist largely of potassium bitartrate, the tartrate extracts from 81 of the 91 single spore isolate cultures were checked against this salt. These included 26 of the 30 cultures that were previously checked against tartaric acid. The incubation time necessary to obtain a heavy growth of mycelium of the different organisms under test varied from 2 to 7 days at 30° C. As can be seen in Table 6, tartrate activity was considerably lower against potassium bitartrate than tartaric acid, ranging from 1.3 to 34.2 per cent decomposition after incubation of substrate and enzyme at 30° C. for 20 hours. Analysis of the data showed that the mean decomposition was 22.3 per cent and the standard deviation was 6.05 per cent. The fungus that produced the most active enzyme was strain #76. This strain was used as a source of tartrate for the remainder of the work.

Production of Mutants of *Aspergillus versicolor* by treatment with

Radio-Active Phosphorus

In an effort to obtain fungus strains which would produce still more active tartrate, several single spore cultures were inoculated on

solid medium containing radio-active phosphorus. This work was done by Dr. Richard S. Davidson, formerly of the Botany and Plant Pathology Department of The Ohio Agricultural Experiment Station. After growing on this radio-active medium, the organisms were subcultured on dextrose, yeast extract, potassium acid phosphate slants. The tartrase from eight of these cultures was tested immediately after and again six months later. From Table 7, it can be seen that immediately after subculturing, the eight strains produced a more active enzyme (an average increase of 15%) than before treatment. However, six months after treatment with radio-active materials, the activity of the enzymes dropped back to what it was in the original single spore isolates.

Retention of Physiological Constancy of Strain #76, *A. versicolor*

After several subcultures of strain #76 had been used, it was observed that the enzyme was losing its activity. In order to regain the original activity of the enzyme, strain #76 was cultured on slants that contained potassium bitartrate as a major source of carbon. After four subculturings on these slants, the activity of the enzyme was greatly improved. From Table 8, it can be seen that the tartrase activity increased from 17.5 per cent decomposition in the first subculture to 33.1 per cent decomposition in the fourth subculture.

Activation of Tartrase by Addition of Metallic Salts and Vitamins to Growth Medium

Because it was observed that tartrase activity was variable when the enzyme was used alone, a study was made of materials that might possibly act as enzyme activators. The first work of this nature was a

TABLE 5

DECOMPOSITION OF TARTARIC ACID BY ENZYMES EXTRACTED FROM
SINGLE SPORE CULTURES OF ASPERGILLUS VERSICOLOR STRAIN #67

Single Spore Culture Number	Initial Tartrate Content mg/ml	Final Tartrate Content mg/ml	Per Cent Decomposition
2	11.0	3.9	64.5
3	11.0	4.2	61.8
9	11.0	6.2	43.6
10	11.0	6.0	45.2
13	11.0	5.2	52.8
14	11.0	5.0	54.5
15	11.0	5.2	52.8
16	10.8	5.6	48.1
17	10.8	7.0	35.1
18	10.8	7.5	30.6
19	10.8	7.2	33.4
20	10.8	6.0	44.5
21	10.8	7.4	31.5
23	11.4	6.1	46.5
24	11.0	6.3	42.6
25	10.9	5.0	54.1
27	11.4	6.2	45.6
29	11.4	6.5	43.0
30	10.9	5.1	53.3
31	11.0	5.8	47.3
32	10.9	5.1	53.3
33	11.2	5.6	50.0
34	11.0	6.2	43.6
35	11.0	6.2	43.6
36	10.9	5.1	53.3
39	11.0	5.4	50.9
40	11.4	6.4	43.8
41	11.4	5.4	52.6
42	11.4	6.2	45.6
43	13.0	9.0	30.8

MEAN DECOMPOSITION - 46.6 per cent
STANDARD DEVIATION - 9.26

TABLE 6

DECOMPOSITION OF POTASSIUM BITARTRATE BY ENZYMES EXTRACTED
FROM SINGLE SPORE CULTURES OF ASPERGILLUS VERSICOLOR STRAIN #67

Single Spore Culture Number	Initial Tartrate Content mg/ml	Final Tartrate Content mg/ml	Per Cent Decomposition
2	8.0	5.8	27.5
3	8.0	6.0	25.0
4	8.3	6.4	22.9
5	8.2	6.4	22.0
6	12.2	8.6	29.5
8	8.3	6.7	19.3
9	8.3	6.4	22.9
10	8.3	6.2	25.3
11	8.3	6.4	22.9
12	8.0	6.0	25.0
13	8.3	6.6	20.4
14	8.3	6.2	25.3
15	8.3	6.7	19.3
16	8.2	6.4	22.0
17	8.2	6.1	25.6
18	9.2	7.6	17.4
19	8.2	6.0	26.8
20	9.2	7.2	21.7
21	9.2	7.0	23.9
23	9.2	7.3	20.7
24	9.2	6.9	25.0
25	9.2	7.3	20.7
26	8.2	6.7	18.3
27	8.6	7.0	18.6
28	8.6	7.0	18.6
29	8.6	6.7	22.0
32	8.6	6.3	26.8
33	8.6	6.7	22.0
34	8.6	6.4	25.5
36	8.6	6.4	25.5
37	8.6	6.3	26.8
38	8.6	6.7	22.0
39	8.6	6.1	29.1
41	9.2	6.7	27.2
42	9.2	7.2	21.8
43	9.2	6.6	28.3
44	9.2	6.8	26.1
45	9.2	6.7	27.2
46	9.2	6.4	30.4

-- continued

TABLE 6 continued

Single Spore Culture Number	Initial Tartrate Content mg/ml	Final Tartrate Content mg/ml	Per Cent Decomposition
47	8.0	6.8	15.0
48	8.0	6.4	20.0
49	8.4	6.2	26.2
50	8.0	7.0	12.5
52	8.4	7.0	16.7
53	8.0	6.9	13.3
54	8.4	6.2	26.2
55	7.9	6.8	13.9
56	7.9	5.8	26.6
57	7.9	6.6	16.4
58	7.9	6.4	19.0
59	7.9	5.8	26.6
60	7.9	6.2	21.5
61	7.9	6.1	22.8
62	7.9	6.4	19.0
63	7.9	6.6	16.4
64	7.9	6.4	19.0
65	8.0	6.4	20.0
66	8.0	6.8	15.0
67	8.0	6.9	13.7
68	8.0	6.2	22.5
69	8.0	6.9	13.7
71	8.0	6.2	22.5
72	8.0	6.0	25.0
73	8.0	6.2	22.5
74	8.0	6.1	23.8
75	8.2	5.6	30.8
76	8.2	5.4	34.2
77	8.2	6.0	26.8
78	8.0	6.2	22.6
79	8.0	6.4	20.0
90	8.0	6.1	23.8
81	8.0	5.8	27.5
82	8.0	7.9	11.3
83	8.0	7.9	1.3
84	8.0	7.9	1.3
85	8.0	6.1	23.8
87	8.0	5.8	27.5
88	8.0	5.5	31.2
89	8.8	7.0	20.3
90	8.0	6.0	25.0
91	8.8	6.9	21.6

MEAN DECOMPOSITION - 22.3 per cent; STANDARD DEVIATION - 6.05

TABLE 7

TARTRASE ACTIVITY OF ENZYMES EXTRACTED FROM STRAINS OF
ASPERGILLUS VERSICOLOR GROWN ON MEDIA CONTAINING RADIO ACTIVE
 PHOSPHORUS

Strain Number	Before Irradiation	Tartrase Activity*	
		Immediately After subculturing from Irradiation Medium	Six Months after Irradiation
4	22.9	37.6	22.9
8	19.3	39.3	18.3
9	22.9	32.8	22.5
10	25.3	39.3	25.7
11	22.9	32.8	23.0
13	20.4	45.0	20.6
14	25.3	37.6	25.7
15	19.3	41.8	19.5

*Expressed as per cent tartrate decomposition.

TABLE 8

TARTRASE ACTIVITY OF ENZYME FROM STRAIN #76
CULTURED ON GELATIN-BITARTRATE SLANTS

Sub-Culture	Tartrase Activity*
1	17.5
2	20.5
3	27.1
4	33.1

*Measured as per cent tartrate decomposition.

study of the activity of tartrase from fungi grown on media containing various metallic salts or vitamins. The concentrations of the metallic salts used and the tartrase activity of the extracts obtained are shown in Table 9. Tartrase extracted from fungi grown on a medium containing zinc, copper, molybdenum, and manganese was significantly more active at the 5% level than that produced by the control samples. When the tartrase from fungus grown on medium containing iron was tested, it was found that its activity was significantly higher at the 1% level than the control samples. However, there was no significant difference between the activity of tartrase produced by fungi grown on media containing these salts.

A study of the relation between vitamins added to the growth medium and tartrase activity was also made. (The vitamins were made into solutions of known concentration and added to the growth medium before sterilization as shown in Table 14.) After the enzyme had been extracted from the moldy medium, two 50 ml. aliquots were taken. The precipitated enzyme from one aliquot was added to a substrate containing in addition to the potassium bitartrate, 0.1 gram of ferrous chloride and 0.1 gram of zinc sulfate per 100 ml. of solution. The enzyme from the other aliquot was added to a substrate containing only a pure 1% potassium bitartrate. After incubation at 30° C. for 20 hours, the enzyme activity in both substrates was measured. In Fig. 3 and Table 14, it can be seen that tartrase extracted from fungus grown on medium containing all the vitamins under test had a significantly (at the 1% level) lower activity than that from the control samples when tested on a pure potassium bitartrate substrate. Then the tartrase from fungi grown

on media containing only one vitamin there was no significant difference in activity except from the samples extracted from fungi grown in the presence of biotin and calcium pantothenate when tested in a pure substrate. When the enzyme extracts were tested in a substrate containing iron and zinc salts, there was no significant difference in activity except with the samples from fungi grown in the presence of nicotinic acid and calcium pantothenate where the activity was significantly lower at the 5% level. In addition, it can be seen in Fig. 3 that when zinc and iron salts were added to the substrate, tartrase activity averaged 7.9% higher.

Relation Between Metallic Salts Added to the Substrate and Tartrase Activity

When it was found that metallic salts and vitamins had little effect in increasing tartrase activity when added to the growth medium, a study was made of the effect of metallic salts added to the substrate. The first section of this study was concerned with a determination of the relation between the same salts as used in the study of the relation between metallic salts in the growth medium and tartrase activity. The concentration of these salts will be found in Table 15. The enzyme activity as determined after incubation of the potassium bitartrate substrate containing these added salts at 30° C. for 20 hours is presented in Fig. 4 and Table 15. In the presence of these salts, tartrase activity was significantly higher than that of the controls. In the presence of zinc, iron, and copper, the tartrase activity was significantly higher than in the presence of the other salts. Of these three salts, iron was the most effective in increasing tartrase activity.

Since it was found that iron (supplied as ferrous chloride) was the most effective activator, a study was made of the relation between a combination of iron and the other salts under test and tartrase activity. In this study, the same salt concentrations were used as in the previous determinations. To ascertain if the anion of the salt was responsible for part of the activation ferrous sulfate was also used. As can be seen in Fig. 5 and Table 16, tartrase activity in the presence of iron, and zinc, copper, molybdenum, manganese, or magnesium salts was significantly higher than in the control samples. Ferrous sulfate was not as effective as ferrous chloride in increasing tartrase activity.

Two separate studies were made to determine the relation between varying concentrations of these salts and tartrase activity. In the first study a combination of one of the more effective combinations, iron and zinc, was used and in the other varying concentrations of ferrous chloride were used. In Fig. 6 and Table 17 it is shown that as the concentration of iron increased and the zinc concentration decreased, tartrase activity increased significantly. However, when equal concentrations of these salts were used enzyme activity decreased slightly. A similar picture is presented in Fig. 7 and Table 18 where a comparison is made between varying concentrations of iron and tartrase activity. Above a concentration of ferrous chloride of .08 grams per 100 ml. of substrate, the tartrase activity was significantly higher than in the control samples.

Relation Between External Factors and Tartrase Activity

In this work, insofar as possible, all factors were kept constant except the one under study.

TABLE 9

RELATION BETWEEN METALLIC SALTS ADDED TO GROWTH
MEDIA AND TARTRASE ACTIVITY¹

Salt	Concentration g/100 gm.	Per cent Tartrate Decomposition ²
ZnSO ₄	0.10	19.2
FeCl ₂	0.10	22.1
CuCl ₂	0.0005	20.9
(NH ₄) ₂ MoO ₄	0.001	21.0
MgSO ₄	0.250	19.8
MnSO ₄	0.020	18.0
Control	--	15.2

¹Strain #76 used.

²Average of two replications.

LSD at 5% and 1% levels 3.83 and 5.81

Figure 3. Relation between Vitamins added to Growth Medium and Tartrase Activity from Fungi Grown on such Medium

Figure 4. Relation Between Metallic Salts Added to the Substrate and Tartrate Activity

Figure 5. Relation Between a Combination of Iron and Other Metallic Salts Added to the Substrate and Tartrase Activity

Figure 6. Relation between Varying Concentrations of Iron and Zinc and Tartrase Activity Added to Substrate

Figure 7. Relation between Varying Concentrations of Iron Added to Substrate and Tartrase Activity

Time:

Tartrase activity was determined at time intervals of 2, 4, 6, 8, and 26 hours incubation at 40° C. The potassium bitartrate concentrations at the beginning and the end of each interval were calculated as molar concentrations in order to compute the velocity constants of the enzyme catalyzed reaction. These constants were computed using the following first order reaction equation:

$$K = \frac{1}{t} \log \frac{a}{a-x}$$

Where K = velocity constant

t = time in hours

a = initial concentration of substrate in moles

x = concentration of substrate utilized in t time

As can be seen in Fig. 8, 78.8 per cent of the total enzyme activity occurred in the first two hours incubation and then increased slowly. The velocity constants, as presented in Table 19, decreased from a high of .0822 at two hours incubation to a low of .008 at 26 hours incubation. It can also be seen that at the end of 8 hours incubation 96.0 per cent of the total tartrate decomposition had occurred.

Temperature

In this study, substrate-enzyme mixtures were incubated for two hours at 20° C., 30° C., 40° C., 50° C., and 60° C. From Fig. 9, it can be seen that the optimum temperature for this enzyme catalyzed reaction was 50° C. The velocity constants (see Table 20) which were calculated with the same equation used above increased as the temperature increased until a maximum was reached at 50° C. and then decreased as the temperature increased. At

50° C. the per cent tartrate decomposition was significantly higher than at 40° C. or 60° C.

pH

Mixtures of substrates with initial pH of 2.0, 3.0, 4.0, 5.0 and 6.0 and enzyme were incubated at 30° C. for four hours. As can be seen in Fig. 10 and Table 21, the per cent decomposition decreased significantly from a high of 45.0 per cent at pH 2.0 to a low of 23.8 per cent at pH 6.0.

Concentration of Substrate

The substrate containing 0.25, 0.50, 1.0, and 2.0 per cent potassium bitartrate were incubated at 30° C. for four hours. Enzymatic activity was calculated as per cent decomposition and as milligrams of tartrate per milliliter decomposition. In Fig. 11 and Table 22 it can be seen that per cent decomposition decreased significantly from a high of 58.4 per cent at a substrate concentration of 0.25 per cent to a low of 11.1 per cent decomposition at a substrate concentration of 2.0 per cent. When activity was calculated as mg./ml. decomposition, it was found that activity increased from 1.05 mg./ml. at the lowest substrate concentration to a high of 3.20 mg./ml. at a substrate concentration of 1.0 per cent and then decreased as the concentration of the substrate continued to increase.

Concentration of Tartrase

This study was made in several different determinations as enzyme concentrations from 0.005 to 1.00 per cent were used to ascertain whether

enzyme concentration influenced the rate of reaction. From Table 10 it can be seen that tartrase activity is significantly higher when used in concentrations ranging from 0.01 to 0.50 per cent than when used at higher and lower concentrations. The highest activity (34.9 per cent decomposition) occurred when the enzyme was used in a concentration of 0.20 per cent.

Identification of Enzymatic Breakdown Products of Tartaric Acid

During the determination of the tartrase activity of enzyme extracts of various strains of Aspergillus versicolor, total acid and pH measurements of the substrate before and after enzyme action were made. As can be seen in Table 11, there was very little change in either total acid or pH measurements and also there was no definite trend shown towards either greater acidity or alkalinity which indicated that the breakdown product was probably an organic acid. In order to identify this acid, filter paper chromatography techniques were used. After the chromatograms were developed the substrate unknowns had separated into two definite spots. As can be seen in Table 12 the R_f values of these spots checked very closely with those of tartaric and succinic acids.

Action of Tartrase in Concord Grape Juice

Concord grape juice of the 1948 and 1951 seasons was used in determining tartrase action. Some of the results of these determinations are listed in Table 13. When it was found that tartrase activity was being inhibited in the 1951 juice, ion exchange resins were mixed with the enzyme before addition to the juice. One of these resins was the anion

exchange resin DeAcidite supplied by The Permutit Company, New York and the other was the cation exchange resin Amberlite IR-120 supplied by the Resinous Products Division of the Rohm and Haas Company, Philadelphia. As can be seen in Table 13, both of these resins were of limited value in preventing inhibition of tartrase. In the presence of these materials, tartrase activity ranged from 15.2 to 21.2 per cent tartrate decomposition.

Figure 8. Relation Between Incubation Time at 40° C. and Tartrase Activity

Figure 9. Relation Between Two Hours Incubation at Various Temperatures and Tartrase Activity

Figure 10. Relation Between pH of Substrate and Tartrase Activity

Figure 11. Relation between Concentration of Substrate and Tartrase Activity

TABLE 10

RELATION BETWEEN CONCENTRATION OF ENZYME
AND TARTRASE ACTIVITY¹

Enzyme Concentration Per Cent	Initial Tartrate Concentration mg/ml	Final Tartrate Concentration mg/ml	Per Cent Tartrate Decomposition ²
0.005	8.8	6.6	29.6
0.010	8.2	5.5	32.9
0.050	8.2	5.6	31.7
0.100	8.2	5.6	31.7
0.150	8.2	5.6	31.7
0.200	8.0	5.2	34.9
0.500	8.8	5.6	32.9
1.000	8.8	6.3	28.4

¹As determined after incubation at 30° C. for 20 hours.

²Average of three replications.

LSD at 5% and 1% levels 1.84 and 2.55.

TABLE 11

TOTAL ACID AND pH MEASUREMENTS OF POTASSIUM

BITARTRATE SUBSTRATE BEFORE AND AFTER

ENZYME ACTION

Determination	pH		Total Acid ml n/10 NaOH ¹	
	Before Enzyme Action	After Enzyme Action	Before Enzyme Action	After Enzyme Action
1	3.40	3.40	5.30	5.47
2	3.40	3.20	5.30	5.80
3	3.40	3.30	5.40	5.62
4	3.00	3.10	5.30	5.45
5	3.00	3.10	5.30	5.40
6	3.00	3.00	5.30	5.45
7	3.00	3.20	5.30	5.35
8	3.20	3.20	5.50	5.50
9	3.20	3.00	5.50	5.65
10	3.20	3.10	5.50	5.50
11	3.20	3.10	5.50	5.40
12	3.20	3.00	5.50	5.65
13	3.20	2.90	5.50	5.30
14	3.20	2.80	5.50	5.35
15	3.20	2.80	5.50	5.30

¹ ml. NaOH necessary to neutralize a 10 ml. aliquot of substrate.

TABLE 12

FILTER PAPER CHROMATOGRAM RESULTS OF IDENTIFICATION
OF ENZYME BREAKDOWN PRODUCTS OF TARTARIC ACID*

Acid	Rf Value		
	1	2	3
Succinic	0.804	0.760	0.759
Fumaric	0.886	0.852	0.875
Tartaric	0.189	0.188	0.175
Unknown #1	0.196	0.180	0.171
Unknown #2	0.800	0.768	0.761

*Determination made on tartaric acid substrate after 20 hours incubation at 40° C.

TABLE 13

ACTION OF TARTRASE IN CONCORD GRAPE JUICE¹

Determination	Initial Tartrate Content mg/ml	Final Tartrate Content mg/ml	Per Cent Tartrate Decomposition
1	9.9	6.8	31.3
2	9.9	7.5	24.5
3	9.7	6.0	38.2
4	9.9	6.8	31.3
5	8.4	8.4	0.0
6	8.4	8.4	0.0
7	8.4	7.8	7.1
8	7.6	6.1	19.7
9 ²	5.8	4.7	18.9
10 ²	5.8	4.8	17.3
11 ³	9.9	8.4	15.2
12 ³	9.9	7.8	21.2

¹Determination 1-4 made on Concord grape juice of 1948 season.
Determination 5-12 made on Concord grape juice of 1951 season.

²10 mg. DeAcidite (Permutit Products Co.) ion exchange resin per 100 ml. juice added.

³25 mg. Amerlite IR-120 (Resinous Products Co.) ion exchange resin per 100 ml. juice added.

DISCUSSION

Ability of Fungi to Utilize Tartrates:

The data presented herein adds to the list of organisms reported by Fulmer and Werkman (21) and Stadtman, Vaughn, and Marsh (74) as being capable of utilizing tartrates as a source of carbon. As can be seen in Table 1, the fungi isolated by Powers (53) exhibited a considerable range in their tartrate utilizing ability. In addition, in at least one fungus, Aspergillus versicolor, this ability was shown to be adaptive as the capabilities of this organism decreased considerably when cultured for a long period of time on a medium not containing tartrates as a carbon source. The forms in which the tartrates are supplied influenced the ability of these organisms to use them as a nutrient. With the exception of Penicillium expansum and Trichoderma glaucum, the fungi utilized higher percentages of tartrates when supplied in the form of potassium bitartrate than when potassium tartrate was used.

Selection of Media for Producing Tartrase:

In order to be acceptable the medium used for tartrase production had to be able to support heavy and rapid growth of the organism. With the procedures used a liquid medium was not suitable because of the length of time necessary to obtain a heavy growth of the fungus. This was probably due to the fact that the nutrients in solution had to diffuse to the surface of the medium in order to be utilized by the organism.

Liquid medium might possibly be utilized if used where surface area was large in comparison to volume.

While one strain of A. versicolor grew readily in a short period under submerged culture conditions, this procedure was not adopted because of the low activity of tartrase produced this way.

With the procedures and equipment available a solid medium consisting largely of ground grape pomace supported the best growth of Aspergillus versicolor. In addition to offering a large surface area, this material apparently contained sufficient carbohydrates and inorganic substances for rapid growth of the fungus. This is brought out in Table 2 and Table 14 which show that the addition of inorganic phosphate, yeast extract, or vitamins did not aid in the production of a more active enzyme. Also when these constituents were added to the growth medium, mycelial production was not visibly increased or hastened.

As can be seen in Table 3, the pH of the medium was of some importance in the production of an active tartrase preparation. Tartrase activity was highest in the enzyme preparations extracted from fungi cultured on medium with a pH of 2.5 to 3.5 as measured before sterilization. Another factor that favored the use of grape pomace was that it was an inexpensive and readily available material. The principal disadvantage to the use of this solid medium was that in order to get rapid growth the inoculum had to be germinated spores of the organism. This required that a liquid germinating medium be inoculated with fungus spores twenty-four hours before the solid medium could be inoculated. However, using this procedure heavy growth was produced in three days by most of the strains tested.

Ability of Strains of *Aspergillus versicolor* to Produce Tartrase:

There was a wide variation in the tartrase activity produced by the various strains of *A. versicolor*. The tartrase activity of the preparations extracted from the eight original strains obtained from several sources varied from 36.1 to 49.1 per cent decomposition of the tartaric acid originally in solution. This variability of strains is in agreement with the work of Raper, Alexander and Coghill (59) with *Penicillium notatum* in the production of penicillin.

Further evidence was found concerning this variability of different strains when single spore cultures were obtained. In the first place only 91 spores germinated and grew from over 200 single spores that were isolated. In Tables 5 and 6 it can be seen that the tartrase produced by these strains varied considerably. The activity of the enzyme extracts obtained from thirty of these cultures ranged from 30.6 to 64.5 per cent decomposition of tartaric acid. When the enzymes from 81 of the single spore cultures were tested on a potassium bitartrate substrate this variation was even more pronounced. Using this salt, enzymatic activity ranged from 1.3 to 34.2 per cent decomposition. There was considerable difference in enzyme activity when the tartrates were supplied in different forms. The enzymes were more active against tartaric acid than the monopotassium salt.

Production of Mutants of *Aspergillus versicolor* by Treatment with Radio-Active Phosphorus:

Radio-active isotopes were used to produce mutants because efficient sources of ultra violet light or X-rays were not available.

Phosphorus isotopes were used as it was one of the few isotopes that could be safely handled with the equipment available at the time this work was done.

As can be seen in Table 7, mutants were obtained that produced tartrase preparations with higher activity than the original strains. However, these mutants were not stable in this respect and after six months the enzyme activity had decreased to the level of that produced by the untreated cultures.

Retention of Physiological Constancy of Strain #76 as *Aspergillus versicolor*:

As indicated by Virtanen (82), the formation of some enzymes is an adaptive process. That tartrase was an adaptive enzyme can be seen in that when cultured on a medium not containing tartrates as a source of carbon, activity markedly decreased. When strain #76 was cultured on slants containing potassium bitartrate as a major source of carbon for four sub-culturings, enzymatic activity was restored to the original level of this strain.

Activation of Tartrase by Addition of Metallic Salts and Vitamins to Growth Medium:

In selecting the concentration of metallic salts as possible activators, the work of Baumann and Heumuller (2) and Lewis (33), Johnson (30), Linderstron-Lang (36), Nason (45) and Smith and Bergmann (72), were utilized. As is shown in Table 9, the enzyme extracts of fungi grown on medium containing these salts was slightly higher than the control samples.

The work of Clark, Bannister, and Mitchell (87), Jacobsohn (28), Leonian and Lilly (33), Perlmann (52), and Robbins and Ma (64), were referred to in selecting the vitamins and the concentrations that were used in this work. As can be observed in Fig. 3, the use of thiamine, pyridoxine, nicotinic acid and inositol had no effect in increasing tartrase. The tartrase from fungi grown on medium containing calcium pantothenate, biotin, and folic acid was slightly less active than the control samples. When iron and zinc were added to the substrate, enzymatic activity was approximately the same except with the tartrase produced from fungi grown on medium containing calcium pantothenate and nicotinic acid.

Relation Between Metallic Salts added to the Substrate and Tartrase Activity:

In the first work on this study, the same salts and concentrations were used as those added to the growth medium. It can be seen in Fig. 4, that when these salts were added to the substrate, there was an increase in tartrase activity. The reason for this may be one of those suggested by Perlman (51). One of these was that several inorganic salts may be responsible for enzyme activation as there may possibly be several alternative enzymatic mechanisms. The other suggestion was that all of the salts used contain a common contaminant that is responsible for the activation.

In the study of the relation between a combination of iron and other salts and tartrase activity, it can be seen in Fig. 5, that enzyme activity was slightly higher when these combinations were used than when

iron was used alone. The reason for this is unknown but might be explained that with the higher concentrations of salts used a common contaminant responsible for activation would be present in a greater concentration.

The study of the relation between varying concentrations of iron and zinc and tartrase activity indicates that tartrase activity increased with increasing iron concentrations. This would indicate that iron is responsible for activation to a greater extent than zinc. Additional evidence for this is shown in Fig. 7, where the relation between varying iron concentrations and tartrase activity is presented. Here also enzymatic activity increases with increasing iron concentrations. Except for the unexplained dip where equal concentrations of iron and zinc were used, the curve in Fig. 6, is very similar to the one in Fig. 7. This work on the relation between metallic salts and tartrase activity indicates that the greatest enzyme activation occurs when iron in the form of chloride is present in the substrate.

Relation between External Factors and Tartrase Activity:

During the study of these factors, the conditions were not necessarily optimum for enzymatic activity but were such that each factor could be studied. Because of this the effect of each factor studied on tartrase was independent of the other factors influencing enzyme action.

Time:

As can be seen in Fig. 8, the period of greatest tartrase activity was during the first two hours incubation then continued at a much slower rate. This was further indicated when the velocity constants were

calculated. At 2 hours incubation the constant was 0.0822, as incubation time increased this constant became progressively smaller, decreasing to 0.0085 at 8 hours, and to 0.0008 at 26 hours. Because of the very small change in tartrate concentration of the substrate between 8 and 26 hours incubation, it is questionable if this last part of the incubation period was of any significant value. This action of tartrase is in good agreement with the relation between time and diastase activity at 68° C. as presented by Gortner (23).

Temperature:

From Fig. 9 it can be seen that as the incubation temperature increased enzyme activity also increased up to a certain point and then decreased. While this is in general agreement with data presented by Gortner (23) on the relation between temperature and amylase activity, temperature did not play as important a role in tartrase activity. When the velocity constants were calculated it was found that the constants for two hours incubation at 40° C. in both the study of time and temperature were the same. The velocity constants increased as the incubation temperature increased reaching a maximum value of 0.0994 at 50° C. At 60° C. enzymatic activity had decreased because of inactivation of the enzyme at this temperature.

pH:

It can be seen in Fig. 10, that as the pH of the substrate increased from 2.0 to 6.0, enzymatic activity linearly decreased. This is not in agreement with the relation between pH and amylase activity as presented by Gortner (23). There is an optimum pH for the action of most enzymes

and in all probability there is one for tartrase but using potassium bitartrate it cannot be shown. This is due to the fact that as the pH decreased, increasing amounts of this salt were converted to tartaric acid. As indicated in Tables 5 and 6, tartrase was more effective in decomposing tartrates in the form of tartaric acid than as potassium bitartrate. Tartaric acid was not used in this study as the results would not be comparable to those obtained in the other studies made on the action of this enzyme. As has been stated earlier, argols are largely crude potassium bitartrate and this is the principal form of tartrates that this enzyme would decompose when used in commercial practice.

Concentration of Substrate:

Two different interpretations can be obtained from Fig. 11, concerning the relation between substrate concentration and enzyme activity. When activity is expressed as per cent tartrate decomposition, activity decreased with increasing concentrations of the substrate. However, when enzyme activity is calculated as milligrams per milliliter of tartrate decomposed it can be seen that enzyme activity increases with increasing substrate concentration up to a certain point and then decreases. This observation agrees with Joslyn (39) who stated that the initial rate of reaction increases with concentration of the substrate until a maximum reaction rate is reached and then levels off within a wide range.

Concentration of Enzyme:

The initial work on this factor indicated that there was very little difference in tartrase activity when different concentrations of the enzyme were used. Because of this several determinations were made using a wide concentration of the enzyme. As can be seen in Table 10, using enzyme concentrations ranging from 0.005 to 1.00 per cent activity was significantly higher in the concentration range from 0.01 to 0.50 per cent than at higher and lower concentrations of the enzyme. However, the change in activity was not proportional to the enzyme concentration as reported by Gortner (23) for short time reactions. In comparison to other enzymes, however, the tartrase catalyzed reaction is a long term reaction so that it is possible that this general statement does not hold. Another factor that may be applicable here is that in calculating the equilibrium constant for the enzyme-substrate complex formed between invertase and sugar was found to be independent of the concentrations of invertase used as shown by Michaelis and Menton (Gortner 23).

Identification of Enzymatic Breakdown Products of Tartaric Acid:

As can be seen in Table 11, there was very little difference in total acid and pH measurements of the substrate before and after enzyme action. If the breakdown products were not acids the pH values would have increased and the amount of sodium hydroxide necessary for neutralization would have decreased. Since this was not the case, the assumption was made that the breakdown product was another organic acid.

Tartaric acid was used as the substrate to identify the acid being formed, as a salt of the acid would not be resolved as well on a filter

paper chromatogram. The first attempts to separate the acids were made using n-butanol as the solvent but were not acceptable as the acids moved too rapidly to obtain good separation. In order to remedy this, the organic solvent used was twenty-five per cent n-butanol in chloroform. The n-butanol contained one mole of formic acid per liter to suppress ionization of the acids which cause the formation of streaks rather than spots of acid on the filter paper. As can be seen in Table 12, the Rf values for all the acids were slightly greater in the first run than in the others. This was probably due to the fact that the concentration of the acids used in this run was lower than in the others. The reason for this is that higher concentrations of acids slightly retard the movement of the acids on the paper.

The filter paper supports were air dried for 48 hours before spraying the Brom-cresol green indicator in order to obtain complete volatilization of the solvent and formic acid so they would not interfere with the development and identification of the acid spots.

Action of Tartrase in Concord Grape Juice:

When used in Concord grape juice, tartrase activity was quite erratic. Tartrase activity was lower in 1948 season juice than in a pure substrate, but was at least active enough to prevent formation of argol crystals in some of the juice samples. One possible explanation for the lower activity was due to the presence of a compound with similar molecular formation as potassium bitartrate which partially interfered with the formation of the tartrase-tartrate complex. Both malic and succinic acids

(natural constituents of grape juice) have molecular structures similar to tartaric acid and may be responsible for the inhibiting of tartrase.

As can be seen in Table 13, in some of the 1951 juice samples, there was no tartrase activity and in others only slight activity. Because it has been shown that tartrase would act in grape juice, it was believed that an inhibitor was present in the juice. Since it has been shown (Gortner 23) that heavy metals sometimes act as enzyme inhibitors, ion-exchange resins were added to the juice to remove them. Amerlite IR-120 is a cation exchange resin which replaces adsorbed cations with sodium ions and DeAcidite is an anion exchange resin which exchanges adsorbed anions with hydroxyl ions. Since when either of these resins were used, partial enzyme activity took place, it is believed that the inhibitor was either a complex compound or that there was more than one inhibitor present in this juice. This compound was probably not of a colloidal nature because when the juice was treated with a pectinase enzyme (Pectinase W) to remove the pectin and materials held in suspension by the pectin, tartrase activity was as uncertain as in untreated samples.

Since the action of tartrase in grape juice was erratic, it cannot be used to prevent the formation of argol crystals at the present time. It will be necessary to identify the inhibitor and a means of eliminating it before tartrase can be used successfully. Since it has been shown that tartrase is effective in preventing the formation of potassium bitartrate crystals in pure solution, the use of this enzyme could be of considerable importance if the problem of tartrase inhibition can be overcome.

SUMMARY

Six different fungi isolated from grape pomace piles and grape wine lees by Powers were found to be able to utilize significant amounts of tartrates as their sole source of carbon. Of these a strain of Aspergillus versicolor was the most effective utilizing 87.7 per cent of the tartrates supplied as potassium bitartrate during 17 days incubation.

Investigations indicated that liquid medium and submerged culture technique were not suitable for the production of tartrase by A. versicolor but a solid medium of ground grape pomace containing an added 1% potassium bitartrate and an initial pH of 2.5 to 3.5 produced heavy mycelial growth with relatively high tartrase activity. Tartrase was extracted from the medium and mycelia by water extraction, filtration and precipitation with ethyl alcohol. Lyophilization techniques were used to prepare a dry stable enzyme preparation.

The enzyme extracts from eight different strains of A. versicolor decomposed 36.1 to 49.1 per cent of the tartaric acid in solution. Enzyme preparations from single spore isolates from the fungus strain which produced the most active enzyme decomposed 30.6 to 64.5 per cent tartaric acid and 1.3 to 34.2 per cent of the tartrates supplied as potassium bitartrate.

There was a slight increase in the activity of tartrase extracted from fungi cultured on media containing iron, zinc, copper, molybdenum, manganese or magnesium salts. However, there was either no increase or a slight decrease in tartrase activity from enzyme preparations extracted from fungi cultured on media containing added biotin, thiamine, nicotinic

acid, calcium pantothenate, inositol, pyridoxine, or folic acid.

When the metallic salts used above were added to the substrate, tartrase activity increased with the greatest increase occurring in the presence of iron. There was also an increase in activity when combinations of iron and the other salts were added to the substrate. Tartrase activity increased when varying combinations of iron and zinc or iron alone were added to the substrate as the iron concentration increased.

The velocity constant of tartrase was greatest at two hours incubation at 30°C . then slowly decreased with an increase in time. As the pH of the substrate increased tartrase activity decreased when the substrate was potassium bitartrate. The greatest enzyme activity occurred at 50°C . then decreased with an increase in temperature. As the concentration of the substrate increased, enzyme activity expressed as percent decomposition decreased but activity expressed as milligrams of tartrate per milliliter decomposed increased up to a point and then decreased. The concentration of the enzyme had no apparent effect on tartrase activity.

By means of filter paper chromatography, it was found that tartaric acid was converted to succinic acid in the enzyme catalyzed reaction.

Tartrase activity was erratic in Concord grape juice. It was approximately two-thirds of that which occurred in a pure potassium bitartrate substrate in 1948 season juice. In 1951 season juice, there was no activity in some cases and slight activity in others. When anion or cation exchange resins were mixed with the enzyme before addition to the

juice slight tartrase activity occurred. Apparently these materials removed part of the material responsible for tartrase inhibition.

A P P E N D I X

TABLE 14

RELATION BETWEEN VITAMINS ADDED TO GROWTH
MEDIA AND TARTRASE ACTIVITY¹

Vitamin	Concentration g/50gm Media	Per Cent Potassium Bitartrate Decomposition ²	
		A	B ³
All vitamins	712.55	15.8	28.8
Thiamine	12.5	20.7	28.2
Calcium Pantothenate	50.0	18.9	23.9
Pyridoxine	50	20.0	28.1
Nicotinic Acid	50	20.6	22.7
Biotin	0.05	18.8	26.9
Inositol	500	20.6	26.9
Folic Acid	50	19.4	26.9
Control		20.6	26.4

¹Single spore culture #76 used.

²Average of two replications.

³With 0.1 gm. FeCl₂ and 0.1 gm. ZnSO₄ per 100 ml. substrate.

LSD at 5% and 1% levels 1.24 and 1.81 (A)
2.23 and 3.25 (B)

TABLE 15

RELATION BETWEEN METALLIC SALTS ADDED TO SUBSTRATE
AND TARTRASE ACTIVITY¹

Salt	Concentration		Per Cent Potassium Bitartrate Decomposition ²
	Gms/100 ml	Molarity	
ZnSO ₄	0.100	.0062	29.0
FeCl ₂	0.100	0.0079	37.5
CuCl ₂	0.0005	0.000037	26.3
(NH ₄) ₂ MoO ₄	0.001	0.000055	22.5
MgSO ₄	0.250	0.0206	23.5
MnSO ₄	0.020	0.0013	20.5
Control	--	--	15.5

¹Single spore culture #76 used.

²Average of three replications.

LSD at 5% and 1% levels 3.21 and 4.45.

TABLE 16

RELATION BETWEEN COMBINATION OF IRON AND OTHER
METALLIC SALTS ADDED TO SUBSTRATE AND TARTRASE
ACTIVITY¹

FeCl ₂		Metallic Salt	Concentration		Per cent Potassium Bitartrate Decomposition ²
gms/100 ml.	Molarity		gms/100 ml	Molarity	
--	--	FeSO ₄	0.100	0.0066	30.7
0.100	.0079	--	--	--	32.1
0.100	.0079	ZnSO ₄	0.100	0.0062	36.8
0.100	.0079	CuCl ₂	0.0005	0.000037	36.2
0.100	.0079	(NH ₄) ₂ MoO ₄	0.001	0.000055	33.6
0.100	.0079	MgSO ₄	0.250	0.0206	39.5
0.100	.0079	MnSO ₄	0.020	0.0013	37.5
--	--	--	--	--	29.7

¹Single spore culture #76 used.

²Average of three replications.

LSD at 5% and 1% levels 2.86 and 3.97.

TABLE 17

RELATION BETWEEN VARYING CONCENTRATIONS OF
 FeCl_2 and ZnSO_4 ADDED TO SUBSTRATE AND
 TARTRASE ACTIVITY¹

Salt Concentration gms/100 ml		Per Cent Potassium
FeCl_2	ZnSO_4	Bitartrate Decomposition ²
0.04	0.20	22.2
0.06	0.18	24.1
0.08	0.16	28.1
0.10	0.14	29.2
0.12	0.12	26.0
0.14	0.10	31.1
0.16	0.08	31.1
0.18	0.06	31.5
0.20	0.04	33.0
Control	--	18.1

¹Single spore culture #76 used.

²Average of three replications,
 least significant difference 1.45 and 1.99 at the
 5% and 1% levels.

TABLE 18

RELATION BETWEEN VARYING CONCENTRATIONS OF
FeCl₂ ADDED TO SUBSTRATE AND TARTRASE ACTIVITY¹

Concentration of FeCl ₂ gms/100 ml	Per Cent Potassium Decomposition ²
0.04	22.6
0.06	23.5
0.08	26.6
0.10	28.5
0.12	27.3
0.14	30.1
0.16	28.5
0.18	31.6
0.20	31.6
Control	21.5

¹ Single spore culture #76 used.

² Average of three replications.
Least significant difference 3.08 and 4.15 at
5% and 1% levels.

TABLE 19

RELATION BETWEEN TIME AND TARTRASE
ACTIVITY

Time Hours	Initial Tartrase Concentration M	Final Tartrate Concentration M	Percent Decomposi- tion* **	K
2	.0425	.0291	31.6	.0822
4	.0291	.0277	34.8	.0105
6	.0277	.0268	37.5	.0075
9	.0268	.0256	39.6	.0085
26	.0256	.0250	41.3	.0008

*Average of three replications.

Least significant difference 3.98 and 6.04
at 5% and 1% levels.

** Incubation Temperature - 40° C.

TABLE 20

RELATION BETWEEN TEMPERATURE
AND TARTRASE ACTIVITY

Temperature °C	Initial Tartrate Concentration M	Final Tartrate Concentration M	Per Cent Potassium Bitartrate Decomposition* **	K
20	.0442	.0322	27.1	.0683
30	.0442	.0314	28.9	.0746
40	.0442	.0303	31.5	.0822
50	.0442	.0279	36.8	.0994
60	.0442	.0296	33.2	.0865

* Average of two replications

**Incubation time - 2 hours.

LSD at 5% and 1% levels 2.52 and 4.37.

TABLE 21

RELATION BETWEEN pH AND TARTRASE ACTIVITY

pH	Initial Tartrate Concentration mg/ml	Final Tartrate Concentration mg/ml	Per Cent Potassium Bitartrate Decomposed ^{1,2,3}
2.0	8.0	4.4	45.0
3.0	8.0	4.7	41.2
4.0	8.0	5.4	33.1
5.0	8.0	5.6	29.5
6.0	8.0	6.1	23.8

¹Average of two replications.

²Incubation time - 4 hours.

³Incubation temperature - 30° C.

LSD at 5% and 1% levels 2.39 and 3.98.

TABLE 22

RELATION BETWEEN CONCENTRATION OF SUBSTRATE
AND TARTRASE ACTIVITY

Per Cent Potassium Bitartrate	Initial Tartrate Concentration mg/ml	Final Tartrate Concentration mg/ml	Potassium Bitartrate Decomposed ^{1,2,3}	
			Per Cent	Mg/ml
.25	1.8	0.75	58.4	1.05
.50	3.6	2.0	44.5	1.60
1.0	7.7	4.5	40.9	3.20
2.0	15.3	13.6	11.1	1.70

¹ Average of two replications

² Incubation time - 4 hours

³ Incubation temperature - 30° C.

LSD at 5% and 1% levels 4.10 and 7.53.

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AUTOBIOGRAPHY

I, Richard Russell Barton, was born on the 20th of October, 1920, in Springfield, Massachusetts. I attended elementary schools in Springfield and Greenfield, Massachusetts. After graduating from Springfield Classical High School in 1939, I attended the Springfield Junior College for one year. I then entered Massachusetts State College as a sophomore where I majored in Chemistry and minored in Food Technology. Immediately after graduation in 1943, I went on active duty with the United States Marine Corps. On being returned to inactive duty in 1946 I reentered the University of Massachusetts to take graduate study in Food Technology. After receiving my Master of Science degree in 1947, I was employed as an instructor by the Ohio Agricultural Experiment Station and The Ohio State University. While holding this rank, I studied for the degree of Doctor of Philosophy in the Department of Horticulture. I am married and have two children.