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Title: INHIBITION OF MICROBIAL GROWTH IN SEAFOOD BY POTASSIUM SORBATE

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The effect of potassium sorbate on microbial growth in seafood was tested by treating English sole (Parophrys retulus) homogenate with 0, 0.1, and 1.0% potassium sorbate. Viable counts during 1.1°C (34°F) storage revealed that 0.1% potassium sorbate delayed the onset of the logarithmic growth from none, for the untreated, to one day. The generation time of 1.7 days and a maximum growth level of 10^9 cells per g were unchanged by the presence of 0.1% potassium sorbate. The 1.0% sorbate treatment extended the lag period to 6 days. The generation time was increased to 2.1 days and the maximum level of growth reached 10^7 cells per g on the 14th day.

Differenciation for microbial flora revealed that Pseudomonas sp., which comprised 17.1% of the total at 0 day, increased to 96.0% of the microbial population in 14 days at 1.1°C for the untreated, to 100% for the 0.1% sorbate treated sample, and to 98.2% for the 1.0% sorbate treated sample. Potassium sorbate at the concentrations employed did not alter the normal microbial spoilage pattern.

When combined with freezing or mild heating, the antimicrobial effect of potassium sorbate was further enhanced.

Quick freezing to -78°C did not affect the viability of Pseudomonas I strain B-6-I-5 or P. putrifaciens strain A-11-I-24. A sorbate resistant Pseudomonas I strain B-6-I-5 was not affected by the combination of freezing and 0.3% potassium sorbate in tryptone-peptone-extract medium (TPE). The sorbate sensitive P. putrifaciens strain A-11-I-24, however, showed a delay in the onset of logarithmic growth in TPE of 19 hrs, in the presence of 0.05% potassium sorbate after freezing, in contrast to the 6.0 hrs for the similarly treated unfrozen cells.

The delay in the onset of logarithmic growth was more pronounced when heated cells were subjected to potassium sorbate. Heating at 50°C for 1 min extended the lag period of Pseudomonas I strain B-6-I-5 in TPE to 4.5 hrs. In the presence of 0.3% potassium sorbate, however, the delay was for 8 hrs. P. putrifaciens strain A-11-I-24 was also heat sensitive and heating at 45°C for 10 sec alone increased the lag period to 10 hrs, and the presence of 0.05% potassium sorbate in TPE further extended this lag period to 18 hrs.

When either frozen or heated cells were placed in minimal broth Davis (MBD), a basal medium that contained only glucose and a mixture of inorganic salts, the effect of potassium sorbate became even more pronounced. For Pseudomonas I strain B-6-I-5 in MBD after heating at 50°C for 1 min, the lag period was extended to 17 hrs without sorbate, but it was further extended to 41 hrs in the presence of 0.3% potassium sorbate. When P. putrifaciens strain A-11-I-24 was heated at 45°C for 10 sec, its lag period was extended beyond 82 hrs in MBD containing 0.05% potassium

sorbate.

Potassium sorbate at concentrations of 0.1-1.0% acted as a mild antibacterial agent. Bacteria under enzymatic stress appeared to be especially vulnerable to the inhibitory effect of potassium sorbate and mild heating, freezing, and limiting nutrients have all enhanced the effectiveness of this inihibitor.

Inhibition of Microbial Growth in Seafood by Potassium Sorbate

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INTRODUCTION

Sorbic acid is one of many short-chained organic acids, which together with their salts, have been shown to exhibit antimicrobial properties. Sorbic acid and its sodium and potassium salts are collectively known as the sorbates.

The fungistatic property of sorbic acid was first noted by Gooding (1945). In protecting cheese and cheese products from mold spoilage, sorbate has been shown to be markedly superior to sodium benzoate (Smith and Rollin, 1954) which was then used to prevent mold growth in food products. In the first place, a smaller concentration of sorbic acid was required to provide the same degree of protection afforded by sodium benzoate. Secondly, sorbic acid was as readily metabolized by man and animal as any other fatty acids (Deuel et al., 1954a), while benzoic acid must be detoxified by the liver and excreted.

Sorbic acid was harmless to rats and dogs when incorporated into their diets to the extent of 5% on a moisture-free weight basis (Deuel et al., 1954b). This acid was shown not to interfere with food digestion (Deuel et al., 1954a). The U. S. Food and Drug Administration has classified sorbate as an additive Generally Recognized As Safe (GRAS).

Sorbic acid was reported to imparted no objectionable taste, odor, or atypical color to the cheese at the fungistatic concentrations employed (Smith and Rollin, 1954). The potassium salt is practically odorless and colorless and, unlike sorbic acid, is readily soluble in water and

heat stable.

The effect of sorbate on yeasts and molds is well known and sorbates are used in a wide variety of foods today (Luck et al., 1976). Little attention, however, has been given to its antibacterial proper

The limited shelf-life of fresh seafood is a major cause of this excellent protein source plays only a limited role in the American diet. Debevere and Votes (1972) reported that potassium sorbate inhibited bacterial spoilage in prepacked cod fillets by reducing the number of spoilage bacteria.

The present study was undertaken to examine the influence of potassium sorbate on microbial growth in ground fish and to examine the mode of its antimicrobial action.

LITERATURE REVIEW

Factors Influencing Effectiveness of Sorbate

The pH of a medium was found to be a very critical factor in the inhibitory action of sorbic acid (Emard and Vaughn, 1952; Sheneman and Costilow, 1955). Bell et al. (1959) plotted the growth of three species of lactic acid bacteria, Lactobacillus brevis, L. plantarum, and Pediococcus cerevisiae, in sorbic acid broth against the dissociation values of sorbic acid obtainable from pH 3.5 to 7.0, and found that the growth reduction of bacteria correlated with the concentration of the undissociated acid. It was suggested that the undissociated form of sorbic acid was highly lipid soluble (Chichester and Tanner, 1968) and had the greater permeability for the cells (Azukas et al., 1961). Therefore, the antimicrobial activity of sorbic acid depended on the concentration of the undissociated form, rather than on the total concentration of sorbic acid (Bell et al., 1959).

Bandelin (1958) tested the effect of pH on the efficiency of sorbic acid against mold growth and found that the activity was reduced with increasing pH. According to Bell et al. (1959), this was due to the higher level of dissociated form of sorbic acid present at the higher pH.

Shibasaki and Tsuchido (1973) obtained a linear relationship between the lethal rate and the pH of the medium. Since the undissociated form was believed to be the active form of sorbic acid (Bell et al., 1959) and the pK of sorbic acid was 4.76, the effectiveness was depen-

dent on the pH of the medium. The range of optimum effectiveness of sorbic acid extends up to pH 6.5 (Chichester and Tanner, 1968).

Besides pH, the concentration of the preservative and the type of media used, would also influence the effectiveness of sorbic acid (Emard and Vaughn, 1952). All these factors would influence the amount of the undissociated form to be found. With higher concentration of sorbic acid, the undissociated forms of sorbic acid would be in higher concentration even at higher pH than at lower pH with lower initial concentration of sorbic acid. As shown by Bandelin (1958), molds could be inhibited even at higher pH when sorbic acid concentration was increased.

As for the type of medium, Emard and Vaughn (1952) indicated that sorbic acid was less inhibitory when contained in liquid medium than in agar medium. Furthermore the medium containing glucose was more effective than the liver infusion medium. Different food systems also markedly influenced the inhibitory action of sorbate (Beneke and Fabian, 1955; Robach and Hickey, 1978b).

Effect of Sorbate on Microorganisms

The fungistatic property of sorbic acid was first noted by Gooding (1945). Sorbic acid was more effective against some microorganisms than the other. This selectiveness was observed by Phillips and Mundt (1950), Vaughn and Emard (1951), Emard and Vaughn (1952), and Costilow *et al.* (1955).

Sorbic acid and its salts have broad spectrum activity against yeasts and molds (Emard and Vaughn, 1952; Beneke and Fabian, 1955; Bell *et al.*, 1959). Among yeasts and molds tested, *Candida krusei* (Costilow

et al., 1955; Bell et al., 1959), a surface type yeast, Torulopsis holmii (Costilow et al., 1955), a subsurface type yeast, and Zygosaccharomyces globiformis (Bell et al., 1959) were found to be the most tolerant to sorbic acid; while Rhodotorula glutins and a pathogen, Cryptococcus neoformans were the most sensitive (Bell et al., 1959).

In cucumber fermentation, Phillips and Mundt (1950) stated that 0.1% sorbic acid was needed to prevent surface yeast development. Costilow et al. (1957) found that sorbic acid in concentrations of much less than 0.1% would effectively inhibit both surface and film-forming types of yeasts.

Rhizopus and Botrytis, which cause rot in strawberry, were inhibited by sorbic acid at a concentration of 0.025% at pH 3.3 and 4.0 (Beneke and Fabian, 1955). This was equally true against the rot producing fungi in tomatoes, such as Collectotrichum, Fusarium, Rhizopus, and Rhizoctonia. Other fungi, Aspergillus, Mucor, Penicillium, and many others were also inhibited by 0.075% sorbic acid at pH 4.4 (Beneke and Fabian, 1955). The yeasts and filamentous fungi tested by Bell et al. (1959) were inhibited in a medium of 0.67% yeast-nitrogen base that contained 0.1% sorbic acid at pH 4.5, whereas, Emard and Vaughn (1952) found that 0.12% sorbic acid in liver broth or agar inhibited all the actinomyces, molds, and yeasts when the initial pH of the media were in the range of 5.0 to 5.5.

Shibasaki and Tsuchido (1973) reported that sorbic acid enhanced the thermal destruction of Candida utilis at concentrations below 0.1%.

Because sorbic acid and its salts have been used largely as antifungal agents, their antibacterial actions have been generally neglect-

ed. Phillips and Mundt (1950) first showed that sorbic acid caused a selective inhibition of microorganisms and reported that 0.1% sorbic acid did not disrupt the desirable lactic acid fermentation. This observation had prompted an attempt to develop a selective medium useful for enrichment and presumptive identification of the lactic acid bacteria (Emard and Vaughn, 1952). It was noted that sorbic acid did not affect the growth of catalase negative lactic acid bacteria, Lactobacillus and Leuconostoc strains, while inhibiting other bacteria, mold, and yeast cultures (Vaughn and Emard, 1951). Emard and Vaughn (1952) later reported that 0.12% sorbic acid in liver broth or agar would selectively permit the growth of lactic acid bacteria. The tolerance of catalase negative lactic acid bacteria (P. brevis, L. plantarum, and L. brevis) to sorbic acid was further confirmed by Costilow et al. (1955). However, not all the lactic acid bacteria had the same level of resistance. Bell et al. (1959) reported that with 0.1% sorbic acid, none of the lactic acid bacteria species tested had grown at pH 3.5. L. brevis and L. plantarum which could grow at pH 4.0 and P. cerevis which could grow at pH 4.5 were more resistant to 0.1% sorbic acid than L. arabinosus, L. fermenti, and S. lactis, which could not grow at pH 5.0. Growths of the commercial yogurt starters, S. thermophilus and L. bulgaricus, were reduced by 0.05% to 0.1% sorbic acid (Hamdan et al., 1971).

Lactic acid production by certain lactic acid bacteria was not believed to be affected by sorbic acid concentration of 0.1% or below (Phillips and Mundt, 1950; Costilow et al., 1957). This, however, was disputed by Hamdan et al. (1971) who showed that acid production by

S. thermophilus and L. bulgaricus was reduced by 0.05% to 0.1% sorbic acid. Borg et al. (1955) also found that lactic acid productions of P. cerevisiae, L. plantarum, and other species of Lactobacillus were markedly inhibited by 0.1% sorbic acid.

Vaughn and Emard (1951) and Emard and Vaughn (1952) suggested that sorbic acid media might also be useful for enrichment and selective isolation of clostridia. However, as with the lactic acid bacteria, considerable species variation in resistance to sorbic acid was noted. Some cultures of the most tolerant species including Clostridium parabotulinum, C. acetobutylicum, C. felsineum, C. tetani, and C. thermosaccharolyticum grew in the presence of 3.0% sorbic acid at pH 6.7-6.8, while C. perfringens was very sensitive to sorbic acid (York and Vaughn, 1954). Later, York and Vaughn (1955a) demonstrated that C. parabotulinum, types A and B, and C. botulinum, types C, D, and E, could grow in the presence of 0.12% sorbic acid in liver infusion broth at pH 5.6. C. parabotulinum also grew well and sporulated in the presence of 3.0% sorbic acid in liver infusion broth at pH 6.7 when vegetative cells were inoculated. Although sorbic acid did not inhibit the growth of C. botulinum and C. sporogenes in culture media, it did not stimulate the growth, either (Hansen and Appleman, 1955). C. botulinum surviving the cooking process of sausage (Tompkin et al., 1974) and those inoculated into canned cominuted pork (Ivey and Robach, 1978) did not grow as well in the presence of sorbate. Woolford (1975) reported that Clostridium could be inhibited by potassium sorbate at a concentration of 25 mM at pH 6.0.

Botulinal toxin production was not inhibited in media containing

either 0.12% or 1.0% sorbic acid (York and Vaughn, 1955), but its formation was delayed (Tompkin et al., 1974; Ivey and Robach, 1978).

Against aerobic, spore-forming bacilli, sorbic acid was quite effective in suppressing the growth (Emard and Vaughn, 1952; Linderberg et al., 1954; Woolford, 1975), but it did not act synergistically with heat (Shibasaki and Tsuchido, 1973).

As shown by Emard and Vaughn (1952), E. coli was sensitive to 0.12% sorbic acid at pH 5.0-5.5 and the thermal destructon of this organism was enhanced by the addition of sorbic acid (Shibasaki and Tsuchido, 1973). Other bacteria exhibited varying degree of resistances to sorbic acid. Among catalase negative bacteria Streptococci were more sensitive to sorbic acid than Lactobacillus or Pediococcus (Bell et al., 1959). Sorbic acid was an active inhibitor of catalase positive microorganisms in general (Emard and Vaughn, 1952). Among them, Streptococcus faeculis (α-hemolytic streptococci) was less sensitive to sorbic acid than the other catalase positive bacteria tested (Emard and Vaughn, 1952).

Among the catalase positive microorganisms, Micrococcus pyogenes var. aureus (Staphylococcus aureus) exhibited considerably more resistance to sorbic acid than Pseudomonas or Salmonella (Emard and Vaughn, 1952). Tompkin (1974) showed that the growth of Staphylococcus aureus in sausage was inhibited by 0.1% potassium sorbate only during the first day, after which outgrowth was rapid. In contrast, Boylan et al. (1976) found that S. aureus on the intermediate moisture foods, which had an a_w of 0.91 and pH 5.6, was inhibited effectively by 0.3% sorbic acid. They further stated that sorbic acid was not only an effective inhibitor but also an inactivator of Staphylococcus at this concentration.

Emard and Vaughn (1952) first showed that sorbic acid inhibited Salmonella. Recently, sorbic acid has been reported to inactivate Salmonella typhimurium in culture media, in milk (Park and Marth, 1972), and in cheese (Park et al., 1970). Robach and Ivey (1978) reported that 5% or greater sorbate dip left average residues of 0.13% sorbic acid or more on the chicken pieces dipped into it and markedly reduced the growth of Salmonella on chicken. A 10% sorbate dip left average residues of 0.32% sorbic acid and it significantly reduced the total bacterial counts found on freshly processed poultry. Tompkin et al. (1974) also showed that the growth of Salmonella was retarded by 0.1% potassium sorbate in cooked, uncured sausage. Lategan and Vaughn (1964) reported that sorbic acid concentration of less than 0.1% in liquid whole egg at pH 5.5, enhanced the 55°C thermal inactivation of S. typhimurium. Similar effect of sorbic acid on the thermal destruction of E. coli and even greater inhibitory effects on yeast were observed by Shibasaki and Tsudhido (1973).

Emard and Vaugh (1952) had tested ten species of Pseudomonas in liver infusion broth and glucose-yeast extract media containing 0.12% and 0.07% sorbic acid, respectively, and found no microbial growth in 7 days. Cottage cheese treated with 0.075% and 0.1% potassium sorbate and inoculated with P. fragi, spoiled 3 to 5 days later than did the control (Bradley et al., 1962). Hassan and Collins (1969) reported that 0.3% or more potassium sorbate in lactose-yeast extract broth at pH 5.5 retarded the growth of P. fragi and at pH 5.1, even 0.1% of sorbate could extend the lag period by 35 days. Rhee (1974) noted that 0.1% potassium sorbate had little or no effect on the growth of P. putrifaciens but it

inhibited the characteristic H_2S production by this organism.

Recently, Vibrio parahaemolyticus was found to be sensitive to sorbate in culture media, crab meat, and flounder homogenate (Robach and Hickey, 1978a and b). Robach and Hickey (1978a) found that at pH 6.0, 0.2% potassium sorbate in a growth medium inhibited the growth of three strains of V. parahaemolyticus and at pH 5.5, only 0.05% was required to inhibit the growth. They (1978b) also reported that an addition of 0.05% sorbic acid still delayed the growth of all three strains of V. parahaemolyticus in crab meat and flounder homogenate. The number of V. parahaemolyticus in crab meat did not increase and only slight increase in number was observed in the flounder homogenate in the presence of 0.1% sorbic acid.

Utilization of Sorbate by Microorganisms

Sorbic acid and its salts can inhibit the growth of molds effectively, but under insanitary conditions, where the mold population is high in relation to sorbic acid, molds can utilize sorbic acid as a carbon source via beta oxidation (Melnick et al., 1954). Marth et al. (1966) suggested that some Penicillium sp. would deplete sorbate from the media by converting the inhibitory sorbic acid into inactive 1,3-pentadiene through decarboxylation. They also indicated that this mode of resistance by Penicillium was species-dependent and interestingly, P. roqueforti, which is used in blue cheese manufacture, was also the most resistant to sorbate. Bradley et al. (1962) reported that P. frequentans did not utilize sorbic acid, but Marth et al. (1966) observed that 2,000 ppm potassium sorbate was depleted by this organism in 7 days.

Saccharomyces cerevisiae was a sorbate sensitive yeast, while Saccharomyces bailli was a resistant one (Warth, 1977). It was demonstrated that the resistance of S. bailli to sorbic acid was due to an inducible, energy requiring system, which removed sorbate from the cell. No evidence of sorbic acid utilization by a heavy yeast, Torulopsis holmii, was found (Costilow et al., 1955). and similar results were obtained for Geotrichum candidum, Rhodotorula metacaligenes (Bradley et al., 1962), and lactic acid bacteria, P. cerevisiae, L. brevis, and L. plantarum (Costilow et al., 1955).

Some cultures of Clostridium utilized sorbic acid. It was observed that apparent utilization, as measured by the disappearance of sorbic acid from the medium, by C. parabotulinum, types A and B, and a non-toxic strain, were as much as 95.5%, 93.7%, and 62.2%, respectively (York and Vaughn, 1954). It was later found that C. botulinum, types C, D, and E, were not able to utilize sorbic acid (York and Vaughn, 1955a).

Mechanism of Sorbate Action

Mukherjee (1952) found that cyanide has selectively inhibited oxidation of butyric acid, CH_3-CH_2-CH_2-COOH, to ketones by molds. The dehydrogenases responsible for converting butyric acid to crotonic acid, CH_3-CH=CH-COOH, were not affected, but the enzyme system responsible for converting crotonic acid to β-hydroxybutyric acid, CH_3-CHOH-CH_2-COOH, was totally inhibited. Once the unsaturated fatty acid reached a certain level, no further increase in its concentration was noted, due to the end product inhibition of the dehydrogenation reaction. On the basis of this work, Melnick, Luckman, and Gooding (1954) postulated that sorbic

acid inhibited microbial growth through inhibition of the dehydrogenase system.

Martoadiprawito and Whitaker (1963) have shown that potassium sorbate irreversibly inhibited yeast alcohol dehydrogenase by the formation of a covalent bond between the sulfhydryl group and the δ and/or β carbons of the sorbate ion. Based on the structural similarity of sorbic acid, CH_3-CH=CH-CH=CH-COOH, and maleic acid, HOOC-CH=CH-COOH, and on the similarity of their actions on ficin, alcohol dehydrogenase, aldolase, and urease, Whitaker (1959) postulated that sorbic acid would inhibit sulfhydryl enzymes by forming a stable thiohexenoic acid derivative, CH_3-CH=CH-RSCH-CH_2-COOH, in a manner similar to the action of maleic acid to the sulfhydryl enzymes (Morgan and Friedmann, 1938a and b).

Wakil and Huscher (1960) showed that sorbyl-CoA was readily formed from coenzyme A (CoA) and sorbate by the catalytic action of fatty acid activating enzyme. Palleroni and de Pritz (1960) reported that sorbate interfered with the cellular synthesis of citric acid and it was probably due to the formation of sorbyl-CoA. The respiration of cells was hardly inhibited when high level of CoA was present along with the sorbate. Harada et al. (1968) explained that the added CoA had overcome the inhibition due to the formation of sorbyl-CoA.

Coenzyme A and many dehydrogenases are sulfhydryl containing enzymes and they would be inhibited by the similar mechanism postulated by Whitaker (1959). Not all sulfhydryl groups were inhibited to the same degree due to the characteristic of the sulfhydryl groups in the active center (Whitaker, 1959). For instance, aldolase and urease were not

inhibited to the same extent by the sorbate as the alcohol dehydrogenase. The sluggish nature of the sulfhydryl group, which could not react with the mild oxidizing agents and involved in the active center of these enzymes, was thought to be responsible for this difference. Between the aldolase and urease, aldolase activity was inhibited at the sorbic acid concentration of 13.2 mM but the urease activity was not affected with this concentration of sorbic acid (Whitaker, 1959). Azukas et al. (1961) showed that the alcohol dehydrogenase and 3-phosphoglyceraldehyde dehydrogenase activities in a yeast (Saccharomyces cerevisiae) extract was not inhibited even after prolonged incubation of the extract with up to 2.5×10^{-4} M sorbic acid.

Sorbic acid also reacted with enolase in the presence of Mg^{+2} and inactivated this enzyme (Azukas et al., 1961). Enolase was shown to contain no sulfhydryl group (Malmstrom et al., 1959). At lower concentration of up to 2.0×10^{-4} M, the sorbate inhibition was competitive, i.e., the sorbic acid combining with a site or sites adjacent to the active site on the enzyme, thereby reducing the affinity of the enzyme to the substrate; whereas at higher concentrations, the sorbic acid molecules flooded all the active sites of the enzymes and became non-competitive (Azukas et al., 1961). Further analyses revealed that the inhibition of enolase was actually partially competitive and partially non-competitive (Azukas, 1962) and it was concluded that enolase was the primary site of sorbic acid inhibition of alcoholic fermentation (Azukas et al., 1961). Enolase in lactic acid bacteria especially those in Lactobacillus and Pediococcus, however, were protected from sorbic acid inhibition by lactic dehydrogenase (Azukas, 1962).

York and Vaughn (1955b) studied growing cells, intact cell suspensions, and crude purified enzyme preparations and showed that the principal inhibition by sorbic acid was the suppression of fumarate oxidation. Later York and Vaughn (1964) further observed that the inhibition by sorbic acid not only involved fumarase but also aspartase and succinic dehydroN genase.

Addition of sorbic acid influenced both the rate of respiration and the total oxygen uptake of yeast cells (Palleroni and de Pritz, 1960). The total oxygen consumption increased initially when sorbate was added, at the expense of the organic substrates, but the consumption finally dropped when still high concentration of sorbic acid was added. Saccharomyces cerevisiae was an exception in that, both the rate and total oxygen uptake were reduced. Carbon dioxide, however, was still produced in the presence of low concentrations of sorbic acid when glucose was present (York and Vaughn, 1964). York and Vaughn (1964) also pointed out that the shift from an oxidative to a fermentative metabolism would result in sorbate resistance.

Anderson (1963) indicated that oxidative phosphorylation was not likely to be influenced by sorbic aicd since there was no change in the phosphate/oxygen ratio, when sorbic acid was tested with liver mitochondria. Palleroni and de Pritz (1960) also observed that sorbate had no effect on the oxidative phosphorylation of the whole cell in the presence of acetate. The oxidative assimilation was found to be inhibited by sorbic acid in E. coli, S. cerevisiae, and Pseudomonas aerugenosa in a manner similar to that observed with 2,4-dinitrophenol (Clifton,

1946). Sorbic acid was thus suggested to act as an uncoupling agent (York and Vaughn, 1964). Thereby, the organic carbon substrate provided could be almost completely oxidized to CO_2, in the presence of sorbic acid, instead of being assimilated 20 to 50% during respiration and converted into cellular materials, due to the prevention of oxidative phosphorylation (Stanier et al., 1970). York and Vaughn (1964) demonstrated the 30% reduction of oxidative phosphorylation in submicroscopic particles of E. coli, when sorbic acid concentration which diminished the oxidative assimilation of glucose by 79% was added. They also indicated that the sorbate concentration which completely inhibited oxidative assimilation has markedly inhibited the growth.

Similar inhibition of oxidative assimilation was observed with acetate (Palleroni and de Pritz, 1960; Anderson, 1963), pyruvate (Anderson, 1963; York and Vaughn, 1964), malate, fumarate, succinate, glucose (York and Vaughn, 1964), ethanol, acetaldehyde, reduced nicotinamide-adenine-dinucleotide (NAD), reduced nicotinamide-adenine-dinucleotide phosphate (NADP), ascorbate, and lactate (Anderson, 1963).

Harada et al. (1968) reported that sorbate inhibition in the whole cell was competitive with pyruvate or acetate, and inhibitory effect of sorbate on respiration was minimal when glucose was used as a substrate (York and Vaughn, 1964; Harada et al., 1968). Except glucose, the rate of oxidation of acetate, pyruvate, and malate decreased slightly, whereas that of succinate and fumarate was markedly influenced by sorbic acid (York and Vaughn, 1964).

Anderson (1963) suggested that the electron transport system might have been the primary inhibitory site for sorbic acid. Later, with

additional data, he concluded that the cytochrome c oxidase system was the primary site of respiratory inhibition by the sorbate. This may also explain the generally observed selective inhibition of catalase positive organisms by sorbic acid (Vaughn and Emard, 1951; Emard and Vaughn, 1952).

Recently, Shibasaki and Tsuchido (1973) reported that sorbic acid could enhance heat sensitivity of Candida utilis and E. coli during heating and recovery period. It was shown that sorbic acid interfered with the cellular repair following thermal injury. The sorbate inhibited the synthesis of new protein and the respiratory activity during the repair process.

MATERIALS AND METHODS

Potassium Sorbate

Food grade potassium sorbate (Monitor, K, granular) was a gift from Monsanto Industrial Chemicals Company, St. Louis, Missouri.

Potassium sorbate stock solution was prepared in distilled water and the solution filtered through sterile Millipore membrane filter, HAWG, 0.45 μm, just prior to use. Appropriate volume of the solution was added into fish homogenate or sterile liquid media to give a desired final concentration. Concentration was calculated on a dry weight basis and expressed as per cent by weight of potassium sorbate per unit volume of media or fish homogenate. The volume of the stock solution added was standardized throughout the experiments.

Fish Homogenate

English sole (Parophrys retulus), caught off the coast of Newport, Oregon, was obtained immediately after filletting from a commercial processor in Newport, Oregon. The pooled fillets were wrapped in a sterile plastic bag, placed on ice and transported to the laboratory. After overnight storage at 5°C, the fillets were ground in a sterile meat grinder. The ground fish was then aseptically divided into three jars and mixed thoroughly with potassium sorbate solutions to give the final concentrations of 0.1% and 1.0%, respectively. An identical volume of sterile distilled water was added to the control sample. The volume of water added was the minimum that was necessary to facilitate homogenization and subsequent microbiological analyses. Ten grams each

of the fish homogenate, with 0, 0.1, or 1.0% potassium sorbate, was weighed into sterile sample vials. The sample vials were then stored at 1.1°C (34°F), and duplicate samples from each treatment were examined for viable counts at intervals for up to 14 days.

Microbiological Analyses

Viable Count

The entire content of the vial was mixed by shaking with 30 ml of sterile Butterfield's phosphate buffer (pH 7.2) to give an 1:3 dilution. A series of 1:10 dilutions were prepared from this mixture in 9 ml of sterile phosphate buffer (pH 7.2), and 0.1 ml of the proper dilutions were spread plated in duplicate on solidified Tryptone-Peptone-Yeast Extract-NaCl-Glucose (TPE) agar plates. This medium was developed by this laboratory for the optimum recovery of microorganisms from seafoods (Lee and Pfeifer, 1974).

The inoculated plates were incubated at 25°C and the colonies examined after 48 hrs.

Microbial Identification

Viable count plates containing well-isolated colonies of 30 to 100 were chosen and all the colonies on the plates were transferred to master plates on a designated spot with sterile tooth picks. Each master plate containing 30 colonies was then identified according to the replica-plate computer method of Corlett, Lee, and Sinnhuber (1965), as modified by Lee and Pfeifer (1975). The procedure involved simultaneous

transfer of 30 colonies with nichrome wire replicator onto 12 selective and differential media. The growth characteristics of each isolate on these plates were then recorded and the microbial identities determined by a key (Lee and Pfeifer, 1975).

Effect of Potassium Sorbate on Microorganisms in Culture Media

Microorganisms isolated and identified from the fish homogenate were randomly selected and their sensitivity to potassium sorbate tested in TPE broth.

Six of each Pseudomonas I, PseudomonasII, PseudomonasIII, Acinetobacter, Flavobacterium-Cytophaga, Arthrobacter, three Moraxella, and two each of Staphylococcus sp. and yeast cultures were tested. In addition to those microorganisms, seven Pseudomonas putrifaciens sp. isolated and identified from our earlier study were also included. These organisms were streaked on TPE agar plates and incubated at 25°C for 48 hrs. The cultures were grown in TPE broth at 25°C for 48 hrs and 0.1 ml or 0.2 ml of the broth culture, depending on the turbidity, was inoculated into each tube containing 10 ml of TPE broth with varying concentrations of potassium sorbate. Minimal broth Davis (MBD, Difco) was included for P. putrifaciens study. The tubes were then incubated at 25°C and the growth of microorganisms were determined by the visual observation of the turbidity.

Effect of Potassium Sorbate on Freeze Injured Microorganisms

Two strains of Pseudomonas sp., Pseudomonas I strain B-6-I-5 and P.

putrifaciens strain A-11-I-24, were used. The former represented the sorbate resistant and the latter the sensitive strains.

Pseudomonas I strain B-6-I-5 and P. putrifaciens strain A-11-I-24 were cultured with shaking (Psychrotherm incubator shaker) in MBD at 25°C until the cells reached the stationary phase. The cells were harvested by centrifugation at 3,000 XG for 10 min and washed twice with phosphate buffer (pH 7.2). Washed cells were then resuspended in the same buffer to obtain a cell concentration of approximately 10^7 cells per ml. The cell suspension in 6 ml quantities was frozen in a dry ice-acetone bath (-78°C) for 8 min for Pseudomonas I strain B-6-I-5 and 4 min for P. putrifaciens strain A-11-I-24. Subsequently the frozen samples were thawed in a circulating water bath at 8°C for 20 min. One ml of the thawed cell suspension was inoculated into side arm flasks containing 50 ml of TPE or MBD, with and without potassium sorbate, respectively. Extent of microbial growth at 25°C was measured by absorbance with a Bausch & Lomb Spectronic 20 spectrophotometer at 420 nm.

The control was processed in the same manner, except that 1 ml of unfrozen cell suspension of 10^7 cells per ml was transferred into a side arm flask containing no potassium sorbate.

Effect of Potassium Sorbate on Heat Injured Microorganisms

The same Pseudomonas I strain and P. putrifaciens strain A-11-I-24 were used for this experiment. The procedures followed were the same as that for the freeze injury study except that the cells were heated in a water bath at 45 or 50°C.

RESULTS AND DISCUSSION

Effect of Potassium Sorbate on Microbial Growth in Fish Homogenate

Figure 1 shows the microbial counts obtained from fish homogenate, treated with 0, 0.1, and 1.0% of potassium sorbate, and stored at 1.1°C (34°F) for up to 14 days.

The microbial growth inhibition by potassium sorbate was concentration dependent . The inhibitory effect of 0.1% potassium sorbate was barely noticeable while that of 1.0% potassium sorbate was quite pronounced.

Potassium sorbate had extended the lag periods of the microbial growth. There was hardly a lag without sorbate. In the presence of 0.1% sorbate, the microbial growth was inhibited for at least a day and 1.0% sorbate extended the lag period to over 6 days at 1.1°C (Fig. 1).

The rates of microbial growth that followed the lag period appeared to be the same for the control and 0.1% sorbate treated samples, whereas that in the 1.0% sorbate treated sample, was slightly lower. The maximum levels of growth reached in 0 and 0.1% sorbate treated samples were 10^9 cells at the end of 14th day. The growth level in 1.0% sorbate treated sample did not reach this maximum in 14 days.

Microbial Flora Change in Sorbate-treated Fish Homogenate

The identities of microorganisms isolated from English sole homogenate during 1.1°C storage are presented in Table 1.

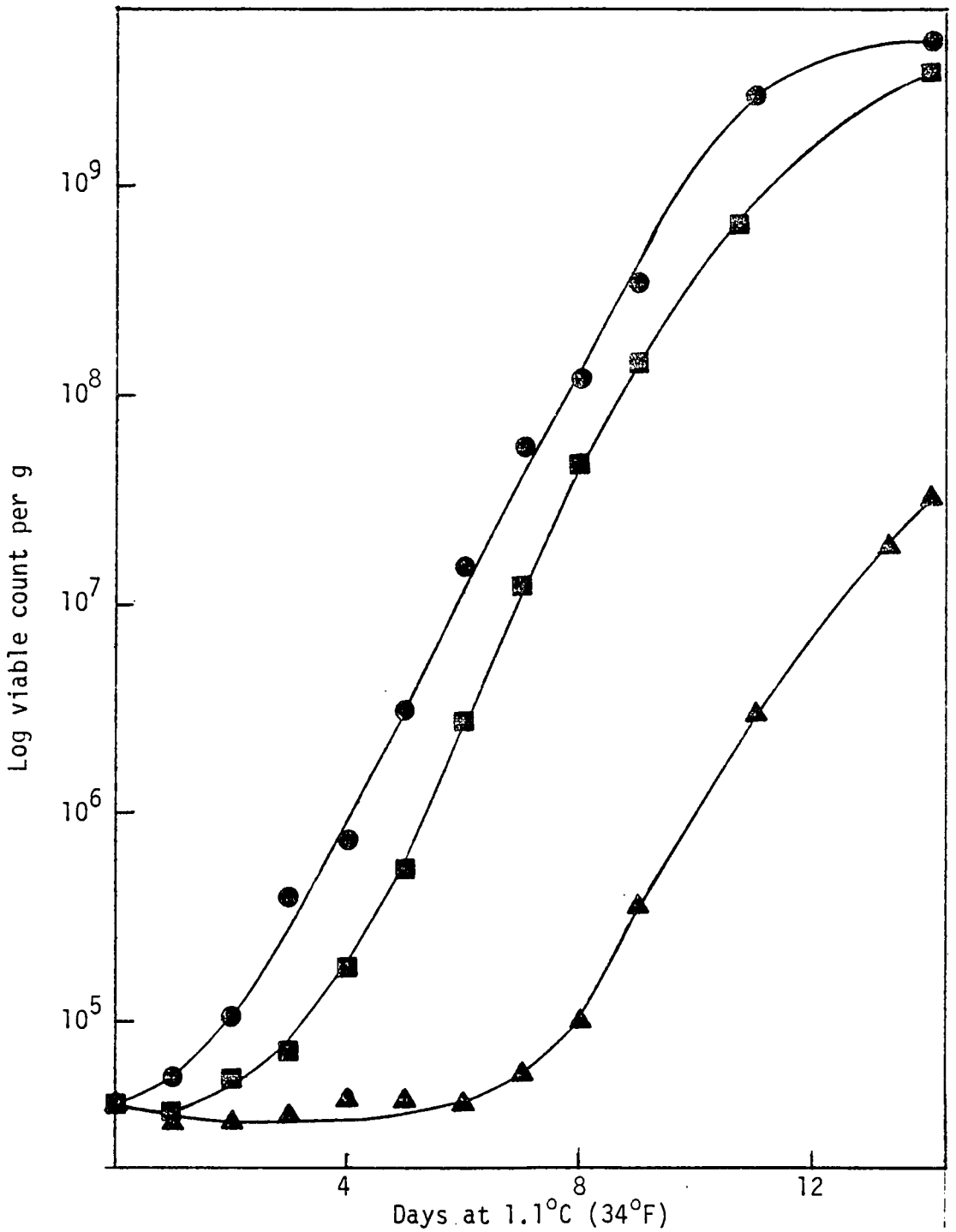

Figure 1. Growth of microorganisms in English sole homogenate treated with potassium sorbate and stored at 1.1°C (34°F). ● = no sorbate; ■ = 0.1% sorbate; ▲ = 1.0% sorbate.

Table 1. Percent microbial flora change in English sole homogenate during storage at 1.1°C (34°F).

Microorganisms	Days at 1.1°C			
	0	6	7	11
Pseudomonas I	5.7	17.9	33.9	12.0
Pseudomonas II	1.4	51.3	48.4	76.0
(P. putrifaciens)	(0)	(5.1)	(9.7)	(14.0)
Pseudomonas III	10.0	10.3	8.1	8.0
Pseudomonas total	17.1	79.3	90.3	96.0
Moraxella	0	2.6	1.6	0
Acinetobacter	7.1	7.7	4.8	0
Flavobacterium-Cytophaga	64.3	10.3	3.2	4.0
Arthrobacter	8.6	0	0	0
Staphylococcus	1.4	0	0	0
Yeast	1.4	0	0	0
Microbial counts of sample ($X10^6$)	0.0402	16.4	56.6	2750
No. of microorganisms identified	70	39	62	50

Flavobacterium-Cytophaga sp. were the most predominant microbial genus at 0 day. In contrast to ocean perch (Lee et al., 1967), no Bacillus, Lactobacillus, Coryneforms, or Micrococcus sp. were isolated from the English sole homogenate. Pseudomonas sp. were the second most predominant genus, followed by Arthrobacter sp. (Table 1). Pseudomonas sp., which occupied 17.1% of the flora, increased to 76.5% after 6 days and to 96% after 11 days. Among Pseudomonas sp., Pseudomonas II sp., which included the H_2S producing P. putrifaciens sp., was primarily responsible for this increase. The rest of the microbial flora, including Flavobacterium-Cytophaga sp. and Acinetobacter sp., were reduced in proportion during this period. Moraxella sp. were isolated from the homogenate even after the 6th day but Arthrobacter, Staphylococcus sp., and yeast were no longer detectable after the 6th day. Hydrogen sulfide producing Pseudomonas sp. appeared on the 6th day and they increased from 5.1% to 14.0% of the total microbial flora of the fish homogenate.

In the presence of 0.1% potassium sorbate, Pseudomonas sp. were the only ones left after 7 days at 1.1°C (Table 2). Among them, Pseudomonas II sp. again were the most predominant. Flavobacterium-Cytophaga sp. disappeared from the flora after the 7th day, Staphylococcus sp., yeast and Moraxella sp. after the 4th day and Acinetobacter sp. after the 6th day.

The 1.0% potassium sorbate treated samples yielded slightly different microbial flora than the 0.1% sorbate treated samples (Table 3). Moraxella, Acinetobacter, and Flavobacterium-Cytophaga sp. were isolated as late as the 9th day. Pseudomonas and Arthrobacter sp. were the only genera left after 11 days. However, Pseudomonas sp. increased from

Table 2. Percent microbial flora change in English sole homogenate treated with 0.1% potassium sorbate and stored at 1.1°C (34°F).

Microorganisms	Days at 1.1°C					
	0	2	4	6	7	11
Pseudomonas I	5.7	38.6	20.6	35.0	44.7	21.1
Pseudomonas II	1.4	16.9	11.8	36.7	39.5	66.7
Pseudomonas III	10.0	6.0	44.1	26.7	15.8	12.2
Pseudomonas total	17.1	61.5	76.5	98.3	100	100
Moraxella	0	3.6	0	0	0	0
Acinetobacter	7.1	9.6	2.9	0	0	0
Flavobacterium-Cytophaga	64.3	21.7	17.6	1.7	0	0
Arthrobacter	8.6	0	2.9	0	0	0
Staphylococcus	1.4	2.4	0	0	0	0
Yeast	1.4	1.2	0	0	0	0
Microbial counts of sample ($X10^5$)	0.402	0.546	1.87	27.9	132	6660
No. of microorganisms identified	70	83	34	60	38	90

17.1% to 98.2% of the total flora on the 11th day while Arthrobacter sp. decreased from 8.6% to 1.8% during this period. It was also noted that the Pseudomonas I sp., instead of Pseudomonas II sp., were the most predominant subspecies in 1.0% sorbate treated sample.

Adam et al. (1964) reported that the most active spoilage microorganisms belonged to the genera Pseudomonas and Achromobacter, when tested on sterile raw fish muscle press juice, prepared from English sole. Our results (Tables 1, 2, and 3) showed that Pseudomonas sp. grew exclusively, either in the presence or absence of sorbate, in English sole homogenate during 1.1°C storage. Surprisingly, none of the H_2S producing P. putrifaciens was found in either 0.1% or 1.0% sorbate treated fish homogenate (Tables 2 and 3). The proportions of non-Pseudomonas sp. remained low and finally most of them disappeared from the flora.

The microbial flora of both sorbate treated and untreated samples were essentially the same. For both samples, Pseudomonas sp. were the most predominant, indicating that sorbate did not disturb the typical microbial spoilage pattern and it also did not exert an appreciable selective influence for one group of bacteria over the other.

The data also indicated that, among Pseudomonas sp., Pseudomonas I sp. were the most sorbate resistant, while P. putrifaciens sp. were the least. Robach (personal communication) has shown a selective elimination of P. putrifaciens sp. from chicken carcasses by dipping the bird into a 5% sorbate solution for 30 sec, to obtain a final concentration of 0.12% sorbate.

Table 3. Percent microbial flora change in English sole homogenate treated with 1.0% potassium sorbate and stored at 1.1°C (34°F).

Microorganisms	Days at 1.1°C					
	0	4	6	7	9	11
Pseudomonas I	5.7	3.2	11.4	53.9	42.7	75.0
Pseudomonas II	1.4	1.6	5.7	16.7	24.0	16.1
Pseudomonas III	10.0	3.2	5.7	8.8	22.7	7.1
Pseudomonas total	17.1	7.9	22.9	79.4	89.3	98.2
Moraxella	0	3.2	2.9	2.0	2.7	0
Acinetobacter	7.1	20.6	11.4	1.0	1.3	0
Flavobacterium-Cytophaga	64.3	58.7	58.6	13.7	5.3	0
Arthrobacter	8.6	4.8	4.3	3.9	1.3	1.8
Staphylococcus	1.4	0	0	0	0	0
Yeast	1.4	4.8	0	0	0	0
Microbial counts of sample ($X10^4$)	4.02	4.29	3.96	5.70	37.2	302
No. of micro-organisms identified	70	63	70	102	75	56

Effect of Potassium Sorbate on Microorganisms in Growth Media

Although the initially predominant microorganism, Flavobacterium-Cytophaga sp., and other minor members of the microbial flora disappeared in fish homogenate treated with potassium sorbate during 1.1°C (34°F) storage and Pseudomonas sp. became the predominant ones, the fish homogenate data did not clearly reveal whether the change in population was due to the difference in sorbate resistance among the bacteria or due to their higher initial number that gave them the numerical advantage. It was especially difficult to determine whether the minor components of the flora, Moraxella, Arthrobacter, Acinetobacter sp., etc. had disappeared from the sorbate treated samples due to their sensitivity to sorbate or being simply overwhelmed by the rapid growth of Pseudomonas sp. Also, there could have been the sorbate resistant species, within a given genus, that were selected.

In order to test the relative sorbate sensitivity of bacteria, randomly selected microbial species were grown in sorbate containing broth and the concentrations of sorbate that produced detectable to complete inhibition of growth were determined (Tables 4 and 5).

The sorbate sensitivity study revealed that Staphylococcus sp. were the most resistant, followed by Arthrobacter, Pseudomonas, Acinetobacter P. putrifaciens, yeast, Moraxella, and Flavobacterium-Cytophaga sp., (Tables 4 and 5).

As expected, Flavobacterium-Cytophaga sp., which predominated the microbial flora of fish homogenate at 0 day but disappeared during

Table 4. Effect of potassium sorbate on microorganisms in TPE broth at 25°C/6 days.

Microorganisms	Strains	O[1]	C[2]	Inhibitory Conc.(%)[3] Min	Max	Microorganisms	Strains	O	C	Inhibitory Conc.(%) Min	Max
Pseudomonas I	B-6-I-5	+	+	0.6	1.5	Acinetobacter	A-6-I-3	-	+	0.2	0.8
	B-6-II-1	+	+	0.4	1.5		A-6-I-9	-	+	0.2	0.8
	B-6-I-1	+	+	0.4	1.6		A-6-I-14	-	+	0.1	0.8
	A-7-I-4	+	+	0.2	1.8		C-4-II-6	-	+	0.2	2.0
	B-7-I-7	+	+	0.3	1.8		C-6-II-24	-	+	0.1	2.2
	B-7-I-24	+	+	0.2	1.5		C-6-II-27	-	+	0.1	0.5
Pseudomonas II	A-6-I-1	+	+	0.2	1.5	Flavobacterium-Cytophaga	C-4-II-22	+	+	0.05	1.2
	A-6-I-15	+	+	0.3	1.3		C-6-II-10	-	+	0.05	1.0
	B-6-I-4	+	+	0.2	1.7		C-4-II-27	-	+	0.05	0.3
	B-6-I-19	+	+	0.2	1.2		A-6-II-19	-	+	0.05	0.7
	B-6-I-30	+	+	0.2	1.2		A-6-II-3	+	+	0.05	0.2
	A-7-I-12	+	+	0.1	1.3		C-6-I-14	+	+	0.05	0.2
Pseudomonas III	B-6-II-23	+	+	0.4	1.3	Arthrobacter	C-6-II-12	-	+	0.05	<3.0
	B-6-I-15	+	+	0.4	1.6		A-0-II-9	-	+	0.05	<3.0
	B-6-II-5	+	+	0.3	1.5		C-4-II-14	-	+	0.2	2.4
	B-6-II-14	+	+	0.1	1.4		C-6-II-8	-	+	0.05	2.6
	A-7-I-12	+	+	0.2	1.4		A-7-III-16	-	+	0.05	2.4
	B-7-I-21	+	+	0.2	1.7		A-0-II-29	-	+	0.05	3.0
Moraxella	A-6-II-7	+	+	0.05	0.8	Staphylococcus	B-2-III-23	-	+	0.6	>3.0
	A-7-III-15	+	+	0.05	0.3		A-0-III-1	-	+	0.4	>3.0
	A-7-III-11	+	+	0.05	0.5	Yeast	A-0-I-12	+	+	0.1	0.8
							B-2-II-2	+	+	0.05	0.6

[1]Cytochrome c oxidase test. [2]Catalase test.

[3]The minimum effect was defined as the concentration which showed observable difference from the control and the maximum effect was for complete inhibition of growth.

Table 5. Effect of potassium sorbate on P. putrifaciens at 25°C/6 days.

Strains	Inhibitory Concentration (%)* in TPE		Inhibitory Concentration (%) in MBD	
	Min	Max	Min	Max
A-11-I-22	0.25	0.6	0.1	0.4
A-11-I-23	0.25	0.8	0.05	0.4
A-11-I-24	0.2	0.9	0.1	0.5
A-11-I-25	0.25	0.9	0.1	0.4
A-11-I-26	0.25	0.7	0.1	0.5
A-11-I-27	0.25	0.6	0.05	0.25
A-11-I-23	0.2	0.7	0.1	0.2

*Same as Table 5.

1.1°C (34°F) storage with sorbate, were the most sensitive to sorbate. Among Pseudomonas sp., P. putrifaciens sp. were the most sensitive. Pseudomonas I sp. were more resistant to sorbate than Pseudomonas II or III species.

It was interesting to note that many sorbate resistant bacteria were cytochrome oxidase negative, while the sorbate sensitive bacteria were cytochrome oxidase positive. This observation tends to confirm Anderson's (1963) hypothesis that cytochrome c oxidase was the target site of sorbate action. While it is true that some of the sorbate sensitive Acinetobacter sp. and Flavobacterium-Cytophaga sp. were cytochrome oxidase negative, the relatively sorbate resistant Pseudomonas sp. and some resistant Flavobacterium-Cytophaga sp. (strain C-4-II-22) were cytochrome oxidase positive. This may be due to the nature of electron transport chains which are much more varied in different bacteria than in all eucaryotic groups, and the components of these chains often differ markedly from those characteristic of mitochondrial systems (Stanier et al., 1970).

Although Staphylococcus and Arthrobacter sp. were the most resistant genera, their growth in the presence of different levels of potassium sorbate showed somewhat different maximum levels after 6 days at 25°C, as were the other more sensitive organisms. This suggests that some systems other than cytochrome c oxidase may be involved in the inhibitory action of sorbate.

York and Vaughn (1954) and Bell et al. (1959) reported a considerable species variation in sorbic acid resistance among catalase negative Clostridium and lactic acid bacteria. Even among catalase

positive microorganisms we tested, a wide variation in sensitivity was noted (Table 4). Nevertheless, enzymatic inhibition must account for the sorbate sensitivity of microorganisms, as the cells in a rich TPE broth required higher concentration of sorbate to inhibit the growth than those in MBD, in which the bacteria had to synthesize all the cellular components from mostly inorganic substrates.

Combined Effect of Potassium Sorbate on Freeze Injured *Pseudomonas*

Since *Pseudomonas* sp. outgrew almost exclusively in the English sole homogenate during storage at 1.1°C and was the most active spoilage organism of English sole (Adam *et al.*, 1964), one of the resistant strain *Pseudomonas* I, B-6-I-5, and one of the sensitive strain *P. putrifaciens*, A-11-I-24, were studied in pure cultures. Since their respective sensitivities to sorbate were observed at 1.1°C, the low temperature survival and growth characteristics were first tested by freezing and thawing the cells and growing them in TPE and MBD, in the presence or absence of potassium sorbate.

Figure 2 shows the growth of *Pseudomonas* I strain B-6-I-5 in TPE and MBD containing 0 or 0.3% sorbate, after being subjected to -78°C for 8 min. There were differences in recovery and growth in TPE and MBD. In a rich TPE medium, no effect of freezing was noted for *Pseudomonas* I strain B-6-I-5. The 0.3% potassium sorbate had little any effect on the growth of either frozen or unfrozen cells (Table 6). The slight inhibitory effect of 0.3% potassium sorbate on freeze injured cells, however, can be noted in Figure 2. In a basal medium MBD, the effects of freez-

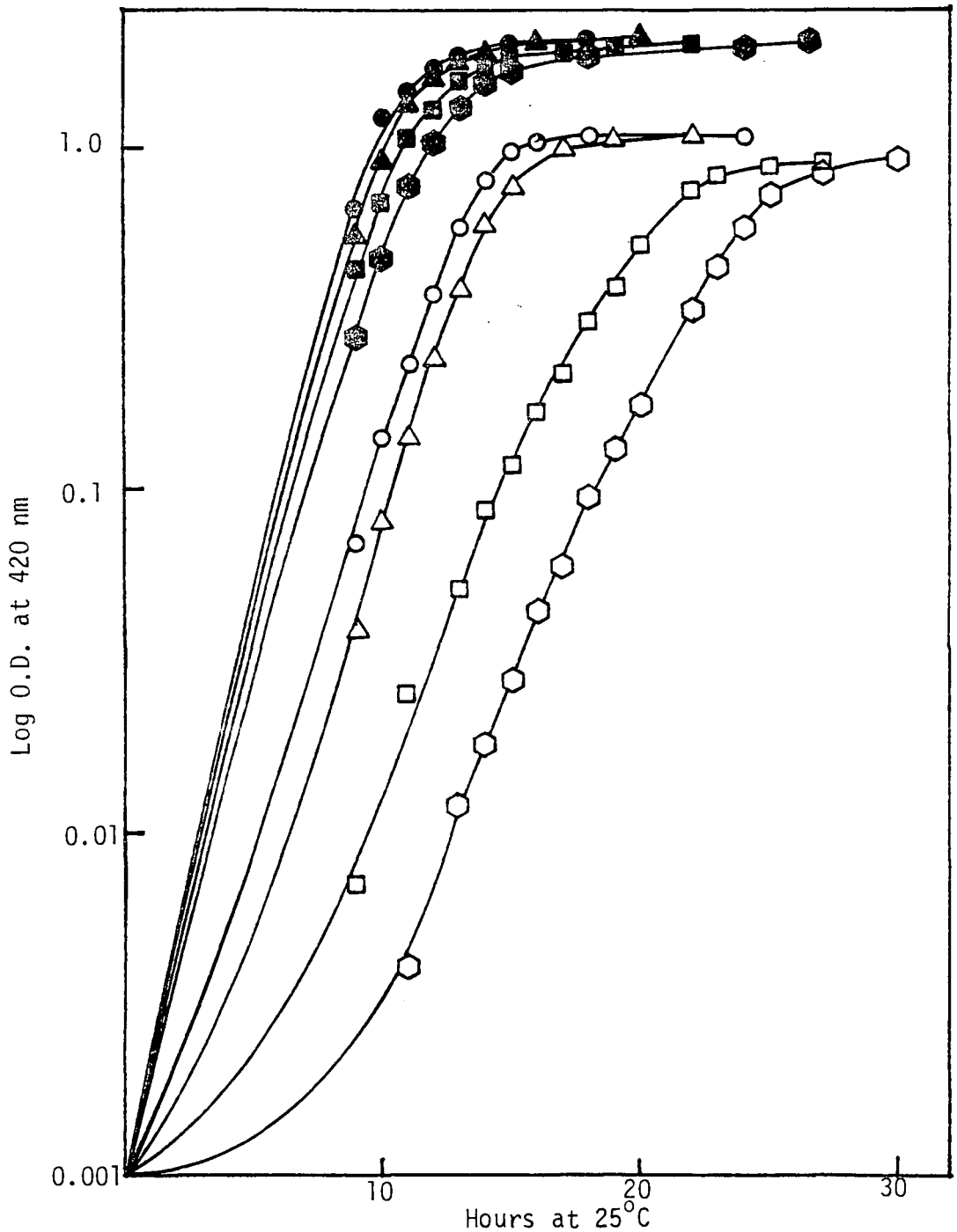

Figure 2. Growth of Pseudomonas I strain B-6-I-5 in TPE and MBD containing 0.3% potassium sorbate after being subjected to -78°C for 8 min.
●= unfrozen, no sorbate, in TPE; ■= unfrozen, 0.3% sorbate, in TPE; ▲= frozen, no sorbate, in TPE; ⬢= frozen, 0.3% sorbate, in TPE; ○= unfrozen, no sorbate, in MBD; □= unfrozen, 0.3% sorbate, in MBD; △= frozen, no sorbate, in MBD; ⬡= frozen, 0.3% sorbate, in MBD.

Table 6. Onset of log phase in growth and generation time of quick frozen Pseudomonas I strain B-6-I-5 in the presence of potassium sorbate (PS).

Treatment*	% PS	in TPE (hrs)		in MBD (hrs)	
		Lag Period	Generation Time	Lag Period	Generation Time
Yes	0	0	3.2	1.8	4.4
	0.3	0	3.6	6.9	5.7
No	0	0	3.2	0.3	4.3
	0.3	0	3.3	3.3	5.7

*Cells were frozen to -78°C in dry ice-acetone slurry for 8 min and thawed in 8°C water bath for 20 min.

ing and sorbate were more noticeable (Table 6). The lag phase was prolonged by nearly 2 hrs by freezing and over 3 hrs by 0.3% potassium sorbate. The combination of freezing and sorbate treatment resulted in nearly 7 hrs of extended lag period. Nevertheless, the growth of frozen cells as measured by the generation time, was nearly identical to that of the unfrozen control. Sorbate reduced the growth of both frozen and unfrozen Pseudomonas I strain B-6-I-5 in MBD.

These observations suggest that greater enzymatic activity within injured cells was necessary for growth and that sorbate, by taxing the already overburdened system, inhibited cell growth more readily. Additionally, the maximum level of growth of cells in TPE was higher than that in MBD, perhaps due to the limiting nutrients.

P. putrifaciens strain A-11-I-24 was more sensitive to sorbate than Pseudomonas I strain B-6-I-5 (tables 4 and 5). Figure 3 shows the marked increases in the lag periods of freeze injured cells in the presence of increasing concentration of potassium sorbate. In a rich TPE broth, injured cells could eventually recover and grow. The lag periods were delayed in proportion to the sorbate concentration, but they eventually reached the same maximum growth levels (Fig. 3 and Table 7). Since the inhibitory action of sorbate was concentration dependent (Emard and Vaughn, 1952), the higher the concentration of sorbate, the longer the onset of the logarithmic growth phase (log phase). This was more pronounced in freeze injured cells. As with Pseudomonas I strain B-6-I-5, the effect of potassium sorbate on growth was greater than that of freezing and thawing (Table 7). In a basal medium MBD, the unfrozen P. putrifaciens strain A-11-I-24 did not start the onset of log phase

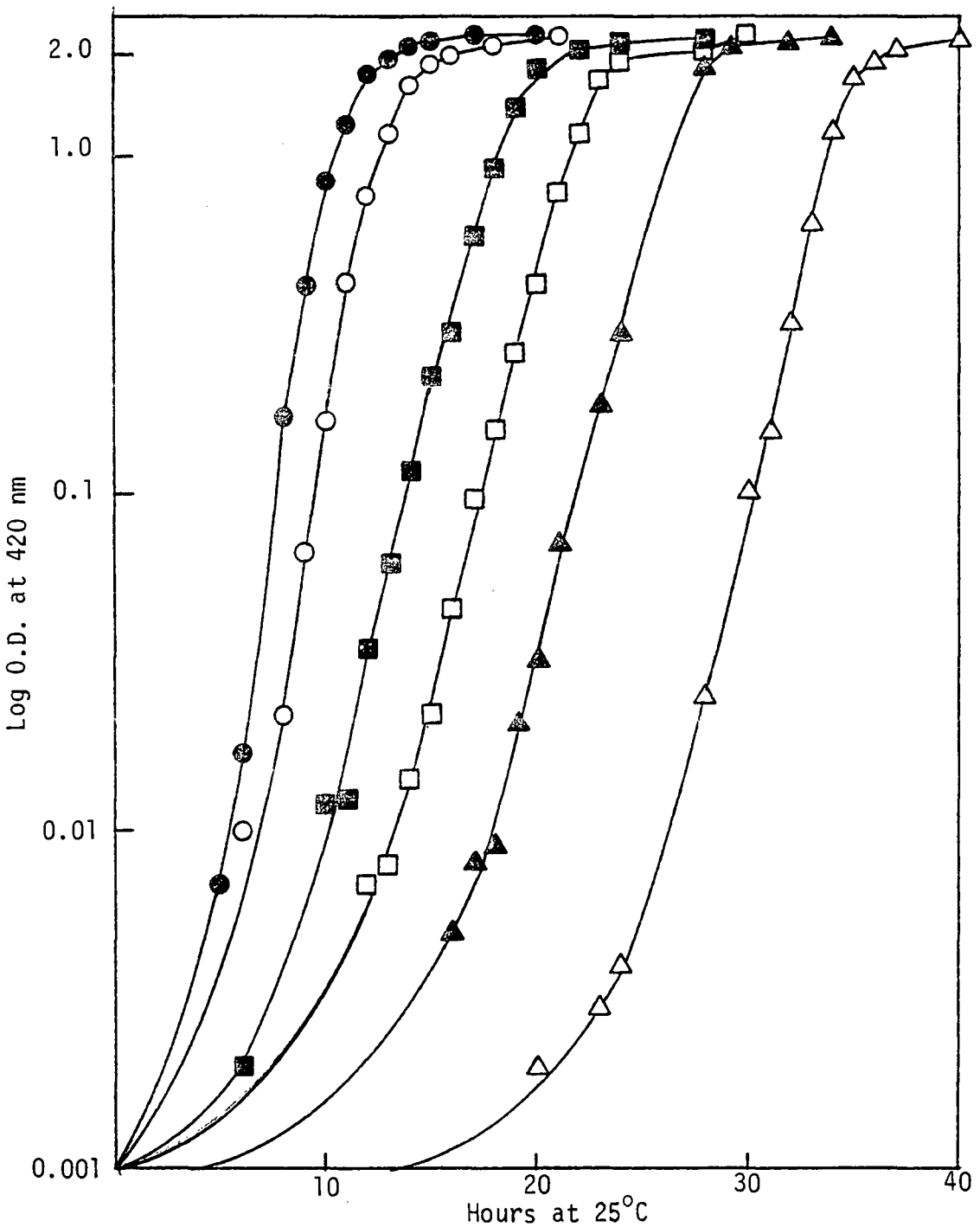

Figure 3. Growth of P. putrifaciens strain A-11-I-24 in TPE containing potassium sorbate after being subjected to -78°C for 4 min. ●= unfrozen, no sorbate; ■ = unfrozen, 0.05% sorbate; ▲= unfrozen, 0.1% sorbate; ○ = frozen, no sorbate; □ = frozen, 0.05% sorbate; △= frozen, 0.1% sorbate.

Table 7. Onset of log phase in growth and generation time of quick frozen P. putrifaciens strain A-11-I-24 in the presence of potassium sorbate (PS).

Treatment*	% PS	in TPE (hrs)		in MBD (hrs)	
		Lag Period	Generation Time	Lag Period	Generation Time
Yes	0	3.5	2.7	36.0	10.0
	0.05	19.0	3.8	-**	-
	0.1	22.7	3.8	-	-
No	0	1.7	2.7	22.0	10.0
	0.05	6.0	3.7	35.0	19.0
	0.1	13.7	3.8	-	-

*Cells were frozen to -78°C in dry ice-acetone slurry for 4 min and thawed in 8°C water bath for 20 min.

**Growth poor.

until 22 hrs' incubation at 25°C and 36 hrs for the frozen cells in the absence of potassium sorbate. In the presence of 0.05% potassium sorbate, the onset of the log phase of the unfrozen cells was delayed to the same extent as that of the frozen cells in the absence of sorbate but the generation time was twice as long (Table 7). When 0.05% sorbate was combined with freezing, poor growth of this organism was shown for at least 100 hrs of incubation at 25°C (Fig. 4).

Freezing and thawing treatment employed for Pseudomonas I strain B-6-I-5 and P. putrifaciens strain A-11-I-24 were mild enough and no inactivation had occurred due to this treatment alone. The injured cells needed greater enzymatic activity than the uninjured cells for repairing. While the rich TPE medium provided more readily utilizable nutrients than the basal medium MBD, which contained mostly inorganic salts, growth and repair were greater in the former than in the latter. The presence of increasing concentration of sorbate increased the retardation of growth and repair, especially for the injured cells, hence, it lengthened the log phase and the generation time. Therefore, sorbate appeared to have enhanced the freeze destruction of cells. It was also obvious that P. putrifaciens strain A-11-I-24 was more vulnerable to freezing, thawing, and sorbate treatment than Pseudomonas I strain B-6-I-5.

Combined Effect of Potassium Sorbate on Heat Injured Pseudomonas

Figures 5 and 6 show the growth of Pseudomonas I strain B-6-I-5 in TPE after heat treatment of 45 and 50°C for up to 10 min.

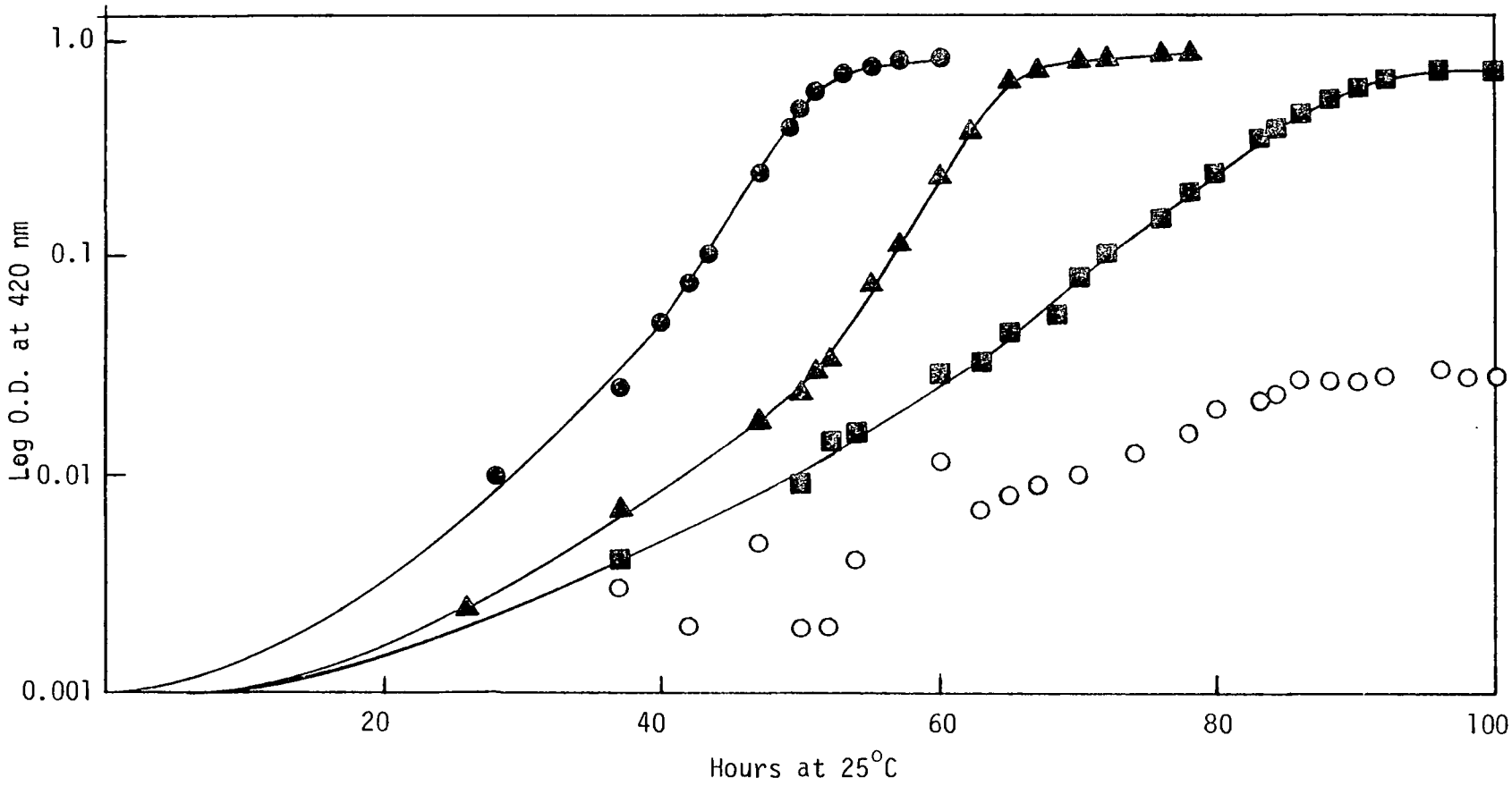

Figure 4. Growth of P. putrifaciens strain A-11-I-24 in MBD containing 0.05% potassium sorbate after being subjected to -78°C for 4 min.

● = unfrozen, no sorbate; ■ = unfrozen, 0.05% sorbate; ▲ = frozen, no sorbate; ○ = frozen, 0.05% sorbate.

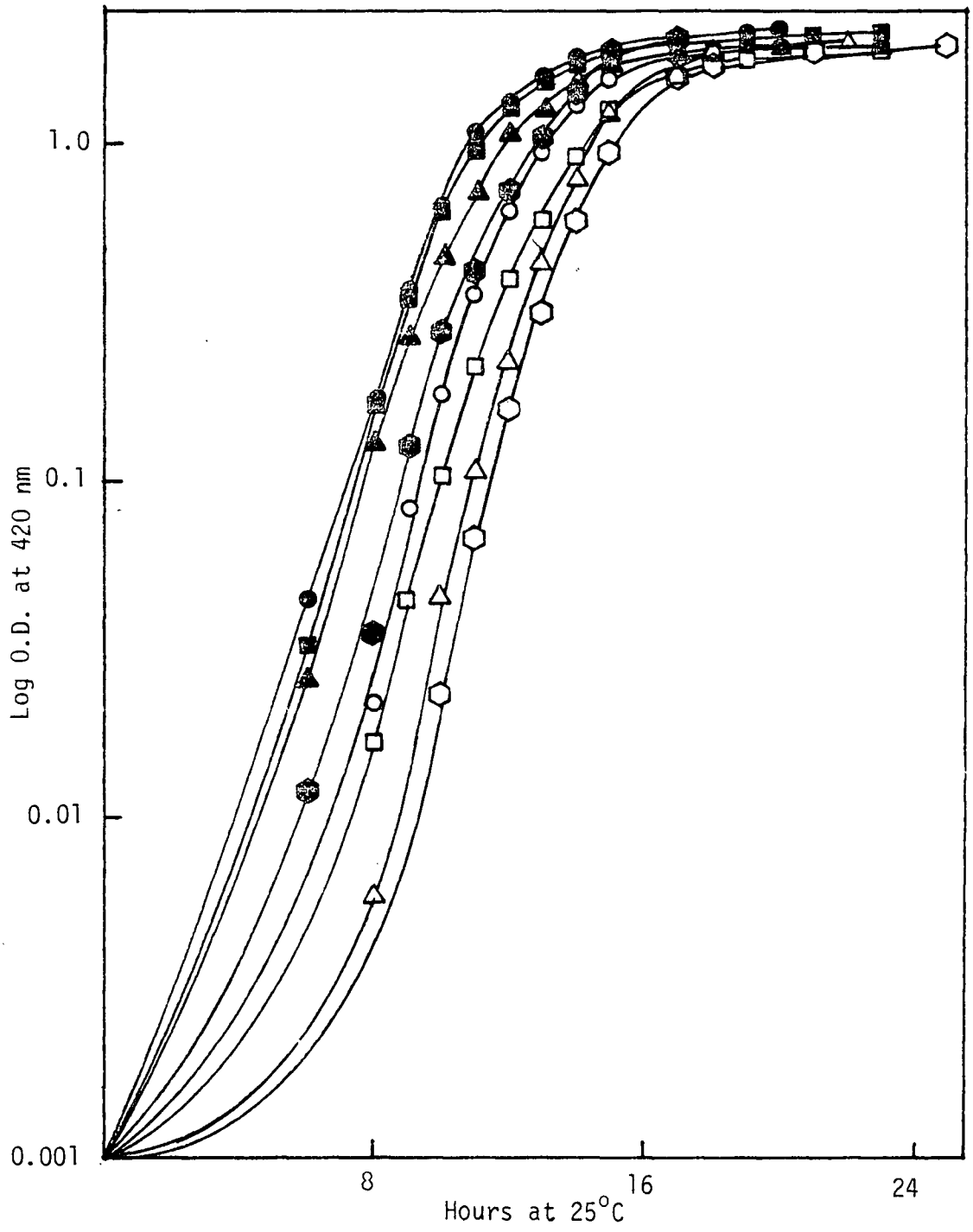

Figure 5. Growth of Pseudomonas I strain B-6-I-5 in TPE containing potassium sorbate after being heated at 45°C.
● = no heat no sorbate; ■ = no heat, 0.1% sorbate; ▲ = no heat, 0.3% sorbate; ⬢ = 5 min heat, no sorbate; ○ = 5 min heat, 0.1% sorbate; □ = 5 min heat, 0.3% sorbate; △ = 10 min heat, no sorbate; ⬡ = 10 min heat, 0.1% sorbate.

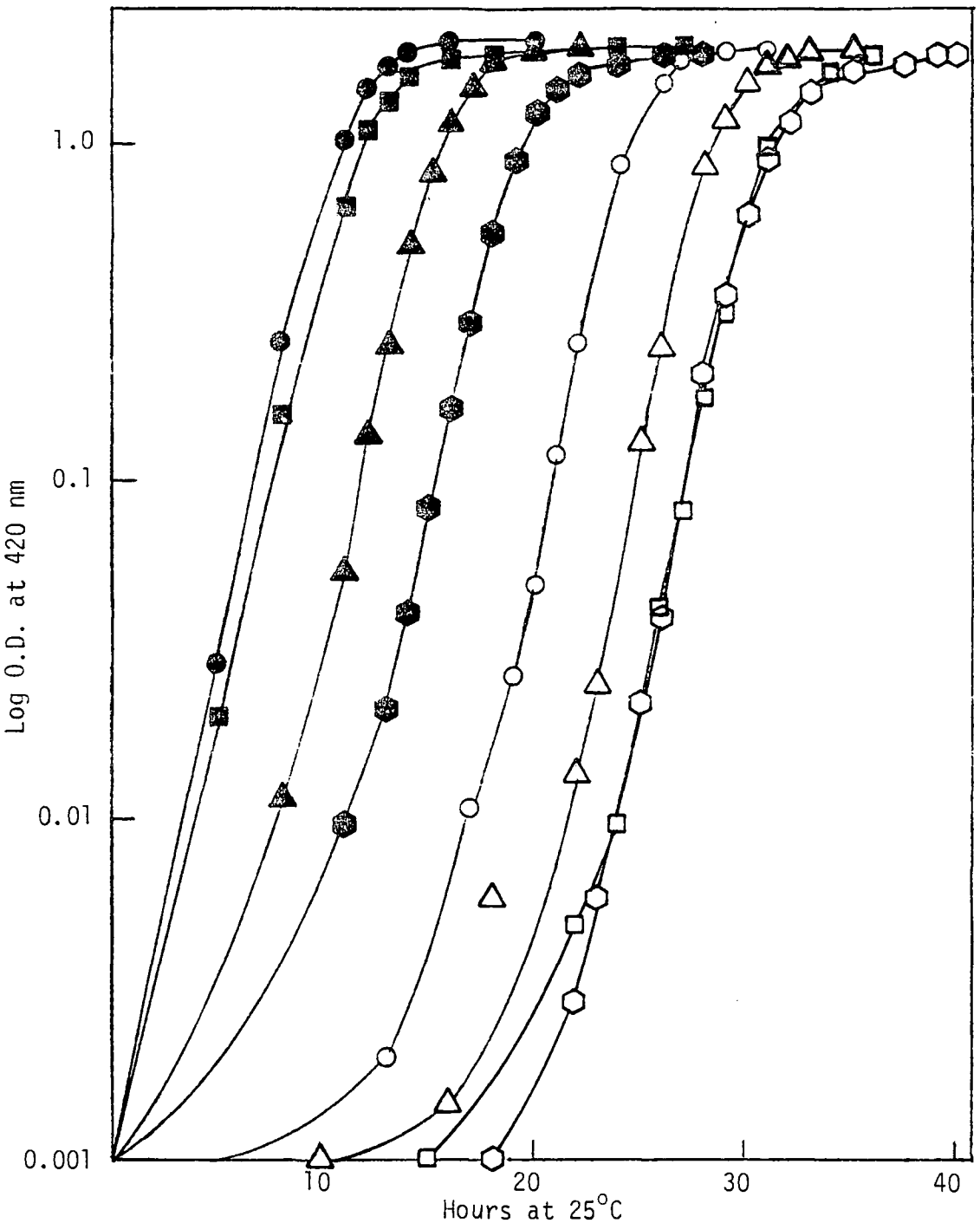

Figure 6. Growth of Pseudomonas I strain B-6-I-5 in TPE containing 0.3% potassium sorbate after being heated at 50°C. ●= no heat, no sorbate; ■ = no heat, 0.3% sorbate; ▲ = 1 min heat, no sorbate; ⬢= 1 min heat, 0.3% sorbate; O= 3 min heat, no sorbate; □= 3 min heat, 0.3% sorbate; △ = 5 min heat, no sorbate; ⬡= 5 min heat, 0.3% sorbate.

Potassium sorbate at 0.1% concentration had no effect on the growth of unheated cells. Similarly, heating at 45°C alone had little effect on growth (Fig. 5). When combined with sorbate, the effect of heating on the growth of Pseudomonas I strain B-6-I-5 became more pronounced, especially at 50°C (Fig. 6). Heating at 50°C for 1 min was sufficient to delay the onset of logarithmic growth for 4.5 hrs, but it nearly doubled to 8 hrs in the presence of 0.3% potassium sorbate (Table 8). The generation time and the level of maximum growth were affected very little by heat or sorbate in TPE. This perhaps indicates that the delayed onset of logarithmic growth represents the period for repair and recovery from the injury. Potassium sorbate seems to have magnified the effect of heat injury.

In MBD, the recovery of the injured cells was much slower because the cells apparently needed to synthesize all the cellular components from mostly inorganic salts, hence greater enzymatic activity was necessary (Fig. 7). When subjected to 50°C for 1 min, the injured cells could recover in MBD containing either 0 or 0.3% sorbate. Both the prolonged lag phase and slower growth rate were observed (Table 8). When the cells were heated at 50°C for 3 min, the growth occurred only in the absence of potassium sorbate. As shown with freezing, the combined effect of sorbate and heat was to prolong the lag phase. Both in TPE and MBD, the generation time of the heated cells slightly increased without the addition of potassium sorbate. As expected, the maximum growth levels of this organism in MBD was lower than that in TPE.

P. putrifaciens strain A-11-I-24 could not survive the heating at 50°C. Figures 8 and 9 show the growth of 45°C heated P. putrifaciens

Table 8. Onset of log phase in growth and generation time of heated Pseudomonas I strain B-6-I-5 in the presence of potassium sorbate (PS).

Heating	% PS	in TPE (hrs)		in MBD (hrs)	
		Lag Period	Generation Time	Lag Period	Generation Time
0 min	0	0	3.2	0	4.3
	0.1	0	3.2	-*	-
	0.3	0.5	3.4	0	6.1
45°C 5 min	0	2.0	3.3	-	-
	0.1	3.0	3.3	-	-
	0.3	3.5	3.4	-	-
45°C 10 min	0	5.0	3.2	-	-
	0.1	6.0	3.5	-	-
50°C 1 min	0	4.5	3.6	17.0	4.9
	0.3	8.0	3.8	41.3	7.1
50°C 3 min	0	13.5	3.6	42.7	5.0
	0.3	19.0	4.0	--**	--
50°C 5 min	0	18.0	3.6	--	--
	0.3	20.4	4.0	--	--

*Experiment not performed.

**Growth poor or absent.

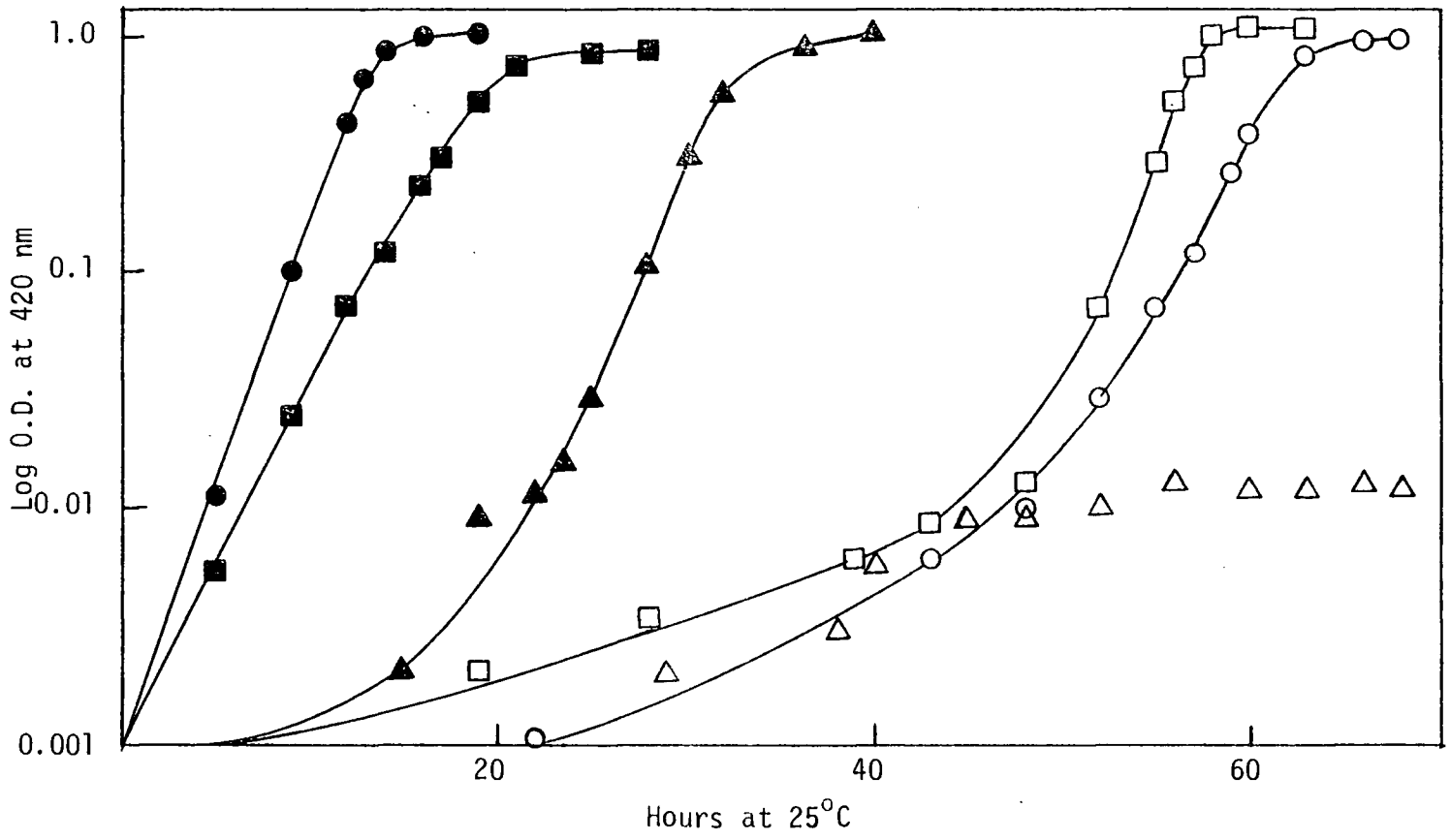

Figure 7. Growth of Pseudomonas I strain B-6-I-5 in MBD containing 0.3% potassium sorbate after being heated at 50°C.

●= no heat, no sorbate; ■= no heat, 0.3% sorbate; ▲= 1 min heat, no sorbate; ○= 1 min heat, 0.3% sorbate; □= 3 min heat, no sorbate; △= 3 min heat, 0.3% sorbate.

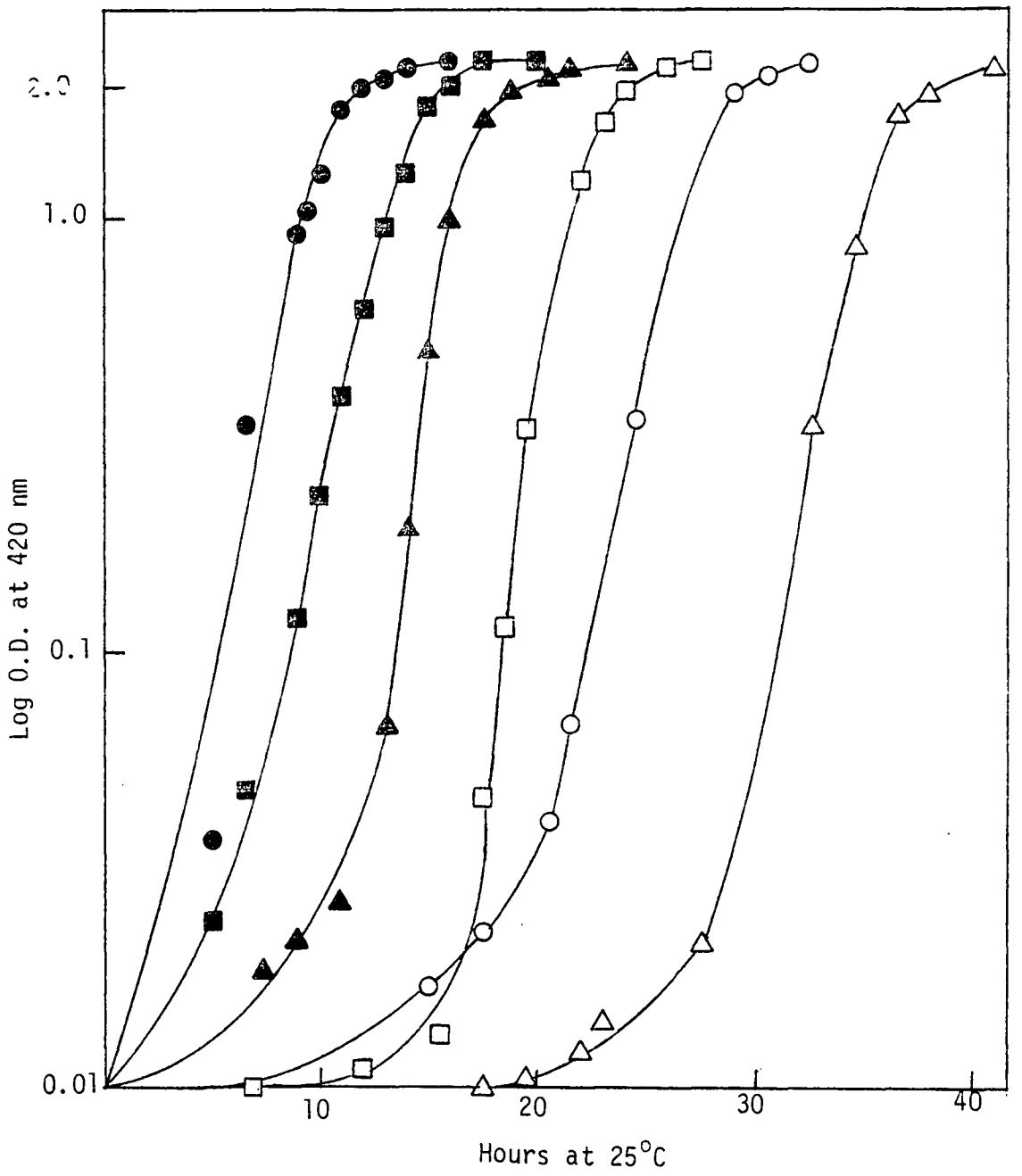

Figure 8. Growth of P. putrifaciens strain A-11-I-24 in TPE containing 0.05% potassium sorbate after being heated at 45°C
● = no heat, no sorbate; ■ = no heat, 0.05% sorbate; ▲ = 10 sec heat, no sorbate; ○ = 10 sec heat, 0.05% sorbate; □ = 2 min heat, no sorbate; △ = 2 min heat, 0.05% sorbate.

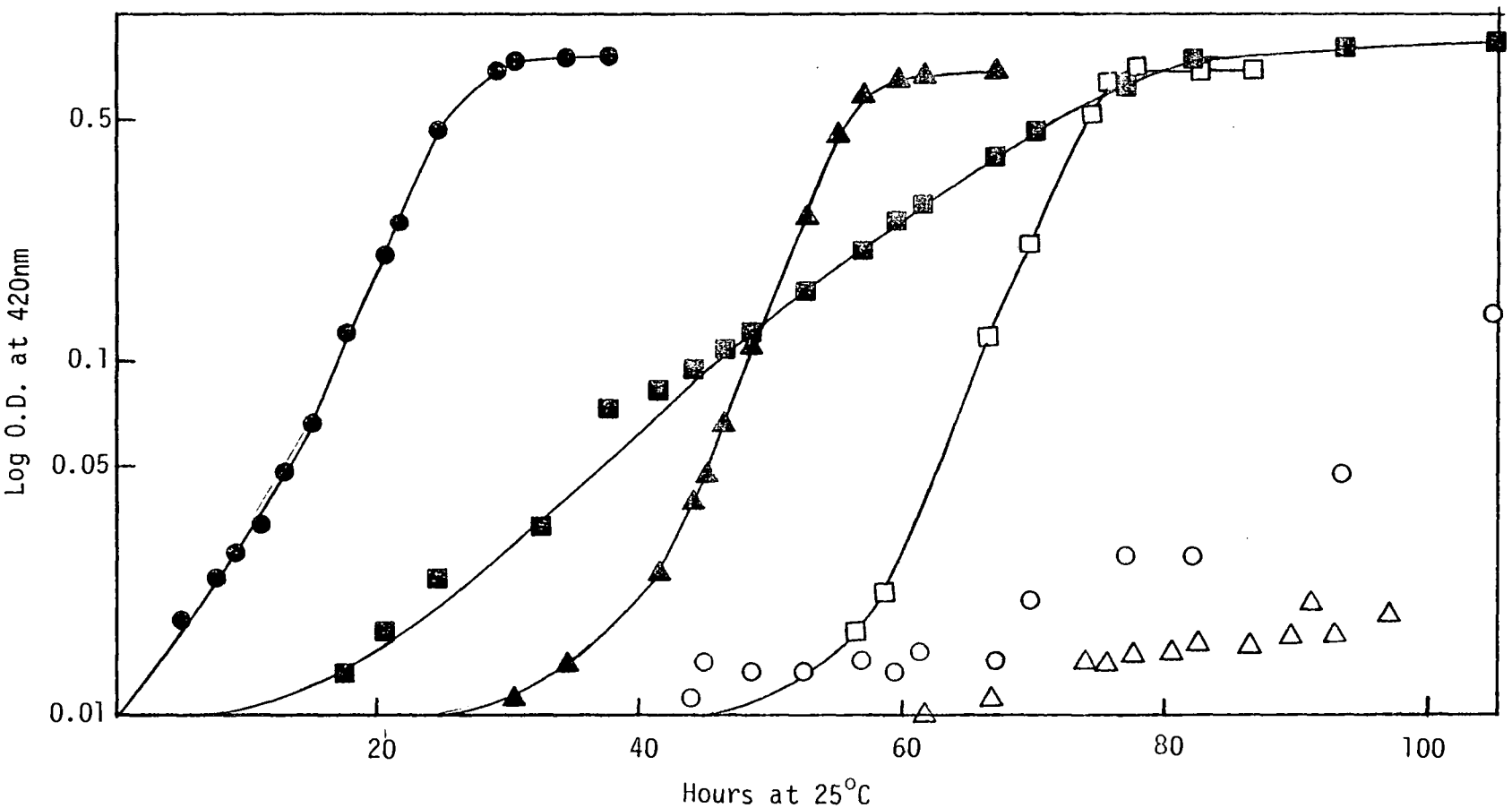

Figure 9. Growth of P. putrifaciens strain A-11-I-24 in MBD containing 0.05% potassium sorbate after being heated at 45°C.

● = no heat, no sorbate; ■ = no heat, 0.05% sorbate; ▲ = 10 sec heat, no sorbate; ○ = 10 sec heat, 0.05% sorbate; □ = 2 min heat, no sorbate; △ = 2 min heat, 0.05% sorbate.

strain A-11-I-24 in TPE and MBD, each containing 0 or 0.05% potassium sorbate. Although heat and sorbate each alone affected the growth, the combined effect of heat and sorbate was more pronounced. Again, the prolonged lag phase characterized the effect of this treatment. Longer heating time resulted in a prolonged lag period and addition of sorbate, at the 0.05% level, dramatically enhanced the effect of heat. In TPE, the growth of P. putrifaciens strain A-6-I-24 was influenced more by sorbate than heat (Table 9). In MBD, the growth was so poor that when 0.05% sorbate was present, it extended the lag period by 3 to 4 times, from 4.6 to 15.0 hrs. The generation time was also tripled from 11.7 to 32.5 hrs (Table 9). When combined with heat, hardly any growth could occur in MBD containing as little as 0.05% potassium sorbate.

Comparison of the Effect of Heating and Freezing on Pseudomonas

Data in Tables 6 and 7 indicate that growth rates at the log phase of both Pseudomonas sp. were not affected by freezng, but sorbate alone had some inhibitory effect. This might suggest that enzymatic activity was not affected by freezing. Most enzymes are heat sensitive, therefore, heating at 50°C for Pseudomonas I strain B-6-I-5 and at 45°C for P. putrifaciens strain A-11-I-24 might have inactivated some of the enzymatic activity. The increased generation time for the heated cells in addition to the inhibitory action of sorbate may be evidence for this (Tables 8 and 9). The onset of log phase was delayed more in heated cells than that for the frozen cells. This might suggest that the psychrotrophic Pseudomonas sp. were more sensitive to heat than to freezing.

Table 9. Onset of log phase in growth and generation time of heated P. putrifaciens strain A-11-I-24 in the presence of potassium sorbate (PS).

Heating at 45°C	% PS	in TPE (hrs)		in MBD (hrs)	
		Lag Period	Generation Time	Lag Period	Generation Time
0 min	0	1.4	3.5	4.6	11.7
	0.05	3.6	4.2	15.0	32.5
10 sec	0	10.0	3.8	38.0	11.6
	0.05	18.2	4.4	--*	-**
2 min	0	15.2	3.8	55.0	11.8
	0.05	26.0	4.8	-	-

*Lag period extended beyond 82 hrs.

**Poor to no growth.

Differences between Pseudomonas I and P. putrifaciens

The cells of frozen Pseudomonas I strain B-6-I-5 recovered very well and all grew to the maximum levels of growth within 30 hrs, either in TPE broth or in MBD, containing 0 or 0.3% sorbate (Fig. 2). The frozen cells of P. putrifaciens strain A-11-I-24, on the other hand, recovered much slower in TPE and MBD. Although all reached the same maximum levels of growth in TPE, it required 40 hrs of incubation at 25°C, when 0.1% sorbate was present (Fig. 3). While in MBD, the frozen cells required 76 hrs and 98 hrs of incubation at 25°C to reach the maximum levels of growth, in the absence and in the presence of 0.05% sorbate, respectively (Fig. 4). Since P. putrifaciens strain A-11-I-24 was more sensitive to sorbate than Pseudomonas I strain B-6-I-5 (Tables 4 and 5), the generation time for P. putrifaciens strain A-11-I-24 in media containing 0.05% or 0.1% sorbate was much longer than that for Pseudomonas I strain B-6-I-5 in media containing 0.3% sorbate (Tables 6 and 7).

Mild heating at 45°C had little effect on Pseudomonas I strain B-6-I-5 grown in TPE but it was quite pronounced for P. putrifaciens strain A-11-I-24 (Figs. 5 and 8). Heating for 10 sec at 45°C for P. putrifaciens strain A-11-I-24 was far more severe than 10 min at 45°C for Pseudomonas I strain B-6-I-5 (Tables 8 and 9). Sorbate had more greatly enhanced the thermal effect on P. putrifaciens strain A-11-I-24 than on Pseudomonas I strain B-6-I-5. This may be due to the greater sensitivity of P. putrifaciens to sorbate and to heat than Pseudomonas I.

Since P. putrifaciens is more vulnerable to heat and freezing, and

more sensitive to sorbate than Pseudomonas I, it is not surprising to find that none of P. putrifaciens sp. was found in the sorbate treated fish homogenate stored at 1.1°C. On the other hand, the overall resistance of Pseudomonas I to heat, freezing, and sorbate, explains how this group of bacteria can predominate the microbial flora of seafoods whether treated or untreated with potassium sorbate.

SUMMARY AND CONCLUSION

Commercially prepared fish fillets of English sole (Parophrys retulus) were ground and mixed with 0.1 and 1.0% of potassium sorbate. Microbial counts were made during storage at 1.1°C (34°F). After identification, representative microorganisms from each genus were tested for their sensitivities to potassium sorbate. Subsequently, combined effects of sorbate on freeze-injured and heat-injured cells were tested on resistant and sensitive Pseudomonas strains. The results and conclusions may be summarized as follows:

1. Potassium sorbate at 0.1% concentration in fish homogenate delayed the microbial growth for a day and at 1.0%, the lag period was extended to over 6 days at 1.1°C. The rate of microbial growth was not affected by 0.1% potassium sorbate and slightly reduced by 1.0% sorbate. Maximum growths for 0 and 0.1% sorbate treated samples were 10^9 cells at the end of 14 days, whereas that for the 1.0% sorbate treated sample was 10^7 cells.
2. Flavobacterium-Cytophaga sp. were the most predominant group of microorganisms found at 0 day and Pseudomonas sp. were the second. However, during storage at 1.1°C, microorganisms that grew in the presence of sorbate were almost exclusively Pseudomonas sp. At 1.0% concentration of sorbate at 1.1°C, Pseudomonas type I reached 75% of the total microbial flora after 11 days at 1.1°C. None of the Pseudomonas putrifaciens sp. was found in any of the sorbate treated samples.
3. Staphylococcus sp. were the most resistant bacteria to potassium

sorbate, followed by Arthrobacter, Pseudomonas (I, II, III), Acinetobacter, P. putrifaciens, yeast, Moraxella, and Flavobacterium-Cytophaga sp.

4. Freezing by itself did not affect the generation time of Pseudomonas I strain B-6-I-5 or P. putrifaciens strain A-11-I-24. Addition of potassium sorbate, however, had increased the generation time of P. putrifaciens strain A-11-I-24. For the resistant Pseudomonas I strain B-6-I-5, no lag period was seen after freezing in the presence of sorbate in TPE, but in MBD which contained glucose and inorganic salts mixture, the lag period was increased in the presence of 0.3% sorbate. Sorbate thus enhanced the freeze injury of microorganisms by delaying the onset of log phase and slowing the growth. More pronounced delays were shown in MBD than in TPE. Growth levels in MBD were always lower than those in TPE.
5. Heat was more injurious than freezing to Pseudomonas sp. perhaps due to inactivation of some enzymes. Heat and sorbate had greater influence on the growth of Pseudomonas sp. than freezing. As with freezing, sorbate delayed the onset of logarithmic growth of heated cells, but heating also increased the generation time, while freezing had no effect.
6. P. putrifaciens was more sensitive to freezing, heating, and to potassium sorbate than Pseudomonas I. Therefore, Pseudomonas I could predominate in sorbate treated fish homogenate, whereas P. putrifaciens could be found only in untreated samples.

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