Prevention of microbial deterioration in salted dried fish

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PREVENTION OF MICROBIAL DETERIORATION IN SALTED DRIED FISH

by

IR. SANTOSO

A Master's Thesis

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the requirements for the award of
Master of Philosophy of
the Loughborough University of Technology

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Studies Humberside College of Higher Education.

Collaborating Establishment : Directorate General of Fisheries. JAKARTA - INDONESIA

December 1989
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Finally, I would like to thank P. Smith for providing accommodation and good companionship during my stay in England.
Dedication

The thesis is dedicated to my wife, Endang who has encouraged me and shown great understanding throughout my study in England, whilst looking after my family in Indonesia. I would also like to dedicate this work to my parents and my daughters, Sukma and Raga.
PREVENTION OF MICROBIAL DETERIORATION IN SALTED DRIED FISH

By Ir. Santoso

ABSTRACT

The reduction of microbial deterioration in salted dried fish by application of the food preservatives, sodium sorbate, sodium benzoate, sodium propionate and sodium bisulphite was assessed using Aspergillus niger and A. penicillioides, Halobacterium salinarium and Staphylococcus sp as test organisms. The two bacteria were grown on complex halophilic medium (CHM) and salted dried fish homogenate (SDFH), whilst the moulds were grown in Czapek Yeast Medium/Malt Extract Medium and 10% salt-Czapek Yeast Medium. Effectiveness of the food preservatives was also tested on one or two-dimensional gradient diffusion systems and on salted dried fish samples.

Sodium sorbate was most effective in controlling growth of A. niger. Growth on both 10% salt-CYA and 10% salt-CYB prevented growth at a level of 0.1% (w/v). Visible growth of the mould was delayed for 3 days when 0.2% sodium benzoate was combined with 10% salt. Total dry weight increase was zero, when 10% salt-CYB was supplemented with a concentration of 0.15% sodium benzoate.

Initiation of A. penicillioides grown on/in 10% salt-MEA or 10% salt-CYB, was delayed by sodium bisulphite, sodium propionate and sodium benzoate at the highest levels tested (0.04%, 0.3%, and 0.2% respectively). Growth was significantly inhibited using 0.1% sodium propionate and 0.1% sodium benzoate in these systems. Sodium sorbate at a level of 0.1% prevented growth of A. penicillioides on 10% salt-MEA and in 10% salt-CYB.

H. salinarium grown on CHM and SDFH was not sensitive to sodium propionate and sodium bisulphite at the highest levels tested 0.3% and 0.03%, respectively, but sodium benzoate at a level of 0.10% was lethal to the bacterium grown in the two media. The lag phase was extended for 7 days, in CHM but no growth was observed in SDFH. Salted dried fish previously dipped in a 1% solution of either sodium benzoate or sodium sorbate showed no sign of spoilage by H. salinarium after 17 days incubation. Sodium sorbate was also effective against the bacterium grown on SDFH when growth remained in lag phase until the end of the incubation period of day 7.

Growth of Staphylococcus sp was totally inhibited using either 0.05% sodium sorbate, 0.03% sodium bisulphite or 0.15% sodium benzoate. At the highest level of 0.3%, sodium propionate did not significantly inhibit growth of this bacterium.
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I. INTRODUCTION.

1.1 Background.

Curing by salting or drying or a combination of these techniques is the main traditional fish processing technique employed in South East Asian Countries.

In Indonesia, 50% of total fish catch is cured and 32% of this comprises salted dried fish (Directorate General of Fisheries, 1987). Such a processing technique is not only cheap and relatively easy to practise, but also yields an end product with a relatively long shelf life. Fish curing is often the only method available to processors in rural areas where there is no electricity, refrigeration etc.

1.2 Problems of traditional fish processing.

There are some problems associated with traditionally processed fish products mainly resulting from the growth of xerophilic, and or halotolerant moulds and bacteria. Growth of moulds on the surface of salted and or dried fish leads to an undesirable appearance, and also presents the possibility of mycotoxin production. Aerobic, halophilic bacteria also grow only on the surface of salted dried fish producing a pink to red colour,
resulting in an undesirable appearance and strong smell. It has been reported that moulds, such as *Polypaecium pise*, *Aspergillus penicillioides*, *Aspergillus niger*, are found on the salted dried fish (Wheeler, et al. 1988). Bacteria including *Micrococcus* spp, *Pseudomonas* spp., *Staphylococcus* spp. and *Halobacterium* spp are also reported to be major microflora (Sanderson et al. 1987). Losses of cured fish due to microbial deterioration and other problems, such as insect infestation, are thought to exceed 20 % to 25 % (Directorate General of Fisheries, 1987).

It is evident that consumption of salted fish may increase the level of salt intake leading to cardiovascular problems.

Administration of permitted food preservatives, such as sodium sorbate, sodium propionate, sodium benzoate and sodium sulphite, may either inhibit or stop growth of moulds and bacteria on salted dried fish. The application of preservatives during processing might reduce the necessity for a high salt concentration in extending shelf life, resulting in a healthier product.
2. LITERATURE SURVEY

2.1 Salted Dried Fish (SDF).

2.1.1 Nutritional importance, composition and production.

Fish is a major source of animal protein in tropical areas, particularly in South East Asia. Fish is rich in protein, with an amino acid composition very well suited to human dietary requirements, comparing favourably with eggs, milk and meats in the nutritional value of protein (Borgstrom, 1962). Fish also contains vitamins, minerals and is of a considerable nutritional significance as a supply of animal protein (FAO, 1981). Sachitharanthan (1977) analyzed 20 species of Srilankan salted dried fish and found that protein content ranged from 41.4 % to 52.8 %; moisture content from 20.7 to 37.1 %; crude fat from 1.01 to 5.8 %; and mineral ash from 17.6 to 28.5 %. The composition of salted dried fish samples collected in South East Asia is presented in Table 1. Salted dried fish contains 27 % to 63 % of protein.

Total production of salted dried fish in South East Asia averaged 761,000 metric tonnes (dry weight) in 1980, about 20 % of total world production. Indonesia produced 58 % of the total South East Asian production and consumed 55 % of it, leaving for export about 3 %. In 1986, Indonesia, Malaysia, Philippines and Thailand increased production to 992,700 MT (metric tonnes) (FAO, 1986).
<table>
<thead>
<tr>
<th>Types</th>
<th>Composition in %</th>
<th>Moisture</th>
<th>Protein</th>
<th>Lipid</th>
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<td>18.7</td>
<td>15.6</td>
<td>0.70</td>
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<td>13.9</td>
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<td>11.9</td>
<td>3.4</td>
<td>0.70</td>
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<td></td>
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<td>Red snapper</td>
<td>49</td>
<td>34</td>
<td>0.2</td>
<td>19.2</td>
<td>17.4</td>
<td>0.74</td>
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<tr>
<td>Thailand</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td>Queenfishes</td>
<td>60</td>
<td>27</td>
<td>3.4</td>
<td>12.3</td>
<td>6.6</td>
<td>0.92</td>
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<tr>
<td><em>Scomberoides spp</em></td>
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<tr>
<td>Anchovies</td>
<td>23</td>
<td>57</td>
<td>5.4</td>
<td>16.2</td>
<td>8.7</td>
<td>0.70</td>
<td></td>
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<tr>
<td><em>Stolephorus spp</em></td>
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<td></td>
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<td></td>
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</tr>
</tbody>
</table>

Source: Hanson and McGuire, 1982.

South East Asia is one of the World's leading areas of
production and consumption of salted dried fish. The Philippines consumed the largest amount of this product (4.4 kg/capita/year) followed by Hong Kong, Indonesia, Thailand and Singapore (Maynard, 1980).

2.1.2 Salted dried fish and Processing Techniques.

Salted dried fish is described as fish preserved by a combination of salting and drying extended by reducing water activity (Directorate General of Fisheries of Indonesia, 1986).

Fish can be salted in three main ways:

1. Kench curing (dry salting).
Granular salt is rubbed into the fish flesh, which is then stacked, while the salt penetrates the flesh and the extracted moisture is allowed to drain away. This technique is suitable for white fish such as cod, red snapper, ling and cat fish.

2. Pickling (pickle salting).
It is similar to kench curing, but the moisture is not allowed to drain away, but, in forming, covers the fish. Saturated brine may be added to completely immerse them. This is the most commonly used salting technique in Indonesia.

Fish are soaked in a concentrated, usually saturated salt solution. Total immersion in brine protect the fish against blowfly infestation during salting.
Salt uptake and water loss are influenced by the fattiness of the fish, thickness, freshness, temperature, chemical purity of the curing salt, concentration and salting time (Burgess, 1967). Brine concentration is the main factor affecting salt uptake and water loss (Souness, 1988). Fish moisture content decreases at approximately the inverse rate of salt uptake. Souness (1988) also found that salt uptake by Clupea sp was most rapid in the first 10 hrs and no benefit was observed in brining fish longer than 10-12 hrs.

Drying usually implies the removal of water vapour by evaporation, but water can also be removed from fish by pressure or by the use of absorbent pads or by addition of salt (Burgess, 1967). Simple drying in the sun is one of the commonest methods of drying used in tropical countries, where the sunshine is abundant. The water evaporated is removed by the wind, thus making drying faster. Being a cheap practice, it is suitable for most developing countries (INLO, 1982). Natural drying is relatively uncomplicated and also gives good results, provided the weather is suitable. After the fish have been split, washed and salted, they are spread on tables or mats (Van Klaveren and Legendre, 1962). Roessink (1988) introduced drying racks made from wood, bamboo, rope mesh or vinyl coated wire. He also designed a solar tent drier which resulted in greater
reduction of water rather than the former design. A maximum
drying temperature of 45°C resulted in the best quality
salted dried fish, with higher temperature leading to case
hardening. Flesh surface hardening blocks the movement of
water from deeper part of the flesh (Souness, 1988).

Processing technique employed varies within species and
origins. The following steps are commonly practiced for
curing large fish, in particular catfish (Arius thalassinus)
in Indonesia.
1. The head is removed.
2. The gut contents, including liver, intestines and
   membrane are removed, before carefully cleaning the
   fish with fresh water.
3. Fermentation in fresh water for 24 hrs.
4. Salt is placed in the gut cavities, and the fish are
   layered with salt in a tank and pickle cured in fresh water
   for 24 hrs.
5. The gutted, headed and salted fish are split and washed
   into butterfly shapes.
6. Sun drying is performed by placing them out on bamboo
   trays for 2 to 3 days, depending on the weather.
   A further split might be necessary, to accelerate drying
   process, particularly in wet weather.
2.1.3 General microbial spoilage.

2.1.3.1 Bacterial spoilage of raw fish.

Fish flesh contains nutrients supporting growth of a wide range of microorganisms. The flesh of healthy fish is generally considered to be sterile (Shewan, 1961). However, slime, gills and the intestines of living fish contain millions of bacteria and other organisms, and many of which are potential spoilers (Burgess, et al., 1967). Soon after the fish dies, spoilage begins. The spoilage bacteria enter the flesh through the gills and kidney, along veins and arteries, and directly through the skin and peritoneum.

Table : 2. Percentage of Bacterial flora of freshly caught fish.

<table>
<thead>
<tr>
<th>Genera</th>
<th>Species of fish</th>
<th>Cod</th>
<th>Herring</th>
<th>Skate</th>
<th>Lemon sole</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas</td>
<td></td>
<td>47</td>
<td>32</td>
<td>53</td>
<td>61</td>
</tr>
<tr>
<td>Achromobacter</td>
<td></td>
<td>37</td>
<td>26</td>
<td>19</td>
<td>22</td>
</tr>
<tr>
<td>Flavobacterium</td>
<td></td>
<td>4</td>
<td>17</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>Vibrio</td>
<td></td>
<td>5</td>
<td>3</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Coryneforms</td>
<td></td>
<td>5</td>
<td>10</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Micrococcus</td>
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<td>1</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td></td>
<td>1</td>
<td>9</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

Bad handling, processing practices and poor sanitation and hygiene in the processing units increases the initial population of microorganisms in raw fish. This leads to poor quality of the end product. Ironically, it can be a common practice to deliberately allow fish to spoil before processing starts, to give the required flavour to salted dried fish (Guillon, 1976 cited by FAO, 1981). *Pseudomonas*, *Achromobacter*, and *Flavobacterium* groups are encountered in marine animals (Shewan, 1961). In warmer waters, genera of mesophilic bacteria, such as *Micrococcus*, *Coryneforms* and *Bacillus* predominate (FAO, 1981). Percentage of bacterial flora of freshly caught fish is presented in Table 2. The total number of bacteria is $10^4$ to $10^6$ cells/cm$^2$ of the skin and up to $10^6$ cells/g of the intestines of freshly caught fish (Hobbs, 1982).

2.1.3.2. Microbial agents in salt.

Microorganisms have an absolute requirement for water in order to grow, although the levels needed vary, depending on the organism. The use of salt and drying are mainly aimed at reducing the water activity (Aw) of fish flesh. Hence, most microorganisms, previously dominant on fresh fish, disappear during salting and the bacterial composition of salted dried fish changes, with the relative growth of halophilic and halotolerant bacteria and xerophilic moulds. The salt itself is in fact a source of these microorganisms. Bain, Hodgkiss and Shewan (1958) reported that *Bacillus* and
Micrococcus groups formed a large percentage of the flora of solar salt. A large number of halophiles are present in solar salt commonly used for salting fish in developing countries. Red halophiles are mainly responsible for the discolouration encountered on salted fish. Owing to cheapness, availability and better "striking" qualities, as a result of more even penetration, and no hard salt crust, solar salt is still commonly used but reddening remains a source of serious losses to salted fish industries (Dussalt, 1957). Halophiles in salt can only be eliminated by heat treatment of $180^0\text{C}$ for 40 minutes (Lamprecht, Vermaak, and Topliss, 1984). No viable halophiles were recovered from the brine treated at $120^0\text{C}$ for 30 minutes, but UV treatment did not effectively sterilise salt. Recrystallisation of salt brines at low pH was reported to inhibit growth of red halophiles initially present in the salt, but did not completely destroy them (Lamprecht and Vermaak, 1984). Storing salt at low humidity for 12 to 18 months can also significantly reduce the number of the bacteria (Dussalt, 1957).

2.1.3.3 Microbial agents on salted dried fish.

Halotolerant and halophilic bacteria mainly responsible for spoilage of Indonesian salted dried fish are *Halobacterium salinarium*, *Halococcus morhuae*, *Halomonas* spp, *Staphylococcus xylosus*, Staphylococcus spp and *Planococcus*.
halophylus (Sanderson, et al. 1988). Surendran and Gopakumar (1984) reported that salted sardine and mackerel contained 80 % gram negative bacteria: Vibrio, Pseudomonas, Moraxella, Acinetobacter and Flavobacterium, and 20 % of gram positive bacteria: Micrococcus and Arthrobacter. Salted sardine was found to contain $4 \times 10^6$ to $8 \times 10^6$ cells/g with Staphylococcus aureus of $3 \times 10^3$/g (Zaidan et al. 1984). Ichinoe (1977) examined 25 samples of commercial sliced dried fish and found the TVC ranged from $2 \times 10^2$ to $9.5 \times 10^6$/g. Four of the samples contained coliforms ranging from $2.3 \times 10^3$ to $4 \times 10^4$/g.

Moulds are one of the most important spoilage agents in salted dried fish during storage. The most prevalent fungus causing a major problem on Indonesian salted dried fish is Polypaecilum pisce which appears as a conspicuous white growth over large areas of the fish surface. Other frequently isolated species are Eurotium rubrum, E.repens, Eurotium amstelodani, E.chevalieri, A.niger, A.flavus, A.sydoti, A.penicillioiides and A.wentii (Wheeler, et al. 1986). Okafor (1968) reported that Penicillium spp (71.4 %) including P.citrinum and P.notatum; Aspergillus spp (16.8 %) including A.candidus, A.wentii, A.flavus, A.meleus, and A.chevalieri; and Scopulariopsis spp are the most frequently isolated fungi from dried fish.

Aspergillus species above grow well in NaCl-based media, but Eurotium species are not generally well adapted to growth in the presence of high concentration of salt,
ie: 15 to 25% (Wheeler and Hocking, 1988). Studies on water relations of xerophilic fungi (Andrew and Pitt 1982) have shown that Basipetospora halophila, Polypaecilum pisce and Exophiala werneckii, isolated from salted fish grew more rapidly on media containing NaCl in comparison with glucose/fructose. Hence, these three moulds are considered to be halophilic. A. penicillioides could grow on media saturated with NaCl, but grew equally well on other solutes.

2.2 Characteristics and occurrence of Halobacterium salinarium and Staphylococcus sp.

2.2.1 Taxonomy and characteristics of the test bacteria.

2.2.1.1 H. salinarium

In the Bergey's Manual (Buchanan and Gibbons, 1974) and Dictionary of Microbiology (Singleton and Sainsbury, 1986), H. salinarium is classified as catalase-positive, oxidase-positive and gram-negative bacterium, motile, typically rod-shaped cells which grow aerobically or anaerobically in the light, and occurring singly with diameter of 0.6 - 1.0 by 1 - 6 μm. Glycine and cystine (GC) contents for its minor and major nutrients are 57 - 60% and 66 - 68%, respectively. Colonies on agar are small (less than 2 mm), round, convex, entire, translucent and pinkish mauve.
2.2.1.2. *Staphylococcus* sp.


"Cells are spherical with 0.5 - 1.5 µm in diameter, occurring singly or in pairs, characteristically dividing in more than one plane to form irregular clusters and non-motile. The bacteria are gram-positive and catalase-positive, chemoorganotrophic, asporogenous and non-motile. Carbohydrate may be utilized, particularly in the presence of air, with production of acid, but gas not detectable by standard procedures. Under anaerobic conditions, the main product of glucose fermentation is lactic acid, and is acetic acid under aerobic condition. GC content of DNA ranges from 30 - 40 moles %"

2.2.2 Occurrence.

Extreme halophilic bacteria, including *H. salinarium* regularly occur on sea salt and impart a red colour to the ponds used to prepare salt from sea water. Until recently, they were still evident in salterns such as those around San Francisco Bay and in certain salt lakes, e.g. the Great Salt Lake (Kushner, 1985). In Indonesia, they are regarded as one of several spoilage agents on salted dried fish in which red colour on the surface and its characteristic unpleasant odour are evident. Sanderson, et al. (1986) found that bacterial spoilers of Indonesian salted dried fish (*Rastrilliger neglectus*), apart from *H. salinarium* were, in addition to *H. salinarium*, *Halococcus mollraeae* and *Staphylococcus xylosus*. Halophiles are also found in sausage casing, hides preserved with solar salt, meat-curing brines, beef-curing brines, bacon-curing brines and salted snoek (Kushner, 1985; Gibbons, 1968; Ingram et al. 1957; Simonds and Lamprecht, 1985).
Owing to its ability to adapt to saline environments, such as salted foods, *Staphylococcus* is regarded as an halotolerant bacterium. Reed (1986) defined halotolerant bacteria as any type capable of sustained growth at a salinity which is double that of sea water, but with no absolute requirement for salt in amounts greater than 0.5 M NaCl or its equivalent. Dealing with saline stress, halotolerant bacteria may develop genetic adaptations, such as insulation and protection.

Fresh fish originally contain relatively high numbers of gram-negative bacteria, such as *Pseudomonas* which was regarded as a major spoilage agent in fish. Salted fish contain high numbers of halotolerant gram-negative bacteria. Fish soaked in used brines contain more complex types of spoilage bacteria (Liston and Shewan, 1957). Genera of *Micrococcus* predominate in slightly salted cod (Dussault, 1957), but *Staphylococcus sp* and halophiles dominate in Indonesian salted dried fish (Sanderson, et al, 1985). *Staphylococcus sp* is introduced during processing, particularly during filleting, splitting and brining (Liston and Shewan, 1957) and clearly are derived in most cases from fish plant workers (ICMSF, 1980). Hence, normal hygiene practices would greatly reduce the level of contamination. Apart from their occurrence in salted dried fish, they are often found in other types of food products (Banwart, 1980), including fresh meats, processed meats,
vacuum packaged meats, bacon, and poultry (Jay, 1978), and fresh carcases (ICMSF, 1980).

In bacon, this bacterium can rapidly grow to high numbers ($10^6$ cells/g) and may become dominant at high storage temperatures (30°C), but are outgrown by Micrococcus sp, at lower temperatures (20°C) (ICMSF, 1980). In general, Staphylococcus sp do not compete well with other organisms (Banwart, 1980) which leads to them growing mostly in salted foods where most other bacteria are inhibited.

### 2.2.3 Physiological and morphological characteristics

Amino acids are used for carbon and energy, though growth may be stimulated by carbohydrates without acid formation (Singleton and Sainsbury, 1987). Proteins and proteases are preferred for growth in complex media supplemented at least 3 M NaCl plus Mg and K ions (Buchanan and Gibbons, 1974).

There is general agreement that the NaCl requirement of *H. salinarum* is specific and can not be replaced by other salts, such as K⁺ (Larsen, 1961). The bacteria grow rapidly in the media containing 3-5 M NaCl, 0.1 - 0.5 M Mg⁺⁺, 1.3 - 2.5 x 10⁻⁵ M K⁺ and 1.2 x 10⁻⁵ M Fe⁺⁺ (Brown and Gibbons, 1966 cited by Larsen, 1967). *H. salinarum* grows best at Aw of 0.78 (Kushner, 1985). Three enzymes extracted from *H. salinarum*, e.g. succinic dehydrogenase, lactic dehydrogenase and cytochrome oxidase obtained the highest activity in the presence of 3-4 M NaCl. Activity of other 5 enzymes declined. *H. salinarum* may adapt to elevated osmotic
and ionic strength by modifying its cellular metabolism to produce structural stability and optimum physiological functioning (Reed, 1985). The intracellular salt concentration of the extreme halophiles is very high and reaches levels comparable to that in the medium in which the cells are grown (see Table 3).

Table 3. Concentration of Intracellular constituents of *H. salinarium*.

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Concentration (Molar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.37 ± 0.21</td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>4.57 ± 0.12</td>
</tr>
<tr>
<td>Cl&lt;sup&gt;-&lt;/sup&gt;</td>
<td>3.61 ± 0.07</td>
</tr>
</tbody>
</table>

Ratio

Cell Na<sup>+</sup>/Medium Na<sup>+</sup> 0.30
Cell K<sup>+</sup>/Medium K<sup>+</sup> 143

Source: Larsen, 1967

Optimum growth temperature for *H. salinarium* is in the range 40 to 45°C (Reed, 1985). This bacterium grows in media with pH ranging from 5.5 to 8.0 and optimum at 7.2 to 7.4 (Gibbons and Buchanan, 1974).

Baird Parker (1974) described *Staphylococcus* as a facultative anaerobe which grows more rapidly and abundantly under aerobic condition; its temperature optimum for growth...
is 35 to 40°C with a growth range of 6.5 to 46°C; its pH range for growth is 4.2 - 9.3 with optimum between pH 6.8 - 7.5 (Banwart, 1980). This bacterium is capable of growing in media containing 7.5 - 15% salt (Baird Parker, 1974 and Banwart, 1980). At this salt level, *H. salinarium* forms a multitude of bent, club-shaped, and very irregular structures, tending towards spherical shape (Larsen, 1961). *Staphylococcus* is sensitive to chlorine, chloramine, iodine and iodophore, but resistant to radiation (Banwart, 1980). Singleton and Sainsbury (1987) added that the bacteria are typically highly sensitive to lysostaphin.

2.3. Characteristics and Occurrence of *Aspergillus niger* and *A. penicillioides*.

2.3.1 Description of the organisms.

According to Pitt and Hocking (1985): "*A. niger* have conidiophores borne from surface hyphae, 1.0-3.0 mm long, with heavy, hyaline, smooth walls, vesicles spherical, usually 50-75 μm diameter, bearing closely packed metulae and phialides over the whole surface; metulae 10-15 μm long, or some times more; phialides 7-10 μm long; conidia spherical, 4-5 μm diameter, brown, with walls conspicuously roughed or sometimes striate. One the best known of all fungal species, *A. niger* is distinguished by its spherical black conidia, derived from colonies which show little or no other colouring."

"*A. penicillioides* has conidiophores borne from the surface or aerial hyphae, showing optimum development on 25% glycerol nitrate agar (G25N), stipes (150) 300-500 μm long, sometimes sinuous, with colourless, thin, smooth walls, enlarging gradually from the base, rather than abruptly to pyriform or spatulate vesicles, vesicles mostly 10-20 μm diameter, usually fertile over two thirds of the area, bearing phialides only; phialides (7-)8-11 μm long; conidia borne as ellipsoids, at maturity ellipsoidal, 4.0-5.0 μm diameter, with spinose walls. In common with *A. restrictus*, *A. penicillioides* grows very slowly under all standard conditions, and produce green conidia. It defers from
A. restrictus by very weak growth on CYA and MEA, by forming radiate conidial heads from spatulate vesicles, fertile over more than the upper half; and by bearing conidia as ellipsoids, which usually separate in liquid mounts."

2.3.2 Conditions for growth.

Both A. niger and A. penicillioides are regarded as xerophilic moulds, due to their abilities to grow on foods with low water activity. A xerophilic mould is defined as a mould which is capable of growing under at least one set of environmental conditions, at a water activity below 0.85 (Pitt, 1975). Mossel (1975) stated that xerophilic moulds normally grow on foods with an Aw ranging from 0.75 to 0.65, such as rolled oats containing 10% of water. A. niger conidia are capable of germinating at a minimum Aw of 0.77 (Ayest, 1968), but for growth, this mould needs a minimum Aw within the range of 0.8 to 0.84, at 37°C (Banwart, 1980). A. niger had a minimum growth temperature of 6 to 8°C, a maximum temperature of 45 to 47°C and an optimum of 35 to 37°C (Pitt and Hocking, 1985). This mould can grow well at pH 4 to 6.5 at various Aw value and is able to grow at pH 2, at high Aw (Pitt, 1981). The minimum pH for growth is 1.2 and is optimal within the range 3 to 6 (Banwart, 1980).

A. penicillioides is capable of germinating in media with a minimum Aw of 0.77 in which glucose/fructose are used to lower Aw, but the minimum Aw for growth decreased to 0.747 when salt was used instead of glucose/fructose. Compared to 8 species tested, this mould had the lowest Aw growth capability (Andrew and Pitt, 1987). In media
containing NaCl/glucose/fructose, the optimum Aw for growth was 0.91 to 0.93 and at pH of 6.5. *A. penicillioides* did not grow at 5\(^\circ\) or 37\(^\circ\). Pitt (1981) found that it grew well on Dichloran 18 % glycerol agar (DG18).

2.3.3 Occurrence.

*A. niger* and *A. penicillioides* have not only evolved the abilities to grow at low Aw, but have also diversified into variety of subtly differing species, occupying a wide range of low Aw niches (Pitt, 1975). *A. niger* as a leading species is widespread and is of importance in foods (Banwart, 1980). This mould, involved in the spoilage of bread, is normally called "the bread mould". *A. niger* with its greenish or purplish-brown to black conidial heads and yellow pigment diffusing into the bread is also referred to as black mould rot ("smut") (Frazier and Westhoff, 1978; Hawker and Linton, 1979). This mould also causes spoilage in grapes, cherries, peaches, apricots, plums, prunes, cocoa beans, chocolate and desiccated coconut (ICMSF, 1980; Robinson, 1980; Kinderlerer, 1984 and Jay, 1978). It has even been found in several spices, such as allspice, cinnamon and chilli (ICMSF, 1980). Although *A. niger* is not reported to be a major problem in salted dried fish, it is reported to be the second commonest mould isolated from samples of Indonesian salted dried fish. *A. penicillioides* was isolated from 6 % of salted dried fish samples (Wheeler et al., 1986) and is quite rarely isolated from foods, primarily because it does
not grow on the media commonly used for fungal isolation and enumeration (Pitt, 1980). It has been, however, isolated from dried fruit, dried fish, flour, pepper and even chillies (Wheeler, et al. 1986).

Growth of moulds on salted dried fish does not appear to cause objectionable flavour or textural changes, but their presence does affect the visual appearance of the fish, making it less acceptable to consumers and reducing its economic value (FAO, 1981). In addition, the metabolism of the moulds would release water, leading to a localized rise in Aw around the affected parts allowing some bacteria previously inhibited to grow.

As described above A.niger is one of the commonest moulds associated with food spoilage and loss. Ironically, the mould is of importance in food industries, because it produces a lactase enzyme capable of hydrolysing lactose in milk as product a mixture of glucose, galactose and small amount of oligosacharide. In soft drink industries, sucrose can be more cheaply converted to citric acid by the use of A.niger (Robinson, 1988). Lactase is also able to clot the milk and might be a substitute for rennet in cheese-making (Banwart, 1980). The mycelia of this mould grown on brewery wastes contain 29% crude protein and might be used as a feed supplement. Selected strains are commercially used in citric acid production, gluconic acid products and a variety of enzyme preparations (Frazier and Westoff, 1978).
A.niger produces mycotoxins, such as malformine A and C, nigragillin and anrasperone D and oxalic acid which apparently do not have the potential to harm human health. Aflatoxins are considered the most important mycotoxins (Robinson, 1988). The presence of A.niger has been reported to detoxify aflatoxin B to aflatoxin Ro (Banwart, 1980). Robinson added that aflatoxin could not be detected if the ratio A.niger to A.flavus was 19:1, but it was if the ratio was 9:1.

2.4 Food preservatives.

2.4.1 Sodium bisulphite (NaHSO₃).

2.4.1.1 Antimicrobial activity.

There is general agreement that sulphites are more active in solutions of low pH, because undissociated molecules are most effective against microorganisms (Banwart, 1980; Chichester and Tanner, 1983). Effectiveness against A.niger was found to increase 100 times when SO₂ was in the undissociated form. Most bacteria, yeasts and moulds are inhibited by or susceptible to sulphites at a concentration of 200 ppm (0.02 %). The minimum inhibitory concentrations against bacteria are presented in Table 4.

The mechanism of antimicrobial action could be explained by cysteine reacting reversibly with sulphur dioxide to form a thiol ester (Ough, 1983). Its powerful inhibitory action mainly affects enzymes with SH groups
Banwart (1980) also suggested that the presence of sulphite in solution can also inactivate enzyme systems, such as cytochrome oxidase. The cytoplasmic membrane of bacterial cells is also attacked by \( \text{SO}_2 \), leading to an alteration in permeability.

**Table 4** Inhibitory action of sulphur dioxide against microorganism.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>pH</th>
<th>Minimum inhibitory concentration in ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- <em>Pseudomonas spp</em></td>
<td>6</td>
<td>50 - 100</td>
</tr>
<tr>
<td>- <em>Staphylococcus aureus</em></td>
<td></td>
<td>80</td>
</tr>
<tr>
<td>- <em>Lactobacillus spp</em></td>
<td>6</td>
<td>55 - 100</td>
</tr>
<tr>
<td>- <em>Escherichia coli</em></td>
<td>6</td>
<td>100 - 200</td>
</tr>
<tr>
<td>- <em>Aerobacter aerogenes</em></td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>- <em>Bacillus spp</em></td>
<td>6</td>
<td>50</td>
</tr>
<tr>
<td><strong>Moulds:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- <em>Mucor spp</em></td>
<td>2.3 - 3.5</td>
<td>30 - 60</td>
</tr>
<tr>
<td>- <em>Penicillium spp</em></td>
<td>4.5</td>
<td>280</td>
</tr>
<tr>
<td>- <em>Aspergillus niger</em></td>
<td>4.5</td>
<td>220</td>
</tr>
</tbody>
</table>

2.4.1.2 Regulatory status and usage.

In the USA and UK, SO₂ and its salts are permitted in accordance with generally recognized as safe (GRAS) and the Preservatives in Food Regulations, respectively. The maximum level permitted varies according to the country and type of food. In general, foods which are directly consumed, seldom exceed 100 ppm/kg (Lueck, 1980). In the USA, it is not permitted in meats, or other foods, recognizable as a source of thiamine (Jay, 1980), but it is permitted in the UK and Australia. The use of SO₂ and its salts in fresh fish is not allowed in the USA, on the ground, that it could restore the bright colour and appearance of freshness to faded fish (Chichester and Tanner, 1983). In France, it can be used in processed shrimp and salted dried fish.

2.4.2 Sodium propionate (CH₃CH₂COONa).

2.4.2.1 Antimicrobial activity.

In general, sodium propionate is primarily used to control mould growth rather than yeasts and bacteria (Chichester and Tanner, 1983). Propionates are sometimes considered more effective against moulds than benzoates. However, Lueck (1980) reviewed that the antimicrobial action of propionates was very weak relatively compared to other food preservatives. Although, Chieply (1983) did not compare those two compounds, he confirmed that benzoates are more active against moulds than bacteria. Owing to their low activities, in practical food preservation, propionates have
to be used in high doses. Some moulds, such as *Penicillium* still grow in media containing 5% of these compounds. Gram-negative bacteria are more susceptible than gram-positive groups (Lueck, 1980). Growth of *Bacillus mesentericus* which often causes rope in bread is also inhibited (Considine, 1986), but its action is primarily bacteriostatic rather than bacteriocidal (Jay, 1980). Buchanan and Ayres (1976) reported that propionates at a level of 0.1% partially inhibited growth of *A. parasiticus* grown on AMY medium, and completely inhibited growth and aflatoxin production at a level of 0.2%. By Masimango (1978) cited by Rosul *et al.* (1987), however, found that 0.1% sodium propionate did not inhibit growth of *A. flavus* or aflatoxin production. Rosul *et al.* (1987) found that the maximum level that permitted growth of *A. parasiticus* was 0.25% after 3 days of incubation, at pH 5.5.

Propionates have a low dissociation constant, thus allowing food to be preserved at high pH (Lueck, 1980). However, their effectiveness is higher at low pH. The undissociated molecule at pH 4 is 88%, whilst it is only 6.7% at pH 6. The most powerful agent against microorganisms is the undissociated lipophilic acid (Jay, 1986). Propionates accumulate in cells and block metabolisms by inhibiting enzyme activity. They also inhibit growth by competing with other substances for the growth of
microorganisms, such as alanine and amino acids (Lueck, 1980).

2.4.2.2 Regulation and application.

Propionates are widely used in baked goods, such as bread and in dairy products, such as cheese, to prevent mould attack (Lueck, 1980). In the USA and UK its usage is permitted in accordance with GRAS and the Preservatives in Food Regulations 1979, respectively. In the UK the application in processing bread is permitted at a maximum level of 3000 ppm, relative to the weight of the flour, and only 1000 ppm for confectionery flour. In the USA, it may be used in bread production up to a level of 3200 ppm of the weight of the flour (Banwart, 1980). Miller (1944) cited by Chichester and Tanner (1983) reported that immersion of cheddar cheese cuts in 8% propionic acid solution increased the mould-free life from the usual 3 to 5 days to 12 to 18 days. Propionates can also be used in syrup, blanched apple slices, peas and beans.

2.4.3. Sodium benzoate (C₆H₅COONa).

2.4.3.1 Antimicrobial action.

Benzoates are directed mainly against yeasts and moulds and bacteria are partially inhibited (Lueck, 1980). However, Chichester and Tanner (1983) reviewed that these compounds are generally considered to be most active against yeasts and bacteria and less active against moulds.
Chieply (1986) reports that currently, these compounds are primarily used as antimycotic agents, and that most yeasts and moulds are inhibited by 0.05 - 0.1 % of the undissociated acid. Food poisoning and spore forming bacteria are inhibited in the range of 0.01 - 0.2 %. The inhibitory action of this compound is presented in Table 5. Against bacteria, the minimum concentrations needed generally range from 50 to 500 ppm, against moulds they were 300 - 500 ppm and against yeasts were 50 - 500 ppm (Jay, 1986).

Like the food preservatives discussed previously, benzoates are far more active against microorganisms at low pH. Hence, its usages are limited to those foods which are acid in nature (Cheiply, 1983). The pKa of benzoates is 4.2 and at pH 4, the undissociated molecule is 60 %, whilst it is only 1.5 % at pH 6 (Jay, 1986). Rahn and Conn (1944) cited by Chieply (1986) reported that the antimicrobial action of these compound was 100 times as efficient in strongly acid solution than in neutral solution. Marcis (1975) detailed that the undissociated form was the only one taken up by microorganisms and ionized in the cell to produce protons that acidified the alkaline interior of cell (Cheiply, 1986). Another hypothesis is that these compounds interfere with the permeability of the microbial cell membrane and/or inhibit membrane transport of amino acids, leading to nutritional starvation of cells (Freeze, 1973). Benzoates have been shown to inhibit a specific enzyme system, such as lipase in Pseudomonas, and acetic acid metabolism and

Table: 5 Minimum inhibitory action of benzoic acid.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>pH</th>
<th>Minimum level (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Moulds</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhizopus nigrificans</em></td>
<td>5</td>
<td>30 - 120</td>
</tr>
<tr>
<td><em>Mucor racemosus</em></td>
<td>5</td>
<td>30 - 120</td>
</tr>
<tr>
<td><em>Penicillium spp</em></td>
<td>2.6 - 5</td>
<td>30 - 280</td>
</tr>
<tr>
<td><em>Aspergillus spp</em></td>
<td>3.0 - 5.0</td>
<td>20 - 300</td>
</tr>
<tr>
<td><em>Cladosporium herbarum</em></td>
<td>5.1</td>
<td>100</td>
</tr>
<tr>
<td>2. Bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas spp</em></td>
<td>6</td>
<td>200 - 480</td>
</tr>
<tr>
<td><em>Micrococcus spp</em></td>
<td>5.5 - 5.6</td>
<td>50 - 100</td>
</tr>
<tr>
<td><em>Streptococcus</em></td>
<td>5.3 - 5.6</td>
<td>200 - 400</td>
</tr>
<tr>
<td><em>Lactobacillus</em></td>
<td>4.3 - 6.0</td>
<td>300 - 1800</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>5.2 - 5.6</td>
<td>50 - 120</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>6.3</td>
<td>500</td>
</tr>
</tbody>
</table>

2.4.3.3 Regulation and application.

In the USA, benzoates are regarded as safe food preservatives (GRAS, 1977, title 21, section 184.1021 and 184.1733). The maximum permitted level is 1000 ppm (0.1 %). In the UK, benzoates are permitted on a wide scale in accordance with the Preservatives in Food Regulations of 1979. In most countries, the maximum permissible levels range from 1500 ppm to 2500 ppm, depending upon the type of food (Chieply, 1986; and Lueck, 1980). In some countries, for egg yolk, the maximum permitted level is 1.25 % (Chichester and Tanner, 1983).

Benzoates may be applied to a wide range of foods including fat products (cheese), fish products, vegetable products, and drinks, in particular, fruit juices and beverages. Benzoates are added to marinades to protect them from fungal spolage and its action is slightly superior to sorbic acid (Lueck, 1980). Margarine is usually treated at level of 0.08 - 0.15 %. Its level of use in fruit juices and ranges from 0.05 - 0.2 %, depending on the type of juices and shelf life required (Lueck, 1980; and Chichester and Tanner, 1983). The use in fruit juices, at the maximum level of 0.1 % may impart disagreeable tastes which are described as being "peppery" or burning (Jay, 1986). Use at a level of 0.05 % helps preserve carbonated beer. Its activities are more pronounced against aerobic bacteria than anaerobic types (Banwart, 1980).
2.4.4 Sodium sorbate (CH$_3$=CH=COONa).

2.4.4.1 Antimicrobial activity.

Most investigators are in a general agreement that sorbic acid and its salts are more effective against moulds and yeasts, while its activity against bacteria is not comprehensive and appears to be selective (Sofos and Busta, 1986; Lueck, 1980; Banwart, 1980; Chichester and Tanner, 1983 and Anonymous, 1980). A great deal of research focusing on its antimicrobial activity has been performed for past 20 years, especially in combination with reduced nitrite levels, in relation to toxin-producing moulds, and to health (Lanari and Zaritzky, 1988; Robach and Hicky, 1978; Flores et al, 1988; Verelttizis and Buck, 1984; Kareer, et al, 1987 and Taylor, 1984). The most comprehensive reviews of its antimicrobial action are those of Sofos and Busta (1981 and 1986), Lueck (1980) and Liewen and Marth (1984).

The main characteristics of its antimicrobial actions are given below:

1. Several food spoilage bacteria, food poisoning groups, including \( \text{"Parahaemolyticus"} \) (Robach and Hicky, 1978), \( \text{Proteus morganii} \) and \( \text{Klebsiella pneumonaeiae} \) (Taylor and Speckhard, 1984); spoilage fungi and toxin-producing fungi are inhibited.

The minimum inhibitory actions against a number of moulds and bacteria are presented in Table 6.

2. Effective microbial inhibitory concentrations of sorbates in most foods are in the range of 0.05 - 0.30 %.
3. Catalase-positive organisms are less resistant to sorbates than the catalase-negative types. Hence, sorbates could be used as selective agents for culturing catalase-negative bacteria and clostridia.

4. Its activities are more pronounced against aerobic bacteria than anaerobic types.

Table: 6. Inhibitory action of sorbic acid.

<table>
<thead>
<tr>
<th>Organisms</th>
<th>pH</th>
<th>Minimum inhibitory concentration in ppm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudomonas sp</td>
<td>6</td>
<td>100</td>
</tr>
<tr>
<td>Micrococcus sp</td>
<td>5.5 - 6.4</td>
<td>50 - 150</td>
</tr>
<tr>
<td>Lactobacillus sp</td>
<td>4.3 - 6.0</td>
<td>200 - 700</td>
</tr>
<tr>
<td>Acromobacter sp</td>
<td>4.3 - 6.4</td>
<td>10 - 100</td>
</tr>
<tr>
<td>Bacillus sp</td>
<td>5.5 - 6.3</td>
<td>50 - 100</td>
</tr>
<tr>
<td>Moulds:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhizopus sp</td>
<td>3.6</td>
<td>120</td>
</tr>
<tr>
<td>Mucor sp</td>
<td>3.0</td>
<td>10 - 100</td>
</tr>
<tr>
<td>Penicillium sp</td>
<td>3.5 - 5.7</td>
<td>20 - 100</td>
</tr>
<tr>
<td>Aspergillus sp</td>
<td>3.3 - 5.7</td>
<td>20 - 100</td>
</tr>
<tr>
<td>Fusarium sp</td>
<td>3.0</td>
<td>100</td>
</tr>
</tbody>
</table>

Source: Lueck (1980) and Sofos (1986)
5. Some bacteria, such as two strains of *Pediococcus cerevisiae* are tolerant to sorbic acid.

6. In general, the antimicrobial actions of sorbates are pH dependent and increase as the pH of substrate decreases. However, they can be used for preserving foods with high pH due to the low dissociation constant (Ka) \(1.73 \times 10^{-5}\). Sofos and Busta (1986) and Liewen and Marth (1984) independently reviewed the mechanism of sorbates against microorganisms. Their reports can be summarized as follows:

1. Sorbates inhibit some sulfhydryl-containing enzymes, such as fumarase, catalase, succinic acid dehydrogenase, enolase, and co-enzyme A.

2. Oxidative phosphorylation and substrate transport are uncoupled from the electron transport system by sorbates.

3. Unsaturated fatty acids undergo oxidation through a free-radical mechanism which prevents growth.

4. The presence of these compounds can also inhibit aflatoxin biosynthesis.

2.4.4.2 Regulation and application. Sorbic acid and sorbates are permitted in most countries for preserving foods. Owing to more favourable action and physiological harmlessness, they are widely used to replace other food preservatives which may be more
undesirable. In the USA, sorbic acid and sorbates are considered in GRAS (Code of Federal Regulations, Title 21 and 182.3089, 182.3225, 182.340 and 102.3795). In UK, they are permitted for a number of foods in accordance with the preservatives in Food Regulations of 1979. The maximum permissible quantity is between 0.1 - 0.2 % (Lueck, 1980). They are used in fatty products, including margarine (0.05 - 0.10 %), dairy products, such as cheese (0.05 - 0.07 %), meat products, such as sausages which can be treated by dipping in 10 - 20 % solutions, fish products, such as fish sausages and oriental preparations and others (Chichester and Tanner, 1983; Sofos and Busta, 1986 and Lueck, 1980).

2.5 The aims.
The aims of the work detailed in this thesis were to:

(i) evaluate the effectiveness of food preservative in
- preventing reddening in salted dried fish,
- inhibiting growth of A.niger and A.penicillioides,
- inhibiting growth of H.salinarium and Staphylococcus sp isolated from salted dried fish,
and

(ii) to determine possible synergistic effects between sodium sorbate and sodium benzoate using gradient diffusion systems.
3. MATERIALS AND METHODS.

3.1 Materials.

3.1.1 Test organisms.

3.1.1.1 Moulds.

Two species of mould were employed, *Aspergillus niger* (culture number: IMI 17454) and *A. penicilloides* (culture number: IMI 71358), which were obtained from CMI London. The former mould was maintained on Czapek Yeast Agar (CYA) slopes and 10 % salt-CYA slopes, while for the latter either 10 % salt-CYA or 10 % salt-MEA (malt extract agar) was used. They were recultured at 6 day intervals with incubation at 30\(^0\) C.

3.1.1.2 *Halobacterium salinarium* (Culture number NCIMB 768)

This was obtained from the National Collection of Industrial Marine Bacteria (NCIMB), Aberdeen. Halophilic agar slopes were used to maintain this bacterium which was recultured at 6 day intervals with incubation at 37\(^0\) C. Owing to its obligate aerobic nature, tops of universal bottles were left unscrewed slightly. The slopes were then put in a plastic bag, to avoid dehydration during incubation.
3.1.1.3 Halotolerant bacteria.

Bacteria from Indonesian salted dried fish (Jambal Roti) were isolated. One of them, which was thought to be halotolerant was sent to NCIMB for identification where it was identified as Staphylococcus sp. This bacterium was maintained on 12.5 % - 15 % salt- Nutrient agar (NA) slopes and subcultured at 7 day intervals. It was incubated at 30°C.

3.1.2 Media used.

Media used for cultivating moulds included the following:

(i) Czapek Yeast Medium (CYM) (formulated by Pitt and King, 1985).

(ii) Defined medium.

(iii) Malt Extract medium (MEM).

This medium was basically formulated by Oxoid which was supplemented with 5 % sucrose.

(iv) 10 % salt-Czapek Yeast Medium and 10 % - salt Malt Extract Medium.

The basic media were supplemented with 100 g salt/l solution (w/v).

(v) Complex Halophilic medium (CHM).

This medium was designed by Sehgal's Gibbons (1960) and Eimhjellen (1965).

The formulations of the above media are presented in Appendix 1.
15% salt-Nutrient Agar.

Nutrient agar, formulated by Oxoid, was supplemented with 100 g of NaCl/litre medium (w/v). It was dissolved in water and filtered through Whatman no.1 paper before being used.

Salted Dried Fish Powder (SDFP).

Fresh cod (Gadus morhua) was beheaded, eviscerated, soaked in fresh water for 12 hours and washed with tap water. Clean, headless-eviscerated cod was then split into butterfly shapes. These were soaked in 10 to 25% salt solution, depending on the type of salted fish required, for 12 hrs at room temperature (16°C to 22°C). After being washed with tap water, the fish was drained and dried by a mechanical drier at 50°C for 16 to 18 hrs.

The flesh was then separated from the bones and scales and ground until a fine powder was produced. The powder was then filtered through a wire strainer (diameter = 1 mm), sterilized for 15 minutes at 121°C and stored in the 5°C chill room.

3.1.3 Food preservatives.

The four food preservatives evaluated were sodium benzoate, sodium sorbate, sodium propionate and sodium bisulphite. Sodium benzoate, sodium sorbate and sodium propionate were prepared in 10% stock solution (w/v), whilst sodium bisulphite stock solution was prepared in 1%
and 10%. All were sterilized by filtering them through Millipore bacterial filters.

3.1.4 Fish.

Cod (Gadus morhua) was used to make salted dried fish homogenate (SDFH) as well as salted dried fish (SDF). It was collected from the fish dock market, Grimsby, washed, put into trays and stored at -18°C until ready for use.

3.2 Methods.

3.2.1 Experimental Design and Analysis of Data.

Experimental procedures were based on a complete random design (CRD) for total dry weight assessments and split plot design (SPD) for radial growth, and bacterial growth assessments. A factor of the CRD was food preservative applied at various concentrations. The levels of the food preservative used were based on permitted levels for most countries. Two factors of the SPD were food preservative and storage time which divided into interval times of 6, 12 or 24 hours depending on microorganism used. Each treatment (concentration) was replicated either 3 or 4 times. Data were analyzed by analysis of variance (Anova) and the Least Significant Difference (LSD) methods and represented in both graphs and tables.
3.2.2 Radial growth.

Each food preservative was used at 4 concentrations (treatments) and one control (no treatment). The treatments employed are given below:

(i) Sodium benzoate and sodium sorbate: 1) 0.05%, 2) 0.1%, 3) 0.15% and 4) 0.20%.

(ii) Sodium propionate: 1) 0.05%, 2) 0.10%, 3) 0.20% and 4) 0.30%.

(iii) Sodium bisulphite: 1) 0.01%, 2) 0.02%, 3) 0.03% and 4) 0.04%.

3.2.2.1 Preparation of food preservative treated Czapek Yeast Agar (CYA)

Nine grams of yeast extract, 9 cm$^3$ of Czapek concentrate, as described in Appendix 1.1, 27 g of glucose, 0.9 g of KH$_2$PO$_4$ and distilled water to 900 cm$^3$ were mixed thoroughly, brought to boiling and transferred to flasks corresponding to designed treatments. All flasks containing agar were autoclaved for 15 minutes at 121$^\circ$C. When the agar flasks were removed from the autoclave, filter-sterilized food preservative solution was immediately added to give the required concentration. Each flask was shaken gently to homogenize the solution and the contents poured into 4 petri dishes, each one containing 12.5 cm$^3$. In total there were 68 petri dishes.
3.2.2.2 Production of spore suspension and inoculation.

*A. niger* and *A. penicilloides* were grown on CYA and 5% salt-MEA slopes respectively, at $30^0\text{C}$ for 4 days. 0.4 cm$^3$ of 0.01% melted agar and 0.05% detergent, such as polysorbitan 80 (tween 80) was dispersed into a 5 cm$^3$-vial. A needle point of spores of 4 days old culture was stabbed and gently mixed into the above mixture.

![Figure: 1. 40-10 Image Analyser.](image)

Petri dishes containing agar were dried in a Baird and Tatlock "Unitemp" drying cabinet at $25^0\text{C}$, to prevent moisture on dish lids from causing disturbances in measuring radial growth and drops of water on agar surfaces causing uneven colony growth. The dishes were inoculated by stabbing a needle dipped in spore suspension into the centre and incubated at $25^0\text{C}$. Growth was observed and assessed using
Preparing of treatments

Control  Na-benzoate or Na-sorbate  Na-propionate  Na-bisulphite (%)

0.05  1.0  1.5  2.0  0.05  1.0  2.0  3.0  0.01  0.02  0.03  0.04

Dispersed into

Sterilization
at 1210°C for 15 minutes

Adding food preservatives

10% stock solutions of
controls  Na-sorbate/Na-benzoate  Na-propionate

0.25  0.5  0.75  1.0 cm³  0.25  0.5  1.0  1.5

of Na-bisulphite

0.5  1.0  1.5  2.0

shaken gently
Inoculation
Pouring agar into plates:
12.5 cm³ each

Drying agar plates
in the air flow cabinet

Inoculation
a needle loop stabbed
into the centre of plates

Spore suspension
in a bottle

Incubation
at 25°C

Measuring colony diameter
at 1 day intervals.

Figure 2. Radial Growth Observation
40-10 Image analyser (see Figure 1) at 24 hour intervals for 14 to 21 days depending on the mould observed and medium used. Observation was stopped when the moulds completely covered the dishes (see Figure 2).

3.2.3 Radial growth assessment on solid media containing salt and preservatives.

This experiment attempted to investigate the effect of food preservatives in conjunction with salt on growth of either xerophilic or halophilic moulds, such as *A. niger* and *A. penicillioides*. Media used were 5 - 10 % salt-CYM and 5-10 % salt-MEM. Radial growth of those moulds was measured using a 40-10 Image Analyser (Analytical Measuring System Ltd) with TV camera (see Figure 1).

The work was done at 24 hour intervals for 14 to 21 days. Techniques used were similar to those for growth assessment in CYA (Section 3.2.2), but 10 % salt-CYA was used instead.

3.2.4. Determination of total dry weight.

3.2.4.1 Preparation of spore suspension.

A 5 day mould culture slope was harvested by adding sterile glass beads and 5-7 cm$^3$ of sterile distilled water to it. The slope was shaken gently to release spores, which were then aseptically filtered with a 7.5 cm-Whatman paper no.1, in order to separate mycelia. Filtration was expedited by means of a buchner flask, connected to a vacuum pump. Collected spores were counted using a haemocytometer in conjunction with a microscope. The formula used for counting
the concentration of the original spore suspension was as follow:

the average number of spores per cm$^2$  
---------------------------------------- $\times 10^6$ spores/cm$^3$  

A further dilution was sometimes necessary to give a required final concentration of approximately $10^4$ spores/cm$^3$. A formula used to find out the final concentration was as follows

\[
1 \text{ cm}^3 \text{ of mother suspension} \\
---------------------------------------- x \ A = B \\
1 \text{ cm}^3 \text{ of mother suspension} + \ C
\]

where $A$ = number of the known spore concentration of mother suspension; $B$ = number of the final spore concentration required; and $C$ = volume of sterile of distilled water required.

3.2.4.2 Preparation of liquid medium.

Yeast extract (3.5 g), 0.7 of KH$_2$PO$_4$, 7 cm$^3$ of Czapek concentrate, 21 g of sucrose and distilled water to 700 cm$^3$ were mixed and stirred thoroughly. pH was adjusted to 6 by addition of 20% KOH. The broth was then transferred to thirteen 250 cm$^3$-Erlenmeyer flasks corresponding to designed treatments (Figure 3). The flasks were then autoclaved at $121^0$ C for 15 minutes.
3.2.4.3 Inoculation and incubation.

All flasks, except for the control, were supplemented with appropriate volumes of sterile food preservative stock solutions. Finally, all flasks, except the blank, were inoculated with 1 cm³ of $5 \times 10^4$ spore suspension/cm³ to give a final concentration of $10^4$ spores/cm³ of broth, before being incubated in a shaking waterbath at $25^0\text{C}$ for 6 days.

3.2.4.4 Harvesting mould.

A 12.5 cm-Whatman no : 546 paper was folded at the margin to give a disk shape having diameter about 9 cm at the bottom, before being dried at $55^0\text{C}$ for 24 hours. By folding in this manner, the filter paper fitted into a buchner funnel which allowed easy removal of the filter paper and mycelia after filtration. The paper was weighed for the purpose of final calculation of dry weight. Vacuum from an aspirator was applied via a flask. The contents of a 250 cm³-flask (mycelia and liquid medium) were filtered directly into a 250 cm³-separator flask. 10 cm³ of distilled water was used to wash the flask and remove the remaining mycelia. The filter paper containing mycelia was dried at $55^0\text{C}$ for 48 hours. It was cooled in a dessicator and finally weighed. A flask containing broth, as a blank, underwent similar treatment to the flask containing mycelia. The final dry weight was that of the filter paper.
Preparation of treatments

Control

Na-sorbate (%) or Na-benzoate (%)  
0.10  0.15  0.20

Na-propionate (%)  
0.10  0.20  0.30

Na-bisulphite (%)  
0.01  0.02  0.03

48.5  48.0  47.5 cm³

Sterilization
at 121°C for 15 minutes

Cooling

Adding food preservatives

10% Stock solution of
Na-sorbate or Na-benzoate
0.5  0.75  1.0

1% stock solution of
Na-propionate
0.5  1.0  1.5

1.0 cm³ each

Na-bisulphite
0.5  1.0  1.5

1.0 cm³ each

Inoculation
spore suspension
5 x 10⁶ spores/cm³

Inoculation
spore suspension
5 x 10⁶ spores/cm³
Figure 3. Dry Weight Assessment.

Incubation in shaking water bath, at 25° C for days.

Drying Whatman filter paper at 55° C for 48 hrs

Harvesting mycelia Vacuum pump; buchner funnel

Drying mycelia at 55° C for 2 days

Weighing
containing dried mycelia minus the filter paper containing dried broth (as a blank).

All procedures above were applied to the determination of mycelial dry weight using 10% salt-CYB/MEB, except that the incubation period was 6 days, due to the slower growth of moulds in media containing salt (NaCl) (see Figure 3).

3.2.5 Growth of *Halobacterium salinarium* in CHB containing food preservatives.

This experiment applied a split-plot design (SPD) where each of the 4 food preservative treatments was carried out separately. Experiments for each food preservative treatment took approximately 8 days and three concentrations of each food preservative were tested on this bacterium. The concentrations used for both sodium sorbate and sodium benzoate were 0.10%, 0.15% and 0.2%. Three concentrations, 0.1%, 0.2% and 0.3% were also used for sodium propionate. The strong smell and permitted level of sodium bisulphite dictated that, the highest concentration used was 0.03% and others were 0.02 and 0.01%.

3.2.5.1 Preparation of Complex Halophilic Broth (CHB).

For experiments involving either sodium sorbate or sodium benzoate treatment, 650 cm³ of CHB adjusted to pH 7.4, were transferred to 4 groups of flasks, each group
containing 3 flasks. These represented the control, 0.10 %, 0.15 % and 0.20 % treatments, respectively. 49 cm³, 48.5 cm³, 48.25 cm³ and 48 cm³ of broth were added to flasks of groups I, II, III and IV, respectively. The flasks were autoclaved at 121°C for 15 minutes. 0.5, 0.75 and 1 cm³ of 10 % filter sterilized sodium sorbate or sodium benzoate stock solution were added to groups II, III, and IV, respectively.

To give final concentrations of 0.1 %, 0.2 % and 0.3 % of sodium propionate, 0.5 cm³, 1.0 cm³ and 1.5 cm³ of 10 % filter sterilized sodium propionate stock solution were added to 48.5 cm³, 48 cm³ and 47.5 cm³, respectively. On the experiment for sodium bisulphite, 3 concentrations were also used which were 0.01 %, 0.02 % and 0.03 %. For those treatments, 48 cm³, 47.0 cm³ and 46 cm³ of the broth was supplemented with 1.0, 2.0, and 3.0 cm³ of 1 % filter sterilized sodium bisulphite stock solution.

3.2.5.2 Inoculation, standardisation of inoculum and incubation.

A 4 day culture was transferred to a bottle containing 10 cm³ halophilic broth which was gently shaken to achieve a homogeneous suspension. The population of this bacterial suspension was determined by either a colorimeter (PCO 1 and 2) with wave length of 540 nm, which gave the highest absorbance, or by direct epifluorescent filtration technique (DEFT). This technique is a rapid method which has previously...
used to count bacterial loading of milk. This method is detailed as follows:

Approximately $10 \, \text{cm}^3$ of halophilic broth was inoculated with a loop of 4 day old *H. salinarium*, which was stirred using a Whirlimixer to give a homogeneous suspension. A nuclepore membrane filter (0.6 um pore size; 25 mm diameter) was then placed on the base of filter tower. The suspension was put in the filter tower and filtered by means of a vacuum pump with an aspirator. Subsequently, $25 \, \text{cm}^3$ Acridine orange was put in the filter tower, left for 2 minutes and passed through the filter containing bacteria and then rinsed by filtering $2.5 \, \text{cm}^3$ of pH 3.0 buffer, followed by $2.5 \, \text{cm}^3$ of isopropanol through the membrane. It was removed from the filter tower and thoroughly air dried by holding in a gentle current of air. A small drop of non-fluorescent immersion oil was placed on a microscope slide and the membrane filter laid on it. Another small drop of immersion oil was dropped on top of the membrane filter and covered with a coverslip. The DEFT slide was finally examined under the epifluorescent microscope in conjunction with a 40-10 Image Analyser and TV camera (Figure 1) to give the numbers of *H. salinarium/cm*^3^.

The bacterial suspension was diluted to give a required initial population. The 12 flasks, containing food preservative treatments, were inoculated with $1 \, \text{cm}^3$ of inoculum to give final population of $10^3 \, \text{cells/cm}^3$ of broth.
They were all incubated at 37° C in a shaker incubator with speed of 110 RPM.

Enumeration was carried out by spread plate technique and also a colorimeter (PCO 1 and 2), using a wavelength of 540 nm. A 6 point standard curve was produced for both absorbances and bacterial numbers. The procedures were detailed as follows:

1. A mother culture was prepared by inoculating a 250 cm³ flask containing 50 cm³ CHB with a loop of 4 day old inoculum.

2. The flask containing inoculated broth was then incubated in the shaker incubator at 37° C for 5 days in which *H. salinarium* achieved optimum growth and produced a reddish pink colour.

3. Two cm³ of mother culture were transferred to a bottle containing 8 cm³ of broth and shaken gently. This corresponded to the second (1 : 5) dilution. In the same manner, dilutions were performed until six dilutions were obtained (1 : 3125). After absorbances had been determined, the six bacterial suspensions were enumerated by spread plate technique (see Figure 4). The absorbances and the bacterial numbers of the six points (dilutions) were used to obtain a standard curve of *H. salinarium*.

After inoculation with a standardized inoculum and incubation at 37° C, samples were withdrawn at 24 hour intervals and analysis stopped when growth of this bacterium
1. Preparation of dilution series.

2. Inoculation: 0.1 cm$^3$ each

3. Spreading the inoculum using silicon glass spreader

4. Incubation at 30° - 37° C for 2-5 days

5. Counting the colonies: 30 - 300 colonies.

6. Numbers: _______________ cells/cm$^3$

Figure: 4 Spread Plate Technique.
in all treatments had achieved the optimum growth (7 to 8 days).

3.2.6 Growth of *H. salinarium* on Salted Dried Fish Homogenate (SDFH)

To prepare SDFH, 100 g of salted dried fish powder (formulation of which is described in Section 3.1.2.7) and 22.5 % salt-water to 1,000 cm$^3$ were added into a 1 l beaker. The remaining procedures were the same to those described in Section 3.2.4. SDFH was used as growth medium instead of CHM.

3.2.7 Growth of *Staphylococcus* sp.

This bacterium was isolated from Indonesian salted dried fish and found to be tolerant to 17.5 % salt (w/v). The effect of preservatives on its growth was determined by a MR 600 Microplate Reader (Dynatech Laboratories, Inc; Figure 5). The analysis was carried out by measuring the absorbances of samples prepared as described below.

3.2.7.1 Preparation of treatments.

The analysis was carried out by measuring absorbances of sample solutions. This machine was completed with a plate that consisted of 8 rows containing 12 wells each. Row A was regarded as blanks, row B was the control and rows C to H were required treatments. Each row contains 12 wells representing replicates (see Figure 6). 20 g of salt were mixed with distilled water and made up to 200 cm$^3$, which
corresponded to 10 % brine. This concentration was chosen, since most Indonesian salted dried fish contains 5 to 20 % salt. Moreover, the presence of a higher salt concentration might reduce the accuracy of analysis, as far as absorbances were concerned. 6.8 g of nutrient broth powder (Oxoid) was mixed with 10 % brine and made up to 200 cm³ and stirred gently. The pH was adjusted at 6 using 20 % NaOH. The broth was dispensed to 26 bottles, each containing 9 cm³; 24 bottles containing broth were supplemented with food preservatives and the remaining other 2 bottles were used as a blank and the control.

Figure 5. MR 600 Microplate Reader.
The treatments were designed as follows:

1. Sodium sorbate, sodium propionate and sodium benzoate consisted of 6 concentrations which were 0.05 %, 0.1 %, 0.15 %, 0.2 %, 0.25 % and 0.3 %.

2. Sodium bisulphite was used at 6 concentrations: 0.01 %, 0.02 %, 0.03 %, 0.04 %, 0.05 % and 0.06 %. To give the final concentrations shown above, the bottles containing 9 cm³'s were added with appropriate volumes of 10 % stock solutions of sodium sorbate, sodium benzoate, sodium propionate or sodium bisulphite.

3.7.2 Inoculation, incubation and enumeration.

All bottles containing food preservative treated broth and control were inoculated with an 18 hour *Staphylococcus* sp culture to give an initial population of $10^3$ cells/cm³ of broth. They were stirred using a Whirlimixer for a few seconds. Four microtitre plates, each containing 96 wells, were prepared for the food preservatives. Rows A, B, C, D, E, F, G, and H of the microtitre plates were used as blanks, the control, 0.05 %, 0.1 %, 0.15 %, 0.2 %, 0.25 % and 0.3 %.
Stage: 1

Preparation of treatments

Group I (Na-benzoate, Na-sorbate, Na-propionate)

Blank control

0.05 0.10 0.15 0.20 0.25 0.30 %

10 % Stock solution of Food preservatives

0.01 cm3 each

18 hr inoculum 105 cells Inoculation

Dispensing broth into the wells each 0.04 cm3

Group 1 (3 microplates)

A: Blank
B: Control
C: 0.05 %
D: 0.10 %
E: 0.15 %
F: 0.20 %
G: 0.25 %
H: 0.30 %
Stage: 1

Preparation of treatments

Group I (Na-bisulphite)

blank control

0.01 0.02 0.03 0.04 0.05 0.06 (%)

10 % Stock solution of Sodium bisulphite

0.01 cm³ each

18 hr inoculum
10⁵ cells

Inoculation

Dispensing broth into the wells each 0.04 cm³

Group II (1 microplate)

A: Blank
B: Control
C: 0.01 %
D: 0.02 %
E: 0.03 %
F: 0.04 %
G: 0.05 %
H: 0.06 %

55
Incubation at 30°C

Withdrawal at 6 hr intervals

Determination of Absorbances using a Microplate reader at 640 nm

Figure 7. Growth Assessment on Staphylococcus sp.
concentrations respectively, of the three food preservatives. Similarly rows A to H were used as blanks, the control, 0.01 % to 0.06 %, respectively of sodium bisulphite. Each well was filled with 0.4 cm$^3$ broth, corresponding to designed treatments. All wells of rows B to H were inoculated with an 18 hour culture to give a final population of $10^3$ cells/cm$^3$ of broth. The 4 microtitre plates were then incubated at 30$^0$C. Samples were withdrawn at 6 hour intervals and their absorbances were determined using MR 600-Microplate Reader (Figure 5), at 620 nm of wavelength. The enumeration was stopped when growth of cultures were in death phase, which occurred after approximately 5 days (see Figure 7). To determine the numbers of the bacteria, a standard curve of Staphylococcus sp was produced. This was basically done as the previous work on a standard curve of H.salienrium.

3.2.8 Gradient Diffusion Systems.

3.2.8.1 Preparing plate agar.

Each square (10 cm x 10 cm$^2$) petri dish contained 60 cm$^3$ of agar. 60 cm$^3$ of the defined agar (section 3.1.2.2) was added to as many bottles as required. These were autoclaved for 15 minutes at 121$^0$C, cooled to approximately 45$^0$C and poured into petri dishes. Agar plates were then dried in a warm air-flow cabinet for several minutes to remove excessive water from both the
surface of the agar and plate lids. Either one or two well(s) (each), having an approximate volume of 14 (7 x 2 x 1) cm$^3$ as necessary, were made on the edges of the plates at right angles to one another (Figure 8). For the two dimensional gradient diffusion system, well 1 was filled with 4 cm$^3$ one food preservative solution and well 2 was filled with the same volume of another food preservative solution. The concentrations used for the sodium benzoate and sodium sorbate treatments ranged from 0.5 % to 5 %.

![Figure 8. Gradient diffusion plate.](image-url)

This experiment was intended mainly to investigate whether any synergistic effect between two food preservatives existed as assessed. Each treatment
also needed a control in which two wells were filled with the same food preservative.

3.2.8.2 Diffusion, inoculation and observation.

All agar plates were incubated at $25^0 \text{C}$ for 48 hours to allow diffusion. A steel grid was inserted into the agar plate to stop any further diffusion which divided the agar plate into 49 small squares each having an approximate area of $81 \text{mm}^2$. Before use, the steel grids were washed with alcohol, flamed and cooled.

A flamed needle was dipped into a spore suspension prepared as described in Section 3.2.3.1, stirred thoroughly and stabbed into the centre of each small square of agar. One loading of spores could be used for two squares. All the plates were then incubated at $25^0 \text{C}$. Radial growth of the mould was observed at 24 hour intervals for 6 days using a 40-10 Image Analyzer, in conjunction with video-camera and TV screen (Figure 1). Colonies were measured horizontally (i.e. E-W) and vertically (i.e. N-S) to give average diameters.

3.2.9 Growth of \textit{H. salinarium} on Salted Dried Fish (SDF)

Concentrations of the sodium sorbate and sodium benzoate evaluated were 1 %, 2 % and 3 % and the sodium bisulphite was tested at concentrations of 0.2 %, 0.4 % and 0.6 %.
Salted dried fish containing 20% to 25% salt was cut into small pieces of 1 - 2 cm dimension, to fit the petri dishes. To prepare each food preservative treatment, 2 litres of the 3 food preservative solutions, as treatments, and 2 litres of sterile water, as a control, were placed in 4 l beakers. Another 4 4l-beakers containing, 300 g SDF were autoclaved at 100°C for 30 minutes. The 300 g SDF samples were each dipped in the appropriate food preservative solutions for 5 minutes and the other 300 g was dipped into water for 5 minutes. They were all weighed before and after dipping to calculate the amount of food preservative solutions absorbed. In this way, the concentrations of food preservative on SDF could be roughly determined. They were redried for 15 minutes at 50°C, to remove excessive water in the flesh. 10 g of SDF were placed in petri-dishes. Each treatment included 30 petri-dishes. In total, there were 270 petri-dishes, representing 3 treatments (9 concentrations) and 30 petri-dishes, representing the control. Each 10 g of SDF was inoculated with 1 cm³ of \(10^4\) cells, i.e. the initial population of \(H.\) salinarium was \(10^3\) cells/g of SDF. They were incubated in a relative humidity cabinet at 37°C and 75% to 80% RH which is optimal for growth of this bacterium. Samples were withdrawn at 2 day intervals and enumerated by the spread plate technique (Figure 4). Serial dilutions were made in 22.5% salt solution.
3.2.10 Analysis of salt content and water activity.

Since *H. salinarium* will not grow on media, in particular SDF, containing less than 18 % salt, analysis of salt in the SDF was necessary. The analysis was performed immediately after the SDF had been produced. The analysis was as follows:

1. Two g of SDF were accurately weighed and macerated using an homogenizer in distilled water for 2 minutes.
2. The extract was transferred quantitatively into a 250 cm$^3$ volumetric flask and made up to volume.
3. 25 cm$^3$ aliquots of this were titrated against 0.1 N AgNO$_3$ using 4 drops of potassium chromate indicator.
4. The end point was a colour change from yellow to red.
5. Analysis was performed in triplicate.

\[
\text{titration volume} \times 5.8
\]

6. \% NaCl = \frac{\text{titration volume} \times 5.8}{\text{samples weight}} \quad (FAO, 1981)

Analysis of water activity was carried out using Novasina Aw meter (Humitec, Ltd.).
4. RESULTS AND DISCUSSION

4.1 Growth of *Aspergillus niger* in media supplemented with food preservatives.

4.1.1 Introduction.

A number of investigators have reported the effects of various food preservatives on the growth of moulds. Bullerman (1984, 1983a, 1983b, 1987 and 1988), Marth (1984, 1985a, and 1985b) and Sofos (1986) investigated the effects of sorbic acid and its salts on the growth of toxin producing moulds, such as *Aspergillus flavus*, *A. parasiticus*, and *A. ochraceus*. Few have investigated propionates, probably because of their tainting characteristics and reputation of being poor growth inhibitors. Similarly, few observations on the efficacy of benzoates in inhibiting growth of moulds are reported. Benzoic acid and its salts have two advantages, they produce a neutral to more desirable odour and are cheap, compared with sodium sorbate.

Sofos (1986), Tsai and Liewen (1988), Zamora and Zaritzky (1987), and Veultzis and Buck (1984) carried out studies on improvement of ham and poultry quality by using food preservatives, such as sorbic acid. However, no work has been conducted on the effect of sorbic acid and its salts on the growth of xerophilic and or halotolerant moulds which spoil dried and/or salted fish. Indonesian salted dried fish *is* reported to be contaminated with *A. niger* (35 % of samples), and *A. penicillioides* (16.2 %) (Wheeler *et al.* 1988).
4.1.2 Radial Growth of *A. niger* in Czapek Yeast Agar (CYA) supplemented with food preservatives.

4.1.2.1 Effect of sodium bisulphite on radial growth of *A. niger* on CYA.

The effect of sodium bisulphite on radial growth of *A. niger* in CYA was assessed. The mould was grown in CYA at 25°C and growth was assessed at 1 day intervals. Results are presented in Figure 9. None of concentrations tested could stop or delay growth of *A. niger*, which showed visible colonies on the second day of incubation, as did the control.

With respect to growth rate, all treatments and the control showed slightly different effects. The maximum growth rate for 0.01% was reached earlier (on day 9) than the control. The maximum rates for 0.02% and the control were reached on the same day (day 10). Growth was delayed for 1 day for 0.03% (day 11) and 2 days for 0.04% (day 12). This suggests that use of 0.01% of sodium bisulphite might slightly stimulate growth of *A. niger*. If this phenomenon is true, growth expressed by dry weight should confirm this (Section 4.1.3).

Analysis of Variance (Anova) of Split-Plot Design indicated that there were significant differences between treatments tested and the control in most days of incubation, as far as radial growth was concerned. However, some differences were not significant, such as between the control and 0.01% and 0.02% sodium bisulphite on day 1, between 0.01% and 0.02% and 0.03%, between 0.03% and
0.04 % on days 1, 8, 11, 13, and day 14. It is possible to conclude that firstly, growth was not significantly inhibited using 0.01 % and 0.02 % sodium bisulphite. Lueck, (1980) found that growth of bacteria was significantly inhibited using the same concentration. The more pronounced action against bacteria is also reported by Chichester and Tanner (1983), Ough (1982) and Considine (1980). The concentration of 0.03 % was the minimum level of sodium bisulphite to inhibit growth of A.niger. This result is supported by Lueck (1980) that the concentration of 0.022 % was the lowest to inhibit this mould at pH 4.5. Its more pronounced action at lower pH is due to a higher proportion of undissociated sulphurous acid and dissolved sulphur dioxide. The use of 0.04 % bisulphite was not significantly more effective than 0.03 % in inhibiting growth of A.niger. Hence, use of 0.03 % was more economical, if sodium bisulphite was chosen as food preservative. It should, however, be borne in mind that part inhibition has little meaning, since any visible growth, regardless of size, on the surface of foods will far reduce or totally compromise the quality.
Figure 9.  Radial Growth of *A. niger*
on CYA plus Sodium bisulphite

![Graph showing radial growth of A. niger over time with different concentrations of sodium bisulphite.](image)

- **X** concentrations:
  - 0.01 %
  - 0.02 %
  - 0.03 %
  - 0.04 %

*Time in days*

*Concentrations*
4.1.2.2. Effect of sodium propionate on radial growth of *A. niger* grown on CYA

The effect of sodium propionate on radial growth of *A. niger* on CYA was assessed and results are shown in Figure 10. Like sodium bisulphite, use of sodium propionate was not able to stop nor delay growth of the mould for any concentrations tested, i.e. 0.05 %, 0.1 %, 0.2 % and 0.3 %. However, use of the lowest concentration (0.05 %) has significantly inhibited mould growth. A slightly different result was reported by Buchanan and Ayres (1981) that the use of 0.1 % propionic acid partially inhibited growth of *Aspergillus parasiticus* and a complete inhibition was achieved by 0.2 %. Ray and Bullerman (1985) indicated that *A. niger* started to be inhibited at a level of 0.08 % at pH 5 and *Alternaria* spp was similarly affected at 0.06 % at the same pH. At a lower pH, the inhibition should be more pronounced, due to the availability of higher level of undissociated substances in the solution (Chichester and Tanner, 1982).

It is not appropriate to compare the effectiveness between sodium bisulphite and sodium propionate, since the levels tested were not equal. The range of concentrations was based on the maximum permitted levels applied in most countries. Sodium bisulphite is an inorganic substance which is more effective and toxic than sodium propionate at the same concentration, but fatal poisoning with SO₂ is impossible, because vomiting occurs (Lueck, 1980). Sodium propionate is readily absorbed by the digestive tract on
Figure 10  
*Radial Growth of A. niger*

on CYA plus Sodium propionate

Measured by 40-10 Image Analyser
account of its good water solubility. There is no risk of accumulation in the body (Lueck, 1980). It might, however, impart an off-flavour to certain foods. Therefore, its use will not probably increase substantially (Ray and Bullerman, 1982).

Maximum growth rates for all concentrations tested, were attained on the same day (day 9) as the control. The rates for 0.05 %, 0.10 %, 0.20 % and 0.3 % were 5.53 mm/day, 5.48 mm/day, 5.19 mm/day and 4.90 mm/day, respectively.

Table 7. The differences in colony diameter means of A. niger grown on CYA + sodium propionate.

<table>
<thead>
<tr>
<th>Concentrations of sodium propionate</th>
<th>controls 0.05 % 0.1 % 0.2 % 0.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 %</td>
<td>5.5*</td>
</tr>
<tr>
<td>0.1 %</td>
<td>7.0**</td>
</tr>
<tr>
<td>0.2 %</td>
<td>8.9**</td>
</tr>
<tr>
<td>0.3 %</td>
<td>11.1**</td>
</tr>
</tbody>
</table>

Values in mm; *: Significant; o: not significant; ** highly significant.

Anova indicated that differences in radial growth between treatments and the control were highly significant, throughout the incubation period (13 days). Similarly, between days of incubation, the differences in radial growth were highly significant, but there was no significant
differences between replicates. This indicated that whole result was highly reliable. After being analyzed by LSD, most differences in colony diameter means between the concentrations tested and the control were highly significant ($P: 0.05$), but between 0.05 % and 0.1 % sodium bisulphite treatments, the difference in colony diameter means was not significant. Similarly between 0.1 % and 0.2 % and between 0.2 % and 0.3 % the differences were not significant, throughout the incubation period (Table 7). This indicates that increasing the concentration above 0.1 % will not significantly reduce growth further. Hence, the use of 0.1 % was appropriate on economic grounds. The initiation of growth, on day 2 was separately analyzed by LSD. Most differences in diameter means between the concentrations tested were not significant. Nevertheless, the differences between the control and concentrations on day 3 onward were highly significant.

4.1.2.3 Effect of sodium benzoate on radial growth of *A. niger* on CYA

Like sodium bisulphite and sodium propionate, sodium benzoate was not capable of totally inhibiting or delaying growth of *A. niger* on CYA (Figure 11). Growth was significantly inhibited, in particular for 0.15 % and 0.2 % treatments, throughout the incubation period. On day 2, growth for 0.05 % was slightly higher compared to the control. Roland, Beuchat and Hitchcock (1984) pointed out that *Byssochlamys nivea* tolerated up to 0.4 % sodium
benzoate at $30^0\text{C}$ of incubation. However, growth was completely stopped when the temperatures of incubation were either $37^0\text{C}$ or $21^0\text{C}$.

It is interesting to note that growth rate for $0.20\%$ sodium benzoate was not maximal until the end of observation (4.91 mm/day) but was lower than that at the same time and the same level of sodium propionate (5.19 mm/day). This is in contrary to the finding of Chichester and Tanner (1982), who reported that sodium benzoate was considered to be most active against yeasts and bacteria and sodium propionate to be most active against moulds, particularly those which grow on baked products, such as bread and cakes.

Table 8 The Differences in Colony Diameter Means of *A. niger* Grown on CYA Containing Sodium Benzoate.

<table>
<thead>
<tr>
<th>Concentrations of sodium benzoate</th>
<th>controls</th>
<th>0.05 %</th>
<th>0.1 %</th>
<th>0.15 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 %</td>
<td>3.8**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 %</td>
<td>4.5**</td>
<td>0.7⁰</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>0.15 %</td>
<td>8.9**</td>
<td>5.1**</td>
<td>4.4**</td>
<td>-</td>
</tr>
<tr>
<td>0.20 %</td>
<td>11.4**</td>
<td>7.6**</td>
<td>6.9**</td>
<td>2.5*</td>
</tr>
</tbody>
</table>

Values in mm; o: not significant; *: significant; **: highly significant.

Like the previous food preservatives, sodium benzoate is reported to be more active at low pH (Chichester and Tanner,
Figure II. Radial Growth of *A. niger*
on CYA plus Sodium benzoate

![Graph showing radial growth of A. niger on CYA with different concentrations of sodium benzoate.](image)

**Concentrations:**
- Controls
- 0.05 %
- 0.10 %
- 0.15 %
- 0.20 %
1982, and Ough, 1983). A recent report indicated that a minimum concentration needed for inhibiting Aspergillus spp was 0.03 % (Lueck, 1980). This is in agreement with the results given in Table 8 which show that 0.05 % sodium benzoate did not significantly inhibit growth of A.niger.

Anova indicated that the difference in colony diameter between the treatments and the control were highly significant. Similarly, between days of incubation, the differences were highly significant. LSD analysis (Table 8) revealed that the difference in colony diameter means between 0.05 % and 0.1 % sodium benzoate treatments was not significant; but between 0.15 % and 0.2 % treatments, the difference was significant (P: 0.05). The remaining differences in colony diameter means between the treatments and the control were highly significant (P: 0.01).

4.1.2.4 Effect of sodium sorbate on radial growth of A.niger on CYA

Unlike the previous food preservatives, sodium sorbate was capable of delaying growth of A.niger on CYA (Figure 12). Therefore, this food preservative is used in various foods, to increase food quality and extend the storage life. Growth initiation for the 0.1 %, 0.15 % and 0.20 % sodium sorbate treatments were on days 3, 3, and 4, respectively. Visible colonies appeared on day 3 for the 0.15 % treatment, although the colony diameter was very small (0.35 mm) and could only be observed by means of a microscope. However, three replicates of four were not
visible, on day 3. Growth in all treatments was inhibited throughout the incubation period compared to the control. Gourama and Bullerman (1987) obtained slightly different results in that growth of *A. ochraceus* was delayed for one day by addition of 0.05 % sodium sorbate. Results given in Figure 12 shows that when concentrations were increased to 0.10 % and 0.15 %, delays were 3 and 5 days, respectively. Using *A. patulum* as the test organism, Przybylski and Bullerman (1980) found that the initiation of growth using 0.05 %, 0.1 % and 0.15 %, at 12°C of incubation, was observed on days 5, 6 and day 6, respectively. This experiment shows that 0.05 % sodium sorbate had a profound inhibitory effect. This is in agreement with Liewen and Marth (1984) who found that most Aspergilli were inhibited at level of 0.05 % sorbic acid at 25°C. Bandelin (1958) reported that 0.08 % was the minimum inhibitory concentration of *A. niger*, but *Alternaria solani* needed a lower concentration (0.02 %). Ronald *et al.* (1984), using *Byssoclamys nivea* as test organism, reported that a concentration of 0.075 % began to inhibit growth and complete inhibition was achieved at a level of 0.15 % potassium sorbate.

Anova indicated that between the treatments and the control, the differences in colony diameter were highly significant (P:0.01). LSD (Table 9) revealed that the differences in total colony diameter means between 0.05 % sodium sorbate treatment and the control, and between 0.2 %
Figure 12. Radial Growth of *A. niger* Grown on CYA plus Sodium sorbate

Measured by 40-10 Image Analyser
and 0.15 % treatments were significant ( P: 0.05), but the remaining differences, between the treatment and the control were highly significant ( P: 0.01).

Table 9. The Differences in Colony Diameter Means of A. niger Grown on CYA Containing Sodium Sorbate.

<table>
<thead>
<tr>
<th>Concentrations of sodium sorbate</th>
<th>controls 0.05 %</th>
<th>0.1 %</th>
<th>0.15 %</th>
<th>0.2 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 %</td>
<td>6.5*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.1 %</td>
<td>13.4**</td>
<td>6.9**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.15 %</td>
<td>20.6**</td>
<td>14.1**</td>
<td>7.2**</td>
<td>-</td>
</tr>
<tr>
<td>0.2 %</td>
<td>26.7**</td>
<td>20.2**</td>
<td>13.3**</td>
<td>6.1*</td>
</tr>
</tbody>
</table>

values in mm; * : significant ; ** : highly significant

4.1.3 Total Dry Weight of A. niger Grown in Czapek Yeast Broth (CYB) Supplemented with Food Preservatives.

4.1.3.1 Effect of sodium bisulphite on total dry weight.

The use of 0.03 % sodium bisulphite did not totally stop growth of A. niger in CYB (Table 10). The reduction in dry weight averaged 52 %, compared to the control. This value was almost equal to the reduction (50 %) obtained by using 0.1 % of sodium sorbate in the same medium. When the concentration was lowered to 0.01 %, growth was stimulated. These results are in parallel with the results for radial growth determination. It is not clear, however if the use of less than the minimum level for inhibition will stimulate
growth. Lueck (1980) pointed out that sulphurous acid may react to form additional compounds with food ingredients, eg. sulfonates. Galactose, mannose, and arabinose react rapidly with bisulphite (Ough, 1983). This linkage of sulphurous acid to carbonyl compounds in foods reduces or even completely neutralizes the sulphurous acid's antimicrobial actions. Similarly, Gourama and Bullerman (1987) found that growth of A. ochraceus was stimulated at the presence of 0.05 % potassium sorbate, at 250°C. Roland et al. (1984) found that sulphur dioxide was lethal at 50 ppm (0.005 %) to Byssochlamys nivea grown in apple juice, at 21°C; but growth was repressed in grape juice at a level of 100 ppm, at the same temperature.

Table 10. Total dry weight of A. niger grown on CYB containing sodium bisulphite for 4 days.

<table>
<thead>
<tr>
<th>Repl.</th>
<th>Controls</th>
<th>0.01%</th>
<th>0.02%</th>
<th>0.03%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>340</td>
<td>440</td>
<td>350</td>
<td>240</td>
</tr>
<tr>
<td>2</td>
<td>500</td>
<td>670</td>
<td>240</td>
<td>160</td>
</tr>
<tr>
<td>3</td>
<td>410</td>
<td>510</td>
<td>290</td>
<td>200</td>
</tr>
<tr>
<td>4</td>
<td>417</td>
<td>478</td>
<td>280</td>
<td>200</td>
</tr>
</tbody>
</table>

Values in mg.
There was no significant difference in dry weight means either between 0.02 % and 0.03 % sodium bisulphite or between the control and 0.01 %, but between 0.01 % and 0.02 % treatments, the difference in dry weight was significant. The differences were highly significant between the control and 0.03 % and between 0.01 % and 0.03 % sodium bisulphite (Table 11). These facts suggest that use of 0.01 % sodium bisulphite did not exert a significant effect against this mould. The minimum level to give significant effect to growth was 0.02 %.

Table 11 The differences in dry weight means of *A.niger* grown in CYA containing sodium bisulphite, for 4 days.

<table>
<thead>
<tr>
<th></th>
<th>Concentrations of sodium bisulphite</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>controls</td>
</tr>
<tr>
<td>0.01 %</td>
<td>61⁰</td>
</tr>
<tr>
<td>0.02 %</td>
<td>137*</td>
</tr>
<tr>
<td>0.03 %</td>
<td>244**</td>
</tr>
</tbody>
</table>

Values in mg. *: significant, **: highly significant, ⁰: not significant.
4.1.3.2 Effect of sodium sorbate on growth of *A. niger* in CYB.

Total dry weight of the mould was reduced by c.50\% in the medium supplemented with 0.10\% sodium sorbate (Table 12). The mycelial reduction increased to 95\%, compared to the control when the concentration was doubled and mycelium in the broth was hardly observed. The mycelial reduction for 0.15\% sodium sorbate treatment was 83\%. It is important to note that use of up to the maximum level (0.2\%) did not stop growth completely.

Table 12 Total dry weight of *A. niger* grown on CYB containing sodium sorbate, for 4 days.

<table>
<thead>
<tr>
<th>Repl.</th>
<th>Controls</th>
<th>0.1%</th>
<th>0.15%</th>
<th>0.20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>680</td>
<td>340</td>
<td>150</td>
<td>30</td>
</tr>
<tr>
<td>2</td>
<td>720</td>
<td>410</td>
<td>100</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>640</td>
<td>400</td>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>x</td>
<td>700</td>
<td>383</td>
<td>117</td>
<td>40</td>
</tr>
</tbody>
</table>

Values in mg.

In general, growth trends in dry weight were in parallel with those on radial growth (see section 4.1.2.4) where visible growth was only delayed for two days at the same level.
Anova showed that differences between the concentrations and the control were highly significant. Between replicates, the differences were also highly significant which reduces the validity of significance given for treatments. LSD analysis (Table 13) revealed that the differences in dry weight means between all the treatments and the control, and between the treatments themselves were highly significant. The exception was that there was a significant difference, between 0.15 % and 0.20 % sodium sorbate.

Table 13 The differences in dry weight means of A. niger grown in CYB containing sodium sorbate, for 4 days.

<table>
<thead>
<tr>
<th>Concentrations of sodium sorbate</th>
<th>Controls</th>
<th>0.10 %</th>
<th>0.15 %</th>
<th>0.20 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10 %</td>
<td>317**</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.15 %</td>
<td>583**</td>
<td>266**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.20 %</td>
<td>343**</td>
<td>343**</td>
<td>77*</td>
<td>-</td>
</tr>
</tbody>
</table>

Values in mg. ** :highly significant, * : significant.

4.1.3.3 Effect of sodium benzoate on growth of A. niger, in Czapek Yeast Broth (CYB)

The results given in Table 14 show that sodium benzoate at a level of 0.1 % had little effect on growth of A. niger. The reduction in dry weight of 9.8 % was only a quarter of that obtained by the sodium sorbate treatment at
the same concentration. When the concentration was increased to 0.2 %, growth was not totally inhibited, but the reduction exceeded 4 fold that of 0.15 % sodium benzoate. Cheipley and Urah (1980) report that with A.parasiticus, derivatives of benzoic acid, at a level of 0.04 %, reduced total dry weight by 90 %, in the basal medium, at pH 4.5.

Table 14. Total dry wight of A.niger grown in CYB containing sodium benzoate, for 4 days.

<table>
<thead>
<tr>
<th>Rpl.</th>
<th>Controls</th>
<th>0.10 %</th>
<th>0.15 %</th>
<th>0.20 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>640</td>
<td>630</td>
<td>550</td>
<td>250</td>
</tr>
<tr>
<td>2</td>
<td>700</td>
<td>610</td>
<td>560</td>
<td>390</td>
</tr>
<tr>
<td>3</td>
<td>680</td>
<td>580</td>
<td>410</td>
<td>500</td>
</tr>
<tr>
<td>X</td>
<td>673.3</td>
<td>606.7</td>
<td>506.7</td>
<td>380.0</td>
</tr>
</tbody>
</table>

Values in mg.

This more pronounced action might be due to the lower pH, because the antimycotic effectiveness of sodium benzoate is strongly pH dependent (Jermini and Lorenz, 1987). Anova showed that the differences between the treatments and the control were significant (P: 0.05). LSD (Figure 15) revealed that at a level of 0.1 %, the reduction in dry weight was not significant, compared to the control. Similarly, when
the concentration was increased from 0.1 % to 0.15 % and from 0.15 % to 0.2 %. The increases in the total dry weight were not significant. These results suggest that \textit{A. niger} grown in liquid medium is not sensitive to sodium benzoate over the concentration range investigated.

Table 15. The differences in dry weight means of \textit{A. niger} grown in CYB containing sodium benzoate, for 4 days.

<table>
<thead>
<tr>
<th>Concentration of sodium benzoate</th>
<th>Controls</th>
<th>0.1 %</th>
<th>0.15 %</th>
<th>0.20 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 %</td>
<td>66°</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.15 %</td>
<td>166*</td>
<td>100°</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.20 %</td>
<td>293**</td>
<td>227**</td>
<td>127°</td>
<td>-</td>
</tr>
</tbody>
</table>

°: not different; *: significant; **: Highly significant; Values in mg.

4.1.3.4 Total dry weight of \textit{A. niger} grown in Czapek Yeast Broth (CYB) supplemented with sodium propionate

Table 16 shows that 0.1 % sodium propionate did not significantly inhibit growth of \textit{A. niger} grown on CYB, the reduction in total dry weight being only 0.78 %. However, inhibition was highly significant when the concentrations were increased to 0.2 % and 0.3 %, leading to reductions in total dry weight of 55.6 % and 69 %, respectively. The increase of concentration from 0.1 to 0.2 %, significantly reduced total dry weight means, but was not significantly
reduced further when the concentration was increased from 0.2 to 0.3 % (Table 17).

Table 16 Total dry weight of *A. niger* grown in CYB containing sodium propionate, for 4 days.

<table>
<thead>
<tr>
<th>Rpl.</th>
<th>Control</th>
<th>0.1 %</th>
<th>0.2 %</th>
<th>0.3 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>450</td>
<td>450</td>
<td>120</td>
<td>160</td>
</tr>
<tr>
<td>2</td>
<td>390</td>
<td>450</td>
<td>160</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>420</td>
<td>350</td>
<td>280</td>
<td>130</td>
</tr>
<tr>
<td>x</td>
<td>420</td>
<td>416.7</td>
<td>136.7</td>
<td>130</td>
</tr>
</tbody>
</table>

Values in mg.

Table 17. The differences in dry weight means of *A. niger* grown in CYB containing sodium propionate.

<table>
<thead>
<tr>
<th></th>
<th>Concentrations of sodium propionate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.1 %</td>
</tr>
<tr>
<td></td>
<td>0.2 %</td>
</tr>
<tr>
<td></td>
<td>0.3 %</td>
</tr>
</tbody>
</table>

Value in mg, o : not significant; ** : highly significant.

These results were not in parallel with those on radial growth where sodium propionate at a concentration of 0.2 % was less powerful against this mould. However, the result on radial growth was likely to be more
reliable, since the differences in total dry weight means between replications were statistically significant. Moreover, the use of sodium benzoate at a level of 0.1% was more effective than sodium propionate.

4.1.4 Radial Growth of A. niger on 10% salt-Czapek Yeast Agar (CYA) supplemented with food preservatives.

4.1.4.1 Introduction.

Salt has been used to preserve foods from microbial spoilage for a long time. Kushner (1971) pointed out that common salt lowers the water activity of a system and thus render conditions less favourable for microbial life. The use of 10% salt only lowers the Aw to 0.928 in which some microorganisms, such as moulds, can proliferate. A saturated salt solution has a Aw of 0.75 at which some xerophilic fungi are still able to grow. Therefore, 10% salt will not prevent growth of A. niger completely. Pitt and Hocking (1979) reported that Aspergillus flavus, and Eurotium chevalieri grew best at Aw of 0.94 to 0.98 and 0.93 to 0.95, respectively. However, when salt was used to lower the Aw in laboratory media, A. flavus and E. chevalieri did not grow below 0.87 for the former and 0.86 for the later. In contrast, germination and growth still occurred in media supplemented with solutes other than salt, such as glucose, fructose and glycerol. In media containing no salt, A. niger spores could germinate at a Aw of 0.71 (Pitt and Hocking, 1982). The presence of salt in media reduces the solubility of oxygen in water, thus the quantity of oxygen available to
aerobic microorganisms in media (foods) containing high salt levels is only a fraction of that in substances with a low salt content (Kushner, 1973). Larsen (1962) pointed out that sodium chloride may interfere directly with metabolism, e.g. by affecting the catalytic function of individual enzymes.

4.1.4.2 Effect of sodium propionate on radial growth of *A. niger* in 10 % salt-Czapek Yeast Agar (CYA)

Figure 13 shows that growth for both the control and the treatments, was initiated on day 4. The use of 0.05 %, 0.1 % and 0.15 % of sodium propionate had little effect on growth of *A. niger*, particularly in the first few days of incubation. Inhibition was quite pronounced using 0.2 % sodium propionate, resulting in a colony diameter that was only half that of the control. Until the last day of observation, growth for 0.20 % was considerably inhibited. For the first 7 days of incubation, growth in 0.10 % sodium propionate was slightly better than in medium containing 0.05 % sodium propionate. The trend reversed from day 8 until the end of incubation.

Anova showed that the differences between the treatments and the control were highly significant. Similarly, there were highly significant differences between days of incubation. Subsequently, LSD (Table 18) indicated that the difference in the colony diameter means between 0.05 %, 0.10 % and 0.15 % sodium propionate were not significant. The remaining differences in colony diameter
means between treatments were highly significant. This suggests that the administration of 0.20 % sodium propionate in conjunction with 10 % salt was more effective in inhibiting growth of this mould. The evidence of no differences between 0.05 %, 0.1 % and 0.15 % indicated that sodium propionate was in fact less active against A. niger. These results confirmed those obtained on media with no salt. Some investigators have suggested that the lower the pH used, the more effective sodium propionate becomes, but Chichester et al. (1981) reported that the use of sodium propionate at pH 6 or slightly higher was more effective in some foods.

Table 18. The Differences in Dry weight Means of A. niger Grown on 10 % salt-CYA.

<table>
<thead>
<tr>
<th>Concentration in %</th>
<th>controls</th>
<th>0.05</th>
<th>0.1</th>
<th>0.15</th>
<th>0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td></td>
<td>8.5**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td></td>
<td>9.5**</td>
<td>0.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td></td>
<td>11.9**</td>
<td>1.90</td>
<td>2.60</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td></td>
<td>15.5**</td>
<td>5.9**</td>
<td>6.6**</td>
<td>4**</td>
</tr>
</tbody>
</table>

values in mm; 0 : not significant, * : significant, ** : highly significant.
Figure 13. Radial Growth of A. niger Grown on 10 % Salt-CYA + Sodium propionate

by 40-10 Image Analyser
No trends were apparent for the growth rate of the mould. The maximum rates for all treatments and the control were reached on the same day (day 7). After that rates declined for all treatments and the control.

4.1.4.3 Effect of sodium benzoate on radial growth of *A. niger* on 10 % salt-Czapek Yeast Agar (CYA)

Figure 14 shows that the addition of sodium benzoate to 10 % salt-CYA was capable of delaying the initiation of mould growth. Visible growth in 10 % salt-CYA supplemented with 0.2 % sodium benzoate was delayed for 3 days. On day 4, only one of the three replicate plates of 0.2 % sodium benzoate showed growth by this mould and growth did not become apparent on the other two replicates until day 7. Until day 8, mould growth on 0.15 % sodium benzoate was slower than that for 0.20 % sodium benzoate, but the situation reversed from day 9 onward. The average slower growth for 0.15 % sodium benzoate was mainly due to one of the three replicates failing to produce colonies. However, it was not clear whether this was due to the action of food preservative or growth failure for other reasons. Radial growth varied significantly between replicates for most the treatments and the control. It was possible that the spores varied in their ability to germinate and produce mycelia when exposed to media stress, particularly as a result of combining salt and food preservatives, since each colony developed from one spore. If one or two spores were accidentally picked up and inoculated in the agar plates,
colony growth would be uneven, leading to irregular diameters. This seems feasible, since the observations of the same mould grown on CYA with food preservatives alone, did not exhibit such a problem. Nevertheless, to justify this hypothesis, further investigation is needed.

Anova showed that the differences in colony diameter between treatments and the control was highly significant (P = 0.00). The differences in colony diameter means between 0.15 % and 0.20 % sodium benzoate, and between 0.05 % and 0.1 % sodium benzoate were not significant (Table 19). It is, however, not always easy to decide the minimum inhibitory concentration of a certain food preservative, because a particular food preservative is often more effective against particular organisms (Barner, 1983). Cheipley (1983) reported that moulds and most yeasts are inhibited in the range of 0.05 % to 0.10 % sodium benzoate.

Table 19. The differences in colony diameter means of A. niger grown on 10 % salt-CYA supplemented with sodium benzoate.

<table>
<thead>
<tr>
<th></th>
<th>controls</th>
<th>0.05 %</th>
<th>0.10 %</th>
<th>0.15 %</th>
<th>0.20 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 %</td>
<td>6.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.10 %</td>
<td>19.6</td>
<td>2.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.15 %</td>
<td>29.4</td>
<td>12.5</td>
<td>9.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.20 %</td>
<td>30.2</td>
<td>13.3</td>
<td>10.6</td>
<td>0.8</td>
<td>-</td>
</tr>
</tbody>
</table>

values in mm.
Figure 14. Radial Growth of *A. niger*
on 10 % salt-CYA plus sodium benzoate
The results also suggest that the use of sodium benzoate in conjunction with salt was far more effective than sodium benzoate alone against *A. niger*. On day 9, when the maximum rates for mould grown in CYA supplemented with 0.05 % sodium benzoate were reached, the colony diameter, averaged 5.27 cm approximately double that on 10 % salt-CYA supplemented with 0.05 % sodium benzoate, on which radial growth was reduced by 58 %, compared to the control. This reduction increased to 60 % at the end of incubation. These results were in support of those of Molin (1980) who found that sodium chloride has a considerable synergistic effect with sodium benzoate.

The maximum radial growth rate on 0.05 % sodium benzoate (2.78 mm/day) was reached on the same day (day 7) as the control (5.00 mm/day). The maximum rate for the 0.10 % treatment (2.32 mm/day) was one day later. The maximum rates for both 0.15 % and 0.20 % treatments were delayed until day 14. Maximum growth rates for these treatments were 1 mm/day for the former and 0.97 mm/day for the latter. At the end of the experiment, growth rates on all treatments and the control sharply decreased and the rate on 0.20 % sodium benzoate was lower than that on 0.15 %.
4.1.4.4 Effect of sodium sorbate on radial growth of *A. niger* on 10 % salt-Czapek Yeast Agar (CYA).

The effect of sodium sorbate was assessed and results are presented in Figure 15. The use of 0.05 % sodium sorbate in conjunction with 10 % salt slowed down growth of *A. niger*, and delayed the initiation of visible growth for 3 days. On day 6, when growth was visible, two of the four agar replicate plates did not produce visible colonies. The following day, visible colonies were noted on the third replicate plate and finally colonies appeared all plates by day 8, when the reduction in colony diameter averaged 93 %, compared to the control. The mould was not capable of growing on media supplemented with 0.1 % of sodium sorbate, throughout incubation period and the use of sodium sorbate alone as described in Section 4.1.2.4, in fact never prevented growth up to the maximum level of 0.2 %.

The maximum growth rate for the 0.05 % sodium sorbate treatment was reached by day 13, and averaged 0.71 mm/day. On the same day, the maximum rate for the control was far higher (3.38 mm/day) and the average reduction in colony diameter decreased from 93 % to 79 % after day 13.

Statistically the differences in colony diameter throughout incubation period (20 days) were highly significant between the 0.05 % sodium benzoate treatment and the control.
Figure 15. Radial Growth of *A. niger* on 10% salt-CYA plus sodium sorbate

Diameter in mm

Time in days

Concentrations
- controls
- 0.05%
- 0.10%: no growth
4.1.5 Total dry weight of *A. niger* grown on 10% salt-CYB supplemented with food preservatives.

4.1.5.1 Introduction.

During the initial investigation, assessment of mould growth by total dry weight did not produce consistent results, especially on the media supplemented with salt. The experiment was repeated and this time using more accurate procedures. All variables influencing, such as temperature, speed of shaking water bath, quantity of water in the shaking bath and position of flasks on a tray of the shaking water bath were carefully controlled. Unfortunately, statistically significant variations between replicates persisted. The same problem was experienced with the dry weight results obtained from *A. penicilloides*. Most observations, within replications, showed statistically significant differences in total dry weight. It was also noted that mycelium was often visibly evident in broth, but the total dry weight was zero. The varying values within replications might be due to two reasons. Firstly, as described in Section 3, the moulds were incubated in the shaking water bath, which possibly did not properly shake the broth. Secondly, as a result of inhomogenous shaking, some spores may have stayed throughout the incubation period at the bottom or in the middle of flasks where supply of oxygen was not sufficient, thus suppressing or failing to initiate growth. Some spores, which, floated to the surface and/or adhered on the flask's walls grew, as a result of better oxygen supply. Moulds are generally regarded as
obligately aerobic (Pitt and Hocking, 1983). Consequently, growth rates varied between flasks or replicates.

The maximum dry weight varies within species of *Aspergillus*. *A. niger* yielded approximately 600 mg/50 cm$^3$ of CYB. Rosul et al. (1980) obtained a yield of 700 mg in 25 cm$^3$ of glucose yeast salts broth, within 4 days incubation, for *A. parasiticus*. With this mould, Marshal and Bullerman (1986) reported less than 200 mg in 25 cm$^3$ of the broth over the same incubation period. Using a growth medium previously used by Rosul (1980), they found that the dry weight of this mould was 500 mg in 25 cm$^3$. With *A. flavus*, Cheipley and Urah (1980) reported dry weight of 1000 mg in 25 cm$^3$ of the broth. The results obtained in Section 4.2 show that *A. penicilloides* grew very slowly and only yielded 200 mg/50 cm$^3$ of 5% salt-CYB for 4 days of incubation. The time needed for incubation was crucial. *A. niger* grew rapidly in CYB. On one occasion the mould was harvested after 7 days of incubation. Total dry weight obtained for the treatments was very similar to the control. It was possible that in 3-4 days of incubation, growth for the control was more rapid than the treatments. After this period of time (4 days) moulds, *A. niger* in particular, begins to sporulate and produce less mycelia which Yousuf (1981) described as "balance phase". Spores were not apparently trapped on the Whatman paper no. 564 when the mould grown in the broth was filtered. The mould for the control produced more spores than the treatments. After 6 to 7 days of incubation,
growth decreased gradually, described as the "storage phase". In this phase, some mycelia underwent bio-autolysis. El-ghazar and Marth (1987) pointed out that this bio-autolysis may lead to loss of soluble intracellular solutes which were released during filtration step of the analysis. Therefore, the best time for harvesting mould is just before the onset of sporulation.

4.1.5.2 Dry weight of A.niger Grown on 10 % salt-Czapek yeast broth (CYB) supplemented with sodium sorbate.

Results of the investigation into the effect of sodium sorbate on radial growth are presented in Table 20. Mould growth for 0.1 % and 0.15 % sodium sorbate was completely inhibited. Total dry weight for 0.05 % sodium sorbate was very low (20 mg in 50 cm³ of the broth). This value was far lower than that obtained for 0.05 % treatment with no salt (277 mg). The results for dry weight paralleled to those obtained for radial growth which was also completely inhibited at a level of 0.05 % of sodium benzoate. Sodium sorbate, used in conjunction with salt, inhibited growth of A.niger far more effectively than the other three food preservatives tested.

Statistically, the difference in dry weight means between the control and 0.05 % sodium sorbate was highly significant. The difference in total dry weight means within replicates was significant, but this may not influence the significance of the treatment, since the difference in dry weight means between 0.05 % treatment and the control was
extremely high (257 mg). The general conclusion was that the use of sodium sorbate alone up to 0.20% could not totally stop the growth of *A. niger* in CYB, but at a level of 0.1% sodium sorbate combined with 10% salt, growth was totally inhibited.

Table 20. Total Dry Weight of *A. niger* Grown in 10% salt CYB Supplemented with Sodium Sorbate.

<table>
<thead>
<tr>
<th>Concentrations of sodium sorbate (%)</th>
<th>Rpl</th>
</tr>
</thead>
<tbody>
<tr>
<td>controls</td>
<td>0.05</td>
</tr>
<tr>
<td>1</td>
<td>200</td>
</tr>
<tr>
<td>2</td>
<td>310</td>
</tr>
<tr>
<td>3</td>
<td>310</td>
</tr>
<tr>
<td>x</td>
<td>277</td>
</tr>
</tbody>
</table>

Values in mg, rpl: replicate.

4.1.5.3 Total Dry Weight of *A. niger* grown on 10% salt-Czapek yeast broth (CYB) supplemented with sodium benzoate.

The effect of sodium benzoate on growth expressed as dry weight of *A. niger* was assessed and results are presented in Table 21. Sodium benzoate was able to totally inhibit growth of *A. niger* at a level of 0.15%. The presence of both 0.10% and 0.05% of sodium benzoate did not completely stop
growth. Compared to the control, reductions in dry weight for 0.05 % and 0.1 % reached 68.75 % and 29.37 %, respectively. However, these were still lower than those obtained by using 0.05 % of sodium sorbate.

With *A. parasiticus*, Al-Gazzar and Mart (1987) found that the use of sodium benzoate alone up to 0.4 % did stop mould growth. They added that this mould hardly grew in 10 % CYB with the presence of 0.1 % sodium benzoate, during 0-3 days, but this mould was still capable of growing over a 7 - 10 day period.

### Table 21. Total Dry Weight of *A. niger* Grown in 10 % salt CYB Supplemented with Sodium Benzoate.

<table>
<thead>
<tr>
<th>Rpl</th>
<th>Controls</th>
<th>0.05</th>
<th>0.1</th>
<th>0.15</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100</td>
<td>100</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>140</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>130</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>x</td>
<td>113</td>
<td>110</td>
<td>20</td>
<td>0</td>
</tr>
</tbody>
</table>

value in mg; Rpl : replicate.

Anova showed that the difference in dry weight means was highly significant between the treatments and the control. LSD analysis indicated that there was no significant difference in dry weight means between the
control and 0.05 % sodium benzoate. The remaining differences in dry weight means between the treatments and the control were highly significant.

4.1.5.4 Total dry weight of *A. niger* grown in 10 % salt-Czapek yeast broth (CYB) supplemented with sodium propionate.

Table 22 shows that the use of sodium propionate in conjunction with 10 % salt was not capable of totally inhibiting growth of *A. niger*.

Table 22. Total Dry Weight of *A. niger* Grown in 10 % salt-CYB Supplemented with Sodium Propionate

<table>
<thead>
<tr>
<th>Rpl.</th>
<th>Controls</th>
<th>0.05</th>
<th>0.1</th>
<th>0.15</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>110</td>
<td>110</td>
<td>120</td>
<td>130</td>
</tr>
<tr>
<td>2</td>
<td>110</td>
<td>150</td>
<td>80</td>
<td>60</td>
</tr>
<tr>
<td>3</td>
<td>230</td>
<td>90</td>
<td>100</td>
<td>140</td>
</tr>
</tbody>
</table>

| x    | 150      | 117  | 100 | 110  |

Value in mg.

When the broth was supplemented with 0.15 % sodium propionate, dry weight was higher than for 0.05 % and 0.1 % sodium propionate. However, dry weight for the three treatments was lower than that for the control.

Anova indicated that there were no significant differences between the treatments and the control, but
between replicates, the difference were significant. This suggests that these results were very unreliable and LSD analysis was therefore not performed.

4.2 Growth of A. penicillioides on malt extract medium (MEM) or Czapek yeast medium (CYM) supplemented with food preservatives.

4.2.1 Radial growth of A. penicillioides on Czapek yeast agar (CYA) and malt extract agar (MEA) supplemented with salt.

Effects of salt on Aw values of both CYA and MEA and on radial growth of A. penicillioides were assessed. This mould was grown on both CYA and MEA containing 0, 5, 10, 15 and 17.5% NaCl, incubated at 25°C for 23 days and assessed at 1 day intervals. Results for Aw values and radial growth on CYA and MEA are presented in Table 23 and Figure 16 and 17, respectively. The mould hardly grew on MEA and CYA with no salt, but it grew better on CYA. Best growth was found on both MEA and CYA containing each 5% NaCl. Growth was gradually inhibited as the salt concentration increased above 5%. The mould was able to grow on CYA and MEA containing 17.5% NaCl, but visible growth was delayed for 9 days and 7 days, respectively, compared to those on MEA and CYA each containing 5% NaCl. Growth showed on both CYA with no NaCl and 5% on day 3, but growth on MEA with no salt was delayed for 7 days. There was no delay either on both media with 10%, and 12.5% NaCl and MEA with 15%, but it was delayed for 3 days for CYA with 15% NaCl. Kulik and
Hanlin (1968) indicated that high salt was needed to inhibit growth of *A. penicillioides*. Buchanan and Ayres (1976) reported that *A. parasiticus* was not inhibited at low concentrations of NaCl (2%) when this mould was grown on AMY medium.

Table 23. Water Activity Value on CYA and MEA with Varied Salt Concentrations.

<table>
<thead>
<tr>
<th>Salt Concentration</th>
<th>Water activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEA</td>
</tr>
<tr>
<td>control</td>
<td>0.98</td>
</tr>
<tr>
<td>5.0 %</td>
<td>0.932</td>
</tr>
<tr>
<td>10.0 %</td>
<td>0.926</td>
</tr>
<tr>
<td>12.5 %</td>
<td>0.912</td>
</tr>
<tr>
<td>15.0 %</td>
<td>0.884</td>
</tr>
<tr>
<td>17.5 %</td>
<td>0.864</td>
</tr>
</tbody>
</table>

Measured by novasina Aw meter.

A maximum growth rate of 1.68 mm/day was noted on MEA with 5 % NaCl. This value was lower than that for *A. niger* grown on CYA which was 6.8 mm/day. *A. penicillioides* grew slightly slower in both media containing 10 % NaCl, but this concentration was chosen for experiments involving use of food preservatives on growth of *A. penicillioides*, since most Indonesian salted dried fish contained 5 to 20 % salt.
Although radial growth on MEA with 10% NaCl was better than on CYA with 10% NaCl, CYB with 10% NaCl was used for experiments for total dry weight. MEB has a dark-brown colour which interferes with the observation of mycelia. MEA and CYA had Aw values of 0.980 and 0.983 (Table 23), respectively, on which growth of A. penicillioides was very poor. It was not clear whether this poor growth was due to either inappropriate Aw values of those media or an absence of salt. When 5% NaCl was added to MEA and CYA, Aw values were 0.930 and 0.940 respectively. Aw values decreased to 0.864 and 0.853, respectively, as the salt content was increased to 17.5%. Although A. penicillioides is capable of growing in media containing saturated-salt, Andrew and Pitt (1966) tended not to classify this mould as halophile, since this mould also exhibited growth on other solutes, such as sucrose and glycerol. Most xerophilic moulds normally grow better in media containing humectants other than salt, for lowering Aw value. This was demonstrated by Wheeler and Hocking (1988) for xerophilic fungi, such as A. candidus, A. sydowi and Pae cilomyces variotii when the maximum Aw needed for germination of these moulds were 0.806, 0.849 and 0.908, respectively. When salt was used to lower Aw, Aw needed for germination of these moulds were relatively higher than when sucrose and glycerol were used instead of salt.

Because of its suppressed growth on media containing
Figure 16. Radial Growth of *A. penicillioides* on CYA Supplemented with NaCl

Measured by 40-10 Image Analyser
Figure 17. Radial Growth of *A. penicillioides* in MEA Supplemented with NaCl

Measured by 40-10 Image Analyser
10 % salt or above and its ability to grow in media containing Aw modifiers other than salt, *A. penicilloioides* might not be a genuine halophile. Better growth on media with 5 % NaCl than media containing no salt was due merely to a more favourable Aw for growth.

4.2.2 Radial growth of *A. penicilloioides* on 10 % salt-malt extract agar (MEA).

4.2.2.1. Effect of sodium sorbate on growth of *A. penicilloioides*, in 10 % salt-MEA.

Because of its poorer ability to grow in media with no salt, experiments for *A. penicilloioides* were carried out on 10 % salt-MEA. The mould was incubated at 25\(^\circ\) C for 22 days and radial growth was measured at 1 day intervals. Results are presented in Figure 18.

Like *A. niger*, *A. penicilloioides* was considerably inhibited in the presence of only 0.05 % sodium sorbate and growth was totally inhibited at concentration of 0.10 %. The initiation of growth for 0.05 % sodium sorbate was delayed for 6 days. This mould grew very slowly and visible growth for the control appeared after 4 days of incubation. *A. niger* appeared on day 2 when grown on CYA, at the same temperature (25\(^\circ\) C). After 22 days incubation, the colony diameter reached 35.6 mm for the control and 10.6 mm for 0.05 % of sodium sorbate. Maximum growth rates for the control and 0.05 % sodium sorbate were 1.58 mm/day (day 15) and 0.56 mm/day (day 22), respectively.
Figure 18. Growth of *A. penicillioides* on 10% salt-MEA + Sodium sorbate

- Controls
- 0.05%
- 0.1%: No growth

Measured by 40-10 Image Analyser
Statistically, the difference in colony diameter means between the control and 0.05 % sodium sorbate was highly significant (P: 0.01)

4.2.2.2 Effect of sodium benzoate on radial growth of A. penicilloides on 10 % salt-MEA

The mould was incubated at 25°C for 22 days incubation and radial growth was measured at 1 day intervals. Results are presented in Figure 19. Visible growth for all concentrations of sodium benzoate was not delayed, compared to the control, but moderate inhibition was observed throughout the incubation period. Growth for 0.05 % sodium benzoate and for the control was, however, similar, except in the last two days of incubation when growth for the controls was faster than for 0.05 % sodium benzoate. Three days before the complete observation, growth on the three treatments (0.10 %, 0.15 % and 0.20 %) was very close together, even the colony diameter for 0.15 % sodium benzoate was higher than that for 0.10 %, on day 22.

The control and 0.05 % sodium benzoate reached highest growth rates on the same day (day 15), these were 1.57 mm/day and 1.6 mm/day, respectively. Growth for 0.05 % sodium benzoate was slightly better, but this was statistically not significant. Maximum growth rates for 0.10 % and 0.15 % treatments were, however, delayed for 1 and 2 days, respectively. The growth rate for 0.20 % sodium benzoate was not maximal yet at the end of observation.
Figure 19. Growth of *A. penicillioides* on 10% salt-MEA + Sodium benzoate

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Time in days</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05%</td>
<td>0, 5, 10, 15, 20, 25</td>
</tr>
<tr>
<td>0.10%</td>
<td>0, 5, 10, 15, 20, 25</td>
</tr>
<tr>
<td>0.15%</td>
<td>0, 5, 10, 15, 20, 25</td>
</tr>
<tr>
<td>0.20%</td>
<td>0, 5, 10, 15, 20, 25</td>
</tr>
</tbody>
</table>

Measured by 40-10 Image Analyser
Anova showed that there were highly significant differences in colony diameter between the treatments and the control. LSD (Table 24) revealed that there was no significant difference in colony diameter means between the control and 0.05 % sodium benzoate. The differences in colony diameter means between the control and the remaining treatments, and within treatments themselves, were highly significant. This suggests that the minimum concentration of sodium benzoate to inhibit growth of *A. penicillioides* grown on 10 % salt-MEA was 0.10 %.

Table 24. The differences in Colony Diameter Means of *A. penicillioides* Grown in 10 % salt-MEA with Sodium Benzoate.

<table>
<thead>
<tr>
<th>Controls</th>
<th>Concentrations of sodium benzoate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05 %   0.10 %   0.15 %   0.20 %</td>
</tr>
<tr>
<td>0.05 %</td>
<td>0.100     -        -        -        -</td>
</tr>
<tr>
<td>0.10 %</td>
<td>4.90**    4.8**    -        -        -</td>
</tr>
<tr>
<td>0.15 %</td>
<td>7.0**     6.90**   2.10**  -        -</td>
</tr>
<tr>
<td>0.20 %</td>
<td>9.30**    9.20**   2.3**   2.3**   -</td>
</tr>
</tbody>
</table>

Values in mm; °: not significant. **: highly significant.

For both for 0.05 % sodium benzoate and the control, onset of sporulation occurred on the same day (day 7) which was two days after growth initiation. Onset for 0.10 %, 0.15 %
and 0.20 % sodium benzoate were delayed by 1, 2 and 3 day(s), respectively, compared to the control.

4.2.2.3 Effect of sodium propionate on radial growth of *A. penicillioides* on 10 % salt-MEA.

The effect of sodium propionate on radial growth of *A. penicillioides* was assessed and results are presented in Figure 20. Unlike sodium benzoate, sodium propionate was added to 10 % salt-MEA up to a concentration of 0.4 %, because previous results for *A. niger* suggest that at concentration of 0.20 % to 0.30 %, growth inhibition was not pronounced. At this level, however, an off-odour become apparent. Ray and Bullerman (1982) described it as cheesy odour. *A. penicillioides* was still capable of growth and initiation of visible growth was not delayed. Visible growth for all treatments and the control appeared on the same day.

Maximum growth rates for 0.1 % and 0.2 % sodium propionate were delayed by one day and three days, respectively, compared to the control. Growth rates for both 0.3 % and 0.4 % sodium propionate were delayed for 4 days. The fastest growth for all treatments and the control was on day 7. After that, the growth rates gradually decreased or were constant.

Total colony diameters within 22 days of incubation, for 0.1 %, 0.20 %, and 0.30 % sodium propionate were reduced by 12 %, 19 %, and 31 %, compared to the control. Reduction for 0.40 % sodium propionate, i.e. (39 %) was almost equal to that using 0.15 % sodium benzoate (35%).
Figure 20. Growth of *A. penicillioides*
10% salt-MEA + Sodium propionate

Measured by 40-10 Image Analyser
Sporulation for both 0.10 %, and 0.20 % sodium propionate occurred on day 9 of the experiment after four days growth. For 0.3 % sodium propionate, sporulation occurred one day later than the two lower concentrations. For 0.4 % sodium propionate, sporulation occurred on day 14. This suggests that sodium propionate was more effective than other food preservatives in delaying onset of sporulation in both *A.niger* and *A.penicillioides*.

Anova indicated that the differences for treatments and the control were highly significantly different. Similarly, there was a highly significant difference in colony diameters between days of incubation. LSD analysis (Table 25) then revealed that a significant difference in the colony diameter means was evident between 0.1 % and 0.2 % sodium propionate.

**Table 25. The differences in the Colony Diameter means of *A.penicillioides* Grown on 10 % salt-MEA Supplemented Sodium Propionate.**

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>0.10 %</th>
<th>0.20 %</th>
<th>0.30 %</th>
<th>0.4 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.10 %</td>
<td>2.3**</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.20 %</td>
<td>3.7**</td>
<td>1.4*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.30 %</td>
<td>6.0**</td>
<td>3.7**</td>
<td>2.3**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.40 %</td>
<td>7.6**</td>
<td>5.3**</td>
<td>3.9**</td>
<td>1.6**</td>
<td>-</td>
</tr>
</tbody>
</table>

* = Significant; ** = Highly Significant.
Figure 21. Growth of *A. penicillioides* on 10 % salt-MEA + Sodium bisulphite

![Graph showing growth of *A. penicillioides*](image)

**Concentrations**
- Controls
- 0.01 %
- 0.04 %

Measured by 40-10 Image Analyser
The differences in the colony diameter means between the remaining treatments and the control were highly significant.

4.2.2.4 Effect of sodium bisulphite on radial growth of A. penicilloides in 10 % salt-MEA

In Section 4.1.2.1 little effect was demonstrated by sodium bisulphite on growth of A. niger, within the range of permitted levels. The experiment on A. penicilloides used two treatments, 0.01 % and 0.04 % sodium bisulphite (the maximum permitted level). Figure 21 shows that growth for the control and the two treatments were very similar, throughout the incubation period.

The growth rate for 0.04 % sodium bisulphite was slightly higher than for 0.01 % and the control. However, the use of 0.04 % sodium bisulphite did not significantly stimulate growth of A. penicilloides.

Anova indicated that there were no significant differences in the colony diameter means between treatments and the control. Growth of A. penicilloides was not inhibited by the treatments. This also applied to A. niger.

4.2.3 Effect of food preservatives on growth of A. penicilloides as assessed by dry weight

4.2.3.1 Total dry weight assessment on A. penicilloides grown in 10 % salt-CYB supplemented with sodium bisulphite.

Table 26 shows that the use 0.02 % sodium bisulphite, in conjunction with 10 % salt had no effect on growth of
A. penicilloides expressed on dry weight. When the concentration was increased to 0.04 %, it began to have an effect and total dry weight was reduced by approximately 65 %. These results did not parallel those for radial growth, where growth was not inhibited at all, at the same concentration. Some moulds yield long mycelia when they are grown in nutrient lacking-media (Pitt and Hocking, 1984). The moulds which grow rapidly on solid media do not necessarily produce a high quantity of dry weight. The use of 0.06 % sodium bisulphite reduced total dry weight by up to 73 %. This value was equal to the reduction exerted using 0.05 % sodium sorbate or 0.3 % sodium propionate. At this level, this preservative produced a strong odour. Hence, in some countries, its usage is confined to homogeneous products, such as wine and soft drinks (Lueck, 1982).

Table 26. Total Dry Weight of A. penicilloides Grown in 10 % salt-CYB Supplemented with Sodium Bisulphite.

<table>
<thead>
<tr>
<th>Repl. Controls</th>
<th>0.02 %</th>
<th>0.04 %</th>
<th>0.06 %</th>
<th>0.08 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>240</td>
<td>240</td>
<td>70</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>110</td>
<td>90</td>
<td>50</td>
<td>30</td>
</tr>
<tr>
<td>3</td>
<td>170</td>
<td>160</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>x</td>
<td>173</td>
<td>163</td>
<td>60</td>
<td>47</td>
</tr>
</tbody>
</table>
Total dry weight in mg; Rpl: replicate.

When sodium bisulphite at a level of 0.08% was used, reduction of dry weight was up to 92% which was exactly equal to that obtained by using 0.3% sodium benzoate. The use of sodium bisulphite, at this level, however, exceeds the permitted level for the direct consumption (Lueck, 1980), but not for dried foods (Ought, 1983).

**Table 27. The Differences in Dry Weight Means of A. penicilloides Grown in 10% salt-CYB Supplemented with Sodium Bisulphite.**

<table>
<thead>
<tr>
<th></th>
<th>the control</th>
<th>0.02%</th>
<th>0.04%</th>
<th>0.06%</th>
<th>0.08%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.04%</td>
<td>34**</td>
<td>31**</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.06%</td>
<td>38**</td>
<td>35**</td>
<td>40</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.08%</td>
<td>48**</td>
<td>45**</td>
<td>14**</td>
<td>10*</td>
<td>-</td>
</tr>
</tbody>
</table>

Value in mg, 0 = not significant, * = significant; ** = highly significant.

Anova indicated that the differences in total dry weight were highly significant between treatments and the control. The use of 0.02% sodium bisulphite did significantly affect growth of this mould (Table 27). There was no significant difference in dry weight means between 0.06% and 0.04% sodium bisulphite. The difference in dry weight means between 0.06% and 0.08% sodium bisulphite was
significant. The differences in dry weight means between the remaining treatments were highly significant.

4.2.3.2 Total dry weight of *A. penicillioides* grown in 10 % salt-CYB supplemented with sodium propionate.

The effect of sodium propionate on growth of *A. penicillioides* was assessed at the highest level of 0.3 % and results are presented in Table 28. The total dry weight of mould grown in 10 % salt CYB with no sodium propionate (the control) was very much lower than that with no sodium benzoate (the control). The percentage difference between the control for sodium propionate and the control for sodium benzoate experiments exceeded 35 %.

Table 28. Total Dry Weight of *A. penicillioides* Grown in 10 % Salt-CYB Supplemented with Sodium Propionate.

<table>
<thead>
<tr>
<th>Repl.</th>
<th>Controls</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>170</td>
<td>90</td>
<td>130</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>170</td>
<td>100</td>
<td>90</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>160</td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>x</td>
<td>180</td>
<td>117</td>
<td>93</td>
<td>37</td>
</tr>
</tbody>
</table>

Value in mg; Rpl: replication.
Generally, the higher the concentration used, the lower total dry weight yielded, in the range of active levels. Total dry weight for 0.10 %, 0.2 % and 0.30 % sodium propionate was reduced by 35 %, 50 % and 79 %, respectively, compared to the control.

ANOVA showed that between treatments and the control, the differences in total dry weight were highly significant. LSD analysis (Table 29) revealed that there were significantly differences in dry weight means between 0.1 % sodium propionate and the control, and between 0.2 % and 0.3 % sodium propionate. In contrast, there was no significant difference in dry weight means between 0.1 % and 0.2 % sodium propionate.

Table 29. The Difference in Dry Weight Means of A. penicillioides Grown in 10 % salt-CYB Supplemented with Sodium Propionate.

<table>
<thead>
<tr>
<th></th>
<th>controls</th>
<th>0.1 %</th>
<th>0.2 %</th>
<th>0.3 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 %</td>
<td>64*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.2 %</td>
<td>86.7**</td>
<td>23.4°</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.3 %</td>
<td>143.3**</td>
<td>80.0**</td>
<td>56.7*</td>
<td>-</td>
</tr>
</tbody>
</table>

values in mg. * = significant, ° = not significant, ** = highly significant.
4.2.3.3 Total dry weight of *A. penicillioides* grown in 10 % salt-CYB supplemented with sodium benzoate.

The effect of sodium benzoate on growth of *A. penicillioides* was assessed at the highest level of 0.3 % and results are presented in Table 30. Mould growth as assessed by dry weight was not totally inhibited at the highest level tested, but it was very much reduced. The greatest reduction (92 %) was achieved using 0.3 % sodium benzoate and total weight was only 23 mg/50cm³ of broth. Less reduction occurred when the broth was supplemented with lower concentrations, in particular using 0.1 % and 0.2 % sodium benzoate. Total dry weight in both concentrations were 157 mg and 197 mg, respectively, which corresponded to 30 % and 40 % reductions.

**Table 30. Total Dry Weight of *A. penicillioides* Grown in 10 % salt-CYB Supplemented with Sodium Benzoate.**

<table>
<thead>
<tr>
<th>Repl.</th>
<th>the control 0.1 % 0.2 % 0.3 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>280 210 130 20</td>
</tr>
<tr>
<td>2</td>
<td>320 140 170 30</td>
</tr>
<tr>
<td>3</td>
<td>240 240 170 20</td>
</tr>
<tr>
<td>x</td>
<td>280 197 157 230</td>
</tr>
</tbody>
</table>

Values in mg.
Anova indicated that there were highly significant differences between treatments and the control and there were no significant differences in total dry weights between replications. LSD analysis (Table 31) revealed that the difference in dry weight means was not significant between 0.10 % and 0.20 % sodium benzoate. The differences in dry weight means between the remaining treatments and the control were highly significant. This suggests that *A. penicilloides* was less sensitive to sodium benzoate than *A. niger*.

**Table 31. The Differences in Dry Weight Means of *A. penicilloides* Grown in 10 % salt-CYM Supplemented with Sodium Benzoate.**

<table>
<thead>
<tr>
<th></th>
<th>controls</th>
<th>0.1 %</th>
<th>0.2 %</th>
<th>0.3 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 %</td>
<td></td>
<td>83.3**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.2 %</td>
<td></td>
<td>123.3**</td>
<td>40°</td>
<td>-</td>
</tr>
<tr>
<td>0.3 %</td>
<td></td>
<td>256.7**</td>
<td>173.4**</td>
<td>133.4**</td>
</tr>
</tbody>
</table>

Values in mg. **: highly significant; o : not significant.

**4.2.3.4 Total dry weight of *A. penicillioides* grown in 10 % salt-CYM supplemented with sodium sorbate.**

The effect of sodium sorbate on growth of *A. penicillioides* was assessed and results are presented in Table 32. There was no growth in broth supplemented with 0.10 % and 0.15 % sodium sorbate. Reduction in dry weight
averaged 79 % when the broth was supplemented with 0.05 % sodium sorbate. The effect was equivalent to using 0.3 % sodium propionate. Nevertheless, this value was still lower than that exerted using 0.3 % sodium benzoate, but higher than that using 0.2 % sodium benzoate. This suggests that sodium sorbate was more effective against *A. penicilloides* than *A. niger*.

Statistically, the difference in dry weight means between the control and 0.05 % of sodium sorbate was highly significant.

Table 32. Total Dry Weight of *A. penicilloides* Grown in 10 % salt-CYB Supplemented with Sodium Sorbate.

<table>
<thead>
<tr>
<th>Repl.</th>
<th>controls</th>
<th>0.05 %</th>
<th>0.10 %</th>
<th>0.15 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>230</td>
<td>40</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>280</td>
<td>50</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>200</td>
<td>60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>x</td>
<td>253.3</td>
<td>50.0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values in mg; Rpl : replication.

As highlighted in the previous discussion (Section 4.2.1), *A. penicilloides* might be regarded as a xerophilic mould. The presence of 5 % salt in the medium led to its best growth. This was probably due to the achievement of an Aw value conducive to growth. When the level of salt was
increased to 10 %, growth was inhibited. This fact suggests that the presence of 10 % salt and 0.10 % sodium sorbate in the medium had a combined effect. That is why the level of sodium sorbate required to totally inhibit both A. niger and A. penicillioides were the same (0.10 %), when they were grown in 10 % salt-CYB.

4.3 General discussion on the Moulds.

A. niger grew well in CYM, but growth was slightly inhibited in CYM containing 10 % salt. A. niger grew much faster than A. penicillioides in both CYM and CYM containing salt. It might be concluded that A. niger is halotolerant mould. It was not clear whether the superior growth of some moulds on media containing salt was due to the fulfilment of its absolute salt requirement or Aw value achievement. Pitt (1975) discussed the status of fungi which had been claimed to exhibit enhanced growth in salt solutions, ie were considered to be halophilic, and concluded that there was no evidence for halophilism in fungi. Andrew and Pitt (1987) reported that Basipetospora halophila showed a superior growth in media with NaCl, rather than in sucrose and glycerol.

Sodium sorbate was superior to the other food preservatives tested, in inhibiting or delaying growth of moulds, in particular A. penicillioides and A. niger. The application of more than 0.04 % of sodium bisulphite produced a distinctive odour. Other investigators have
mentioned that 0.06% was the limit level producing a strong odour. Compared to the another three food preservatives, sodium bisulphite exhibited the least effectiveness, within the concentrations of the permitted levels. Lueck (1980) confirmed that sulphurous acid and its salt is directed mainly against bacteria, rather than yeasts and moulds. Its action is more effective at low pH, i.e. 3 to 5. As stated in Section 3.24.2, media used were adjusted to pH 6, i.e. the pH of salted dried fish, which might reduce the effectiveness of sodium bisulphite.

Sodium propionate is mainly effective against moulds. The results showed that an application of 0.3% of this food preservative was not able to stop or delay mould growth in either CYM or 10% salt-CYM. This level is actually the maximum level permitted in United Kingdom in foods, but 1000 ppm is the maximum level for raw materials, such as flour.

Growth initiation for A. niger was not delayed when cultured on CYA with no salt, but it was delayed by 2 days on the media containing 10% of salt. The mould was somewhat resistant to both sodium propionate and sodium bisulphite when used at or below permitted levels. This is in agreement with Lueck (1980), who reported that the action of sodium benzoate is primarily against moulds and yeasts, including aflatoxin forming microorganisms. Tanner and Chichester (1972) however, reported this food preservative to be more active against yeasts and bacteria, and less active against moulds.
It was not clear if *A. penicilloides* was halophilic or xerophilic. The investigation was mainly to prevent growth of fungi which are capable of growing in foods containing salt and/or of low water activity, such as salted dried fish. Hence *A. penicilloides* was chosen as one of two test organisms. Pitt *et al.* (1985) identified some new species of fungi from Indonesia. Two of them were *Polypaecilum pisce* and *Basipetospora halophila*. They were isolated from salted dried fish and grew better in medium containing salt (Andrew and Pitt 1987). They reported that *A. penicilloides* was capable of growing in or on media containing high salt, but it grew equally well on the medium containing sucrose and glycerol. The results (Section 4.2.1) shows that this mould hardly grew in CYB/CYA containing 17.5 % NaCl. Andrew and Pitt (1987) reported that this mould was capable of growing in a medium containing saturated salt. Although *A. penicillioiides* was frequently found on salted dried fish, this mould has not yet been isolated from dried fish. Wheeler and Hocking (1988) isolated some fungi from Indonesian dried fish. These were *Eurotium amstelodani*, *A. candidus* and *A. sydowi*. These moulds are regarded as xerophilic, due to capabilities of growing in the low Aw media. However, they did not find *A. penicilloides* in Indonesian dried fish.
4.4 Effect of Food Preservatives on Growth of Halobacterium salinarium in CHM, SDFH and SDF.

4.4.1 Introduction.

Since the Aw value of a saturated salt solution is in the region Aw of 0.75 and a number of microorganisms continue to grow below this limit, it is impossible to completely protect a foodstuff from all microbial attacks by using common salt alone. Some bacteria, such as Halobacterium spp and Halococcus spp need salt for their growth. The type of preservation can influence the total number and type of organism present, thus affecting the level and type of antimicrobial action needed (Branen, 1983). Lowering Aw, in particular using salting and/or drying, can select for those organisms which have the ability to survive and/or grow at a lowered Aw. Also, the possible role of dietary sodium in the development of hypertension in certain individuals has prompted public health and regulatory authorities to recommend reducing dietary intakes of sodium chloride (Sofos, 1986). Both factors suggest that the need for other antimicrobial agents, such as food preservatives, as a supplement to or instead of salt.

With the decline of salt as a common preservative of foodstuffs, much of the economic incentive for studying halophilic bacteria, in particular the genera Halobacterium and Halococcus, has disappeared (Gibbons, 1969). However, Kushner (1985) still indicated optimistically that the
continued interest in these organisms can only be judged by a survey of listings under "halobacteria" and concluded no evidence of a declining interest since 1980.

4.4.2 Growth Media and Methods.

Growth of *H. salinarium* in media, including fish medium supplemented with various concentrations of salt was evaluated at two different temperatures. Results are presented in Table 33 and 34.

Table 33. Growth of *H. salinarium* in Complex Haphilic Agar Supplemented with Different Salts and Temperatures, at 37° and 42° C.

<table>
<thead>
<tr>
<th>Salt</th>
<th>Growth on agar plates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>at 37°</td>
</tr>
<tr>
<td>laboratory NaCl</td>
<td></td>
</tr>
<tr>
<td>15 % + other salts</td>
<td>no growth</td>
</tr>
<tr>
<td>20 % + other salts</td>
<td>fair (7 d)</td>
</tr>
<tr>
<td>20 % no other salt</td>
<td>no growth</td>
</tr>
<tr>
<td>22.5 % + other salts</td>
<td>very good (5 d)</td>
</tr>
<tr>
<td>25 % + other salts</td>
<td>good (6 d)</td>
</tr>
<tr>
<td>30 % no other salts</td>
<td>no growth</td>
</tr>
<tr>
<td>22.5 % + sea salt</td>
<td>very good (5 d)</td>
</tr>
<tr>
<td>25 % sea salt</td>
<td>excellent (4 d)</td>
</tr>
</tbody>
</table>

Other salts: 1 % MgSO₄, 0.05 % FeSO₄, 0.3 % Trisodium citrate, and 0.2 % KCl. Figures in brackets indicate visible colonies.
Webber (1947), Kartznelson (1952), Brown and Gibbons (1960), Dundas et al. (1960), Shegal and Gibbons (1960) and Eimhjellen (1965) have separately formulated complex media for growing extreme halophilic bacteria.

Table 34. Growth of H. salinarium on Fish Agar Supplemented with Various salt concentration.

<table>
<thead>
<tr>
<th>Salt concentration</th>
<th>Growth at 37°C</th>
<th>Growth at 42°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 % + other salts</td>
<td>poor (9 d)</td>
<td>poor (9 d)</td>
</tr>
<tr>
<td>20 % no other salts</td>
<td>no growth</td>
<td>poor (9 d)</td>
</tr>
<tr>
<td>25 % + other salts</td>
<td>good (6 d)</td>
<td>very good (5 d)</td>
</tr>
<tr>
<td>25 % no other salts</td>
<td>very poor (10 d)</td>
<td>very poor (10 d)</td>
</tr>
<tr>
<td>30 % + other salts</td>
<td>no growth</td>
<td>no growth</td>
</tr>
</tbody>
</table>

d: day; other salt: 1 % MgSO₄, 0.05 % FeSO₄, 0.2 % KCl, 0.3 % Trisodium citrate. Figures in brackets indicate visible colonies.

If all the components proposed by those investigators were summed up, there would be 26 items. For the purpose of H. salinarium, the author attempted to produce a formulation that gave a best growth for this bacterium, based on formulations proposed by those investigators. The best growth of this bacterium was observed in CHM containing 22.5 % to 25 % of sea salt, at 42°C C, where visible colonies on agar plates were noted on day 4 (Table 33). Gibbons (1968) suggested that halophilic bacteria grow best in medium containing 25 % to 30 % of salt. However, growth
was not evident when CHM and fish agar was supplemented with 30% of salt.
The results also indicate that sea salt was more desirable than laboratory salt, leading to faster growth. The agar plates containing 22.5% of laboratory salt showed visible colonies on day 5. Laboratory salt was used in the investigation because of the possibility of contaminants interfering with the results obtained by colorimeter. Sea salt was, however, chosen for plating to obtain faster growth times. The faster growth on media supplemented with sea salt might be explained by the fact that the source of this bacterium is sea salt itself (Kushner, 1985 and Gibbons, 1968). Mined salt does not contain these bacteria, but can become infected with Halobacterium during storage or use (Larsen, 1967). The results also indicate that *H. salinarium* still could grow in CHM containing 20% salt, at 42°C and failed to show visible colonies when incubated at 37°C.

The presence of salts, other than common salt, was apparently crucial. 25% salt-fish medium, containing salts other than common salt, showed visible colonies on plates on day 5 as on CHM. Colonies however appeared on day 10, when such salts were excluded (Table 34). Reed (1985) reported that K⁺ was needed for osmoregulation. The gradient of intracellular K⁺ to extracellular K⁺ can be 1000:1. K⁺ may constitute 30 to 40% of dry weight (Sharp and Mushters 1985). Hence, the requirement for K⁺ becomes growth limiting
(Gochnaner and Kushner, 1969). Mg\(^{++}\) plays an important role in colour production. When the Mg\(^{++}\) was omitted, the normal regular rod shape became a coccoid form (Larsen, 1967) and Gibbons (1968) described the shape as "spheres".

When the quantity of casamino-acids added to CHM was reduced from 0.01 % to 0.005 %, visible colonies on both media were noted on the same day (day 5). Even when casamino acids were excluded and yeast extract used instead, colonies appeared on the same day, but were smaller.

The use of 25 % salt-fish medium, supplemented with salts other than common salt, gave promising results for growing H. salinarium, since the visible colonies in both complex medium and fish medium first appeared on the same day (day 5). Some bacteria such as Pseudomonas spp, Vibrio spp and Acinetobacter spp, which are often found on fish, grew faster in fish flesh broth than in the usual artificial media (Chandrasekaran, et al. 1985). To prepare fish agar was extremely time consuming, however, hence fish agar was not used throughout the experiment.

The pigmentation of the extreme halophilic bacteria, in particular H. salinarium, is an outstanding characteristic of these bacteria. The pigments are mainly carotenoids (Larsen, 1967). Within 2 - 3 days, the pink-red colour did not develop in the broth inoculated with H. salinarium, but on day 4, the broth changed to slightly yellow to pink and became completely red on day 5 or 6. This developing pigmentation caused problems when attempting to enumerate.
using a colorimeter. Absorbance values were dependent upon both the quantity of bacteria and the intensity of bacterium's pigment. The more mature the bacteria were, the higher colour intensity was produced. This phenomenon led to higher absorbances for older bacteria than the younger ones of the same population. Hence, in general, enumerating *H. salinarium* by means of colorimetry was not applicable.

4.4.3. Growth of *H. salinarium* in Complex Halophilic Broth (CHB) Supplemented with Food Preservatives.

4.4.3.1 Growth of *H. salinarium* in CHB supplemented with sodium bisulphite.

The effect of sodium bisulphite on the growth of *H. salinarium* in CHB was assessed and the results are presented in Figure 22. None of the treatments delayed initiation of growth, after 3 days of incubation. During this time, the numbers increased by, on average 2 log cycles. Reddening was not yet observed. Subsequently, growth for treatments and the control increased, on average, 1 log cycle and achieved maximum numbers on day 4, when the broth was completely red. On the next day (day 5), growth began to decrease gradually. At this stage, the broth produced a very strong off-odour.

Statistically, there was no significant difference in counts between treatments and the control. This suggests that, at a level of 0.03 % (300ppm) sodium bisulphite, *H. salinarium* was not inhibited. Lueck (1980) reports that most bacteria are sensitive to sodium bisulphite at levels
Figure 22. **Growth of** *H. salinarium*  
**In CHB + Sodium bisulphite**

![Graph showing the growth of *H. salinarium* in CHB with and without sodium bisulphite.](image-url)

- **Y-axis:** Log numbers / cm³
- **X-axis:** Time in days
- **Legend:**
  - *controls*
  - 0.01 %
  - 0.02 %
  - 0.03 %
of 50 to 100 ppm. This food preservative is mainly directed at two groups of bacteria, eg acetic acid producing and malolactic bacteria (Ough, 1983). Sulphite at a level of 100 ppm proves lethal to Acetobacter and Lactobacillus (Amerine, 1980, and Jay, 1986). Salmonellae are also inhibited by sulphite levels of 15-109 ppm, whilst Serratia liquefaciens, S.marcescens and Hafnia alvei are resistant at this level, requiring 185 to 270 ppm free S0₂ in broth to inhibit them (Lueck, 1980).

The inhibitory effect is due to S0₂ attacking the SH group of enzymes. It is not clear whether this bacterium contains less enzyme possessing SH group, but Larsen (1967) clearly indicated that Halobacterium spp contain large numbers of enzymes, such as succinic dehydrogenase, lactic dehydrogenase and cytochrome oxidase, more active in the presence of salt. This shows that the enzymes produced in this organism are different to those of other organisms.

4.4.3.2 Growth of H.salinarium in CHB supplemented with sodium propionate.

The effect of sodium propionate on the growth of H.salinarium was assessed and results are presented in Figure 23. Like sodium bisulphite, sodium propionate was not very effective against H.salinarium. Numbers increased roughly by one log cycle/day and peaked on day 5 for the three treatments and the control. By day 6 growth entered the death phase.
Figure 23. Growth of *H. salinarium*
In CHB + Sodium propionate

![Graph showing growth of *H. salinarium* in CHB with sodium propionate at different concentrations.](image)

- **Controls**
- 0.1%
- 0.15%
- 0.20%

Log numbers/cm³ vs. Time in days.
Statistically, viable counts of the three treatments were not significantly lower than those for the control, throughout the incubation period, suggesting that sodium propionate is not a suitable agent for controlling *H. salinarium*, since the maximum level permitted (3000 ppm) was ineffective. This ineffectiveness against *H. salinarium* might be due to the relatively high pH in the growth medium (pH 6). At this pH value, the undissociated acid is only 6.7 %, when the undissociated molecule of this organic acid is necessary for optimal antimicrobial activity (Jay, 1986). Previous work on moulds (Section 4.1 and 4.2) suggests that the use of this food preservative at a level of 0.1 % gave a pronounced action which indicates that this food preservative is more active against moulds. Jay (1986) stressed that the inhibitory action of propionates is primarily fungistatic rather than fungicidal (Jay, 1986, Anonymous, 1980 and Banwart, 1980).

4.4.3.3 Growth of *H. salinarium* in CHB supplemented with sodium sorbate.

The effect of sodium sorbate on *H. salinarium* grown in CHM was assessed and the results are presented in Figure 24. Sodium sorbate proved very effective against the moulds tested (Section 4.1.2.4, 4.1.3.2, 4.2.2.1 and 4.2.3.4). Sodium sorbate also proved inhibitory towards this bacterium. At a level of 0.1 %, growth was persistently inhibited up to day 4 and peaked on day 5. Viable counts for 0.15 % and 0.2 % of sodium sorbate were consistently lower.
Figure 24. Growth of *H. salinarium* in CHB + Sodium sorbate

![Graph showing growth of H. salinarium in CHB with sodium sorbate at different concentrations.](image-url)
than the control until the end of incubation (day 6). On the other hand, growth for the control was very rapid on day 1 and day 2, and during this time, viable counts increased on average 2 log cycles/day, with only 1-1.5 log cycle increases for the three treatments. The next day (day 3), growth slowed down and reached a peak on day 6 for the 0.15 % treatment. In contrast, the maximum growth for 0.2 % had not been achieved by the end of incubation. Sodium sorbate did not completely stop growth within the range of concentration tested, but merely slowed it down. Robach (1978) reported that sodium sorbate at a level of 0.2 % was lethal to *Pseudomonas fluorescence*, a common agent of fish spoilage. *Listeria monocytogenes*, was inhibited at a level of 0.10 % and totally stopped at level of 0.3 %. *Vibrio parahaemolyticus* (Robach and Aickay, 1978) and other organisms, such as catalase positive groups (Sofos, 1980) were also inhibited by this food preservative. However, it was relatively ineffective against catalase negative bacteria, including *H. salinarium* (Anonymous, 1980).

Anova confirmed that the differences in viable count between treatments and the control were highly significant. Similarly, there was highly significant difference in viable counts between days of incubation.
4.4.3.4 Growth of H. salinarium in CHB supplemented with sodium benzoate.

The effect of sodium benzoate on growth of H. salinarium was assessed and results are presented in Figure 25. Growth for the control was similar to results for previous experiments (Section 4.4.2.3), where 2 log cycles/day increase within the first 2 days of incubation was observed. The increase was 0.5 log cycle, after day 3 and the maximum viable count reached on day 5. On day 6, growth entered into the death phase. It is clear that sodium benzoate considerably inhibited growth of the bacterium, and was even bacteriocidal. Growth for the three treatments was reduced by 0.5 to 1.5 log cycles for the first 24 hrs. Between 2 and 5 days, there was no growth, but the organism appeared again on day 6 for 0.10 % sodium benzoate and day 7 for 0.15 and 0.2 % sodium benzoate treatments. The viable counts were still below the initial inoculum level (4.00), however, the minimum level tested was 0.1 %, so the minimum bacteriocidal level was not discovered.

It is concluded that growth for the 0.1 % was still in log phase by day 6 and it was still in this phase for the 0.15 % and 0.2 % treatments until the end of incubation.

4.4.4. Growth of H. salinarium in salted dried fish homogenate (SDFH) supplemented with food preservatives
Figure 25. Growth of *H. salinarium* in CHB+ Sodium benzoate

---

Control | 0.1% | 0.15% | 0.2%
---|---|---|---

Time in days

Concentrations
4.4.4.1 Growth of *H. salinarium* on SDFH supplemented with sodium propionate.

The effect of sodium propionate on *H. salinarium* grown in SDFH at 37°C was assessed. Results are presented in Figure 26. When this bacterium was grown in SDFH, growth was not as rapid as in CHB. For the first 24 hrs of incubation, increase in viable count approximated 0.5 log for the three treatments and the control, but the viable count for the three treatments were significantly lower than the control. There was no delay in lag phase for the treatments, compared to the control. The next day (day 2) viable counts for treatments and the control were almost equal. However, growth for the 0.2% sodium propionate treatment was consistently inhibited until the end of incubation. The maximum viable counts for the control and 0.10% treatment were achieved on the same day (day 6). Maximum counts for the 0.15% and 0.2% sodium propionate treatments were not obtained until the end of incubation. Although, the use of sodium propionate at the maximum level tested (0.2%) was able to inhibit growth, this was not sufficient to delay the reddening in the homogenate or off-odour which occurred on the same day as the control (day 5). This onset of reddening occurred one day later than in CHB.

Anova shows that the differences in viable counts for 7 days of incubation between treatments and the control were significant. LSD analysis revealed that there were no significant differences in viable count means between 0.10% and 0.15% and between 0.15% and 0.2% treatments, for 7
Figure 26. Growth of *H. salinarium* in SDFH + Sodium propionate

![Graph showing growth of *H. salinarium* in SDFH + Sodium propionate](image)

- **Concentrations**
  - $ullet$ Controls
  - $0.1\%$
  - $0.2\%$
  - $0.3\%$

**Axes:**
- **Y-axis:** Log number of cells per ml
- **X-axis:** Time in days

**Legend:**
- Controls
- $0.1\%$
- $0.2\%$
- $0.3\%$
days of incubation. The remaining differences in viable counts between treatments and the control were significant.

This suggests that the use of sodium propionate significantly reduced viable counts, but the reduction was not enough to delay reddening in SDFH. Hence the use of this food preservative for preserving SDF is of questionable value.

4.4.4.2 Growth of *H. salinarium* in SDFH supplemented with sodium sorbate.

The effect of sodium sorbate on *H. salinarium* grown in SDFH was assessed and results are presented in Figure 27. Growth for the control was slightly different to that obtained on the control during the sodium propionate experiment. This may merely be due to a different initial population obtained. For the first 24 hrs of incubation, day 2, and day 3, viable counts increased by less than 1 log, 2.4 log, and 1.3 log cycles, respectively. On day 4, growth peaked. For all levels of sodium sorbate, growth was inhibited in the first 24 hrs, and bacteriocidal effect resulted in decreasing viable counts. Growth in 0.10 % and 0.15 % sodium sorbate treatment began to increase again on day 2, but was still below the initial population (c. 10^4/cm^3). Growth in 0.2 % sodium sorbate only increased from day 6. Viable counts for both 0.15 % and 0.20 % sodium sorbate did not reach the original level of the inoculum (still in lag phases) by the end of incubation. So, lag phase for 0.1 % was delayed for 4 days, compared to the
Figure 27. Growth of *H. salinarium* in SDFH + Sodium sorbate
control. Reddening did not develop during the incubation period.

Anova indicated that the differences in viable count were highly significant between treatments and the control. Similarly, the differences in viable counts were highly significant between days of incubation. This suggests that sodium sorbate was an effective inhibitor of the bacterium. However its presence at a level of 0.1% was not indefinitely bacteriostatic, so reddening might have occurred few days later. The use of 0.15% and 0.20% sodium sorbate prevented growth more effectively and so would delay reddening.

4.4.4.3 Growth of *H. salinarium* on SDFH supplemented with sodium benzoate.

The effect of sodium benzoate on *H. salinarium* grown on SDFH was assessed and results are presented in Figure 28. Growth trends for the control were similar to that observed during sodium propionate experiments. Viable counts for treatments decreased with increasing concentration of sodium benzoate exerting the greater effect. No growth was detectable on days 2, 3, and 4 for 0.1%, 0.15% and 0.2% sodium benzoate, respectively. These results confirm that when using CHM, sodium benzoate did not only inhibit growth, but was also lethal to *H. salinarium*. Consequently, reddening never occurred on SDFH for all levels of sodium benzoate tested.
Figure 28. Growth of *H. salinarium* in SDFH + Sodium benzoate
4.5 The Use of Food Preservatives in Fish Processing.

4.5.1. Introduction.

The purpose of the experiments described in this section, was to assess potential use of preservatives in processing of fish, to determine roughly the amount of food preservative in fish flesh and to discuss if the results obtained reinforced the results obtained during the previous experiment using CHB and SDFH as the growth media.

Previous results indicated that the order of effectiveness of the antimicrobial agents tested was: sodium benzoate, sodium sorbate, and sodium propionate. Previous results (Section 4.4.2.1) also showed that sodium bisulphite in the range of permitted level had no affect to H. salinarium. However, it was retested, because of its importance as a food preservative in the fish processing industry.

Three methods of food preservative application were considered. The first alternative was application during salting stage, which allows a long exposure (24-24 hrs), leading to penetration into the flesh. However, as Halobacterium and related organisms grow on the surface of salted dried fish, there would be no necessity for this penetration of the food preservative into deep flesh. The third alternative, which was chosen as an appropriate technique, is to dip SDF just after drying into the food preservatives for a period of 3 minutes. Dipping the
products into the food preservative solutions is one of several methods of application available, including spraying and direct addition (Sofos and Busta, 1980).

Results from the preliminary experiments showed that *H. salinarium* hardly grew in the presence of 30% salt (w/v). Hence salting was done by soaking fish in 25% brine for 24 hrs, by which, the salt content in flesh averaged 21.0% at which this bacterium can grow.

Legislation in most countries is designed to produce lists of permitted preservatives and the maximum permitted levels for various food commodities. In most cases, the preservatives are incorporated into homogeneous products, e.g bread, sausages, soft drinks etc, thus allowing producers to easily meet the intentions of legislation in relation to maximum permissible levels (Murray, 1988). However certain fishery products such as salted dried fish and marinades are by nature non-homogeneous. This leads to practical difficulties, regarding legislation. Hence, analysis of residues is crucial. To meet this intention the technique employed in this experiment gave an approximation of residues.

4.5.2 The Use of sodium bisulphite in processing fish.

The use of sodium bisulphite, in processing fish was assessed. Salted dried fish were dipped in this preservative solution for 3 minutes and then inoculated with $10^4$ bacteria
Figure 29. Growth of *H. salinarium*
On SDF+ Sodium bisulphite

![Graph showing growth of *H. salinarium* on SDF+ Sodium bisulphite.]
Samples were assessed at 1 day intervals. The results are presented in Figure 29.

When the three groups of SDF were dipped into 0.2 %, 0.4 % and 0.6 % of sodium bisulphite solutions, preservative residues for the three treatments averaged 0.012 %, 0.023 % and 0.035 %, respectively. In general, treatments moderately inhibited growth of *H. salinarium*, throughout incubation period, leading to lower viable counts than the control. As previously discussed, at a maximum level of 0.03 % (300 ppm), sodium bisulphite did not affect growth when *H. salinarium* was grown in CHB and SDFH. This may be explained that although total residue level in the flesh, for example 0.2 % treatment was only 0.012 %, the concentration would be far higher on the surface layer of SDF where these bacteria normally grow. This suggests that *H. salinarium* might after all be susceptible to sodium bisulphite at high concentration.

Growth for the control increased by, on average, 1 log cycle/day within 6 days of incubation and viable counts for day 8 and 10 were almost equal. From day 10 onwards, viable counts slightly fluctuated and peaked on day 13.

The viable count for 0.6 % sodium bisulphite treatment was slightly lower than those for 0.2 % and 0.4 % treatments. Maximum counts for 0.2 % and 0.4 % treatments were achieved on day 13 and day 15, respectively. Despite lower numbers being present, reddening in 0.2 % 0.4 % and 0.6 % treatments occurred in the same day which was one day
earlier for the control (day 10). This suggests that sodium bisulphite should be no longer used as an agent to combat the reddening on salted dried fish.

4.5.3 The use of sodium sorbate in processing fish.

The effectiveness of sodium sorbate in preventing reddening of salted dried fish as a result of H. salinarium growth was assessed and results of bacterial growth are presented in Figure 30. Sodium sorbate residues for 1%, 2% and 3% of sodium sorbate treatments were 0.058%, 0.117% and 0.175%, respectively. Growth inhibition of H. salinarium for the three treatments was highly significant (P < 0.0). The log phase for 1% sodium sorbate treatment was delayed for 2 days and growth increased on average 0.22 log/day from day 3 to day 15. Maximum viable count was achieved on day 15. On the last day, growth was in the death phase. The use of higher than 1% sodium sorbate was not only bacteriostatic but also bactericidal to H. salinarium, since viable counts for 2% and 3% of sodium sorbate treatments were lower than initial inoculum within 4 days of incubation. Growth for these two treatments fluctuated from 3 to 4.5 log, throughout the incubation period. Up to day 15, there was no evidence of reddening on the surface of SDF for 1% treatment, but a slight off-odour was recognized. The 2% and 3% treatments prevented the formation of off odour in addition to preventing reddening. These results confirm
Figure 30. Growth of *H. salinarium* in SDF + Sodium sorbate

![Graph showing growth of *H. salinarium* in SDF + Sodium sorbate](image.png)
results on CHB and SDFH, that sodium sorbate was effective against *H. salinarium*.

4.5.4 The use of sodium benzoate in processing fish.

The use of sodium benzoate to prevent bacterial growth and consequent reddening in salted dried fish was assessed. Results for bacterial growth are presented in Figure 31.

Sodium benzoate residues determined for 1 %, 2 % and 3 % sodium benzoate treatments approximated 0.058 %, 0.117 % and 0.35 %, respectively. Viable counts for the three treatments decreased from $10^4$ cfu/cm$^3$ (initial inoculum) to $10^3$ cfu/cm$^3$ (1 log cycle decrease) on day 2. Subsequently, this bacterium was not detectable until the end of incubation (day 18). In other words, salted dried fish, dipped into 1 % sodium benzoate solution, contained no *H. salinarium* after 3 days of incubation. This suggests that the most effective preservative for preventing reddening in salted dried fish was sodium benzoate.

4.5.5 General discussion on *H. salinarium*.

*Halobacterium salinarium* is classified as an extreme halophilic bacterium, since its minimum requirement for salt is 20 % and its optimum salt concentration for growth is in the range of 25 % - 30 % (Larsen, 1967). Results (Section 4.4.2) showed that the halophilic medium supplemented with 22.5 % salt gave the best growth for this bacterium.
Figure 31  Growth of *H. salinarium*
On SDF + Sodium benzoate

![Graph showing the growth of *H. salinarium* on SDF + Sodium benzoate, with different concentrations of Sodium benzoate over time.]
The previous results on moulds (Section 4.1 and 4.2) clearly indicated that *A. niger* and *A. penicillioides* demonstrated broadly similar responses to the presence of food preservatives. Sodium sorbate and sodium benzoate exhibited the most activity against them, in the range of permitted levels. Against *H. salinarium* relative effectiveness of the preservatives evaluated differed, in that sodium benzoate was the most effective. The use of sodium benzoate, within the permitted range, was not able to totally inhibit growth of *H. salinarium* in complex halophilic medium at 37° C within 7 days of incubation. When this bacterium was, however, grown in SDFH, it was incapable of growing at the minimum level of 0.05 % sodium benzoate. Growth on salted dried fish, by inoculating the bacterium onto the surface of salted dried fish, reinforced evidence that the bacterium failed to proliferate or was even killed by using sodium benzoate.

Sodium sorbate was not only effective against the two moulds, but was also effective against *H. salinarium*. Some investigators have described that sodium sorbate is mainly active against catalase-positive bacteria. Against *H. salinarium* its efficacy was lower than sodium benzoate, but it was more effective than sodium propionate and sodium bisulphite. Supplementing halophilic medium with sodium sorbate considerably inhibited growth of this bacterium, but it was not bacteriocidal.
Until recently, sodium bisulphite was used in fish food preservation as one means of preventing reddening in salted dried fish. From the results obtained (section 4.4.3.1 and 4.5.2), there was no evidence that this food preservative at a maximum level of 0.03 % is reliable enough to do so. Growth of the bacterium on both Complex halophilic medium and SDFH was not inhibited.

4.6. Growth of *Staphylococcus* sp in 10 % salt nutrient broth (NB) supplemented with food preservatives.

4.6.1 Introduction.

*Staphylococcus* sp was isolated from Indonesian SDF ("Jambal roti") which contained 11 % salt (w/w). This bacterium was chosen, since it represented a major part of the bacterial population of the product when isolated on 10 % salt-NA and and NA with no salt. Sanderson, Indriati, Anggawati and Sudradjat (1988) reported that a gram-positive bacterium isolated from Indonesian salted dried fish was identified as *Staphylococcus xylosus*. In the preliminary experiment, *Staphylococcus* sp was grown on NA supplemented with increasing concentrations of salt and found to be capable of growing on NA containing 17.5 % of salt. Larger colonies and faster growth were observed on NA rather than on 10 % salt-NA.

As described in Section 3.2.3, growth was determined by a M 600 microplate reader which was satisfactory method for monitoring growth of *Staphylococcus*
sp when large numbers of treatments were tested. However, three main drawbacks were experienced using this equipment. Firstly, like other optical methods, the microplate reader was not capable of interpreting decreasing growth. Hence, any lethal action of food preservatives was not clearly shown. Secondly, bacterial absorbances fluctuated by up to 3 points which was equal to $0.67 \log \text{cells/cm}^3$. To simplify the results obtained, numbers fluctuating between 3.8 and 4.5 log were taken to represent the lag phase and any negative absorbance was interpreted as total inhibition.

4.6.2 Effect of sodium propionate on growth of *Staphylococcus* sp

The effect of sodium propionate on *Staphylococcus* sp grown on 10% salt-NA at 25°C was investigated. Samples were assessed at 6 and 12 hr intervals and results are presented in Figure 32.

Growth for the control had passed through the lag phase and was in the exponential phase, and numbers increased by $1.6 \log$ cycles, after 6 hrs incubation. During the second 6 hours, growth slowed down and increased only $0.4 \log$ cycles. After 30 hrs, growth was in stationary phase and entered during the death phase, in the following 6 hrs. Growth in 0.05%, 0.1%, and 0.15%, 0.2%, 0.25% and 0.3% sodium propionate treatments was significantly inhibited for the first 12 hrs, but was in exponential phase, after 24 hrs incubation. After 30 hrs, the differences in numbers between treatments and the control were not significant. This
Figure 32. Growth of Staphylococcus sp in 10% salt NB + Sodium propionate

Log numbers per cm³

Time in hours

Concentrations
- Controls
- 0.06 %  
- 0.10 %  
- 0.15 %
- 0.20 %  
- 0.25 %  
- 0.30 %

by Microplate Reader
suggests that the use of sodium propionate, at the highest level of 0.3 %, was not completely effective against *Staphylococcus* sp grown in NB.

### 4.6.3 Effect of sodium bisulphite on growth of *Staphylococcus* sp

Results are presented in Figure 33. The use of sodium bisulphite at a level of 0.01 % extended the lag phase of *Staphylococcus* sp growth until 6 hrs and growth was significantly inhibited, leading to lower numbers throughout incubation period. When the concentration was increased to 0.02 % and 0.03 %, lag phases were extended until 12 hrs and 66 hrs, respectively. Doubling the concentration, extended delay by 5.5 times. As a result, numbers were significantly lower than those for 0.01 % sodium bisulphite and the control. Growth was completely inhibited using sodium bisulphite at a level of 400 ppm and did not appear until the end of incubation. Compared with *H. salinarium*, *Staphylococcus* sp was more susceptible to this food preservative in the range of 100 to 400 ppm. Lueck (1980) reports that *Staphylococcus* aureus was inhibited at a minimum level of 80 ppm (0.008 %). Banwart (1980), however, generalized that Gram negative bacteria are more susceptible than Gram positive bacteria to this food preservative.

It is difficult to conclude that the use of sodium bisulphite alone could prevent spoilage in SDF, since this preservative did not significantly inhibit growth of
Figure 33. Growth of *Staphylococcus* sp in 10% salt-NB + Sodium bisulphite

![Graph showing growth of *Staphylococcus* sp in different concentrations of sodium bisulphite.](image)
4.6.4 Effect of sodium benzoate on growth of *Staphylococcus* sp

Figure 34 shows that the powerful action of sodium benzoate against *H. salinarium* did not manifest itself against *Staphylococcus* sp. Its usage at a level of 0.05% significantly \( P=0.01 \) inhibited growth, leading to lower numbers than the control until 66 hrs of incubation period. Range levels of 0.02 to 0.04% sodium benzoate are reported as minimum inhibitory concentrations against *Staphylococcus* spp, at pH 5.2 - 5.6 (Lueck, 1980). Growth rates during the first 24 hrs of growth for the control and 0.05% treatment were 0.1 log/hr and 0.07 log/hr, respectively. Growth for 0.1% and 0.15% sodium benzoate treatments was delayed for 24 hrs and 48 hrs respectively and significantly \( P=0.00 \) inhibited. When the concentration was increased by 0.05%, the delay was doubled. Growth was completely stopped, when the concentration was further increased to 0.20%. Since absorbances for 0.2% to 0.3% treatments were negative (lower than blank value), the use of sodium benzoate at a level up to 0.2% might be lethal to this bacterium. Regulations in the USA (GRAS), permit a maximum level of 0.1% of sodium benzoate for preserving foods, at which concentration effectiveness against *Staphylococcus* sp was observed. In salted dried fish where its Aw is very low (0.76 - 0.86) and nutrients are not as comprehensively
Figure 34. Growth of *Staphylococcus* sp in 10 %-NB + Sodium Benzoate

![Growth graph showing the effect of different concentrations of sodium benzoate on the growth of *Staphylococcus* sp.](image)

**Concentrations**
- Controls
- 0.05 %
- 0.1 %
- 0.15 %
- 0.20 %

by Microplate Reader.
available as in nutrient broth, action of sodium benzoate against *Staphylococcus* sp may be more effective, thus requiring a lower concentration. A conclusion was that the use of sodium benzoate for preventing spoilage in SDF was promising.

4.6.5 Effect of sodium sorbate on growth of *Staphylococcus* sp

Figure 35 shows that numbers for 0.05 % and 0.1 % sodium sorbate treatments fluctuated from 4.03 to 4.35 log throughout the incubation period (120 hrs). Growth was still in the range of initial inoculum (4 log). This means that growth may be still in the lag phase and totally inhibited until the end of incubation. Absorbance values for 0.15 % to 0.3 % sodium sorbate were negative (less than blank absorbance values). Hence, sodium sorbate may be lethal to *Staphylococcus* sp at those levels. Salmonellae can be killed by 0.1 % of undissociated sorbic acid (Anonymous, 1980). Other investigators report that sorbic acid and its salts are mainly effective against catalase positive bacteria, including *Staphylococcus* spp (Anonymous, 1980; Lueck, 1980; Furia, 1980; Banwart, 1980 and Sofos, 1983). Sodium sorbate proved the most promising agent for preserving salted dried fish, as a result of its wide spectrum of activity against several bacteria and moulds. It might prove even more powerful if used in conjunction with sodium benzoate.
Figure 35. Growth of *Staphylococcus* sp in 10% salt-NB + Sodium sorbate

Log numbers/
Log numbers/
Log numbers/
Log numbers/

Time in hours

concentrations
Controls

0.05 %

0.1 %

0.15 %: No growth

by a Microplate Reader
4.7 Growth of A. niger on Gradient Diffusion Systems.

4.7.1 Introduction.

Two-dimensional plates have potential in examining synergism between chemical effectors, including biocides and antibiotics. Waters and Lloyd (1985) used the techniques to assess the effect of salt, pH and temperature on the growth of luminous bacteria. Sacks et al. (1986) examined growth of moulds on pH gradient plates. Recent work conducted by Gadd et al. (unpublished) examined the toxicity of some heavy metals to Aspergillus pullulans.

In this experiment, gradient diffusion systems were used to determine the effects of sodium sorbate and sodium benzoate on growth of A. niger and to assess any possible synergistic effects between the two preservatives.

4.7.2 Effect of food preservatives on radial growth of A. niger as assessed by one-dimensional diffusion gradient system.

4.7.2.1 Method.

Growth of A. niger on one-dimensional gradient plate diffusion system was assessed. A 7 x 1.25 x 0.5 cm well in the agar plate had 4 cm$^3$ sodium sorbate or sodium benzoate solution (0.5 to 4.5 %) added to it. The agar plate was then incubated at 25$^\circ$C for 48 hrs to allow the preservative to diffuse into the agar. A steel grid plate was inserted to stop further diffusion and to divide the agar plate into 49 small agar squares (0.81 Cm$^2$ each). The actual concentration gradient of sodium sorbate or sodium benzoate
produced was not quantified (for technical reasons, although attempts to develop an HPLC method for the preservatives were made during the project). Spores were inoculated into each small agar square (see Section 3.23). Radial growth in each row containing 7 small agar squares was measured by a 40-10 Image Analyser at one day intervals. Figure 37 shows the arrangement of the system used.

4.7.2.2 Effect of sodium sorbate on growth of *A. niger* assessed by gradient diffusion plate.

The effect of sodium sorbate on growth of *A. niger* was assessed and results for radial growth measurement after 72 hrs incubation are presented in Figure 37. After 72 hrs incubation, growth for 0.5 % sodium sorbate was not visible on row I (1 cm from the well). Colonies on row I gradually appeared in row 1, after 5 days, respectively. Results overall suggest that there was no complete growth.
Figure 37. Effect of sodium sorbate on
Growth of A. niger Assessed by Gradient Diffusion Plate
Assessed after 72 hrs incubation
inhibition, using 0.5 % sodium sorbate, after 5 days of incubation, since colonies appeared on row I at this time. When the concentration was increased to 1 %, growth trends were similar to that for 0.5 % sodium sorbate. When the concentration was further increased to 4 %, a larger zone of inhibition was observed from rows I to IV, after 72 hrs of incubation. The size of the inhibition zone was not extended, when the concentration was further increased to 5 %, but growth on all rows was slower than for 4.0 % sodium sorbate treatment. This suggests that the size of inhibition zone and colonial growth were affected by the concentration of food preservatives used and incubation period. The higher the concentration used, the wider the inhibition zone and the slower growth became. Barry (1980) proposed 9 main factors influencing diffusion tests, including inoculum, nature of the zone edge, agar depth, composition of the agar medium, growth characteristics of the test strain, temperature of incubation, incubation time and concentration of antibiotics or food preservatives in the reservoir. In this experiment, all those factors were well controlled.

4.2.3. Effect of sodium benzoate on radial growth of *A. niger* as assessed by gradient diffusion agar systems.

The effect of sodium benzoate on radial growth of *A. niger* on gradient diffusion plate was assessed. Results for radial growth of *A. niger* incubated for 72 hrs are presented in Figure 38. When 0.05% and 1 % sodium benzoate
Figure 38. Effect of Sodium Benzoate on Growth of A. niger Assessed by Gradient Diffusion Plate

After 72 hrs incubation
were used in the well, *A. niger* was able to grow on row I of the agar plate, after 72 hrs incubation. When the concentration was further increased to 3 %, growth was not visible on rows I and II, after the same time interval. As far as size of inhibition zone was concerned, there were no significant differences observed using 3 %, 3.5 %, 4 % and 4.5 % sodium benzoate treatments where inhibition zones reached row II in 72 hrs. Results indicate that for all concentrations tested, a consistent gradient was noted up to row 5 as assessed by colonial diameter. Radial growth on row 6 and 7 was not consistent. The use of 4.5 % produced a straight line relationship between colonial diameter and distance from well.

These results indicate that sodium sorbate was more effective than sodium benzoate against *A. niger*.

4.7.3 Effect of sodium sorbate and sodium benzoate on radial growth of *A. niger* assessed by two-dimensional gradient diffusion systems.

A two-dimensional gradient diffusion system was obtained by adding 4 cm$^3$ of sodium sorbate into well 1 and another 4 cm$^3$ into well 2 in the agar plate at the same concentration (Figure 39). The agar plate was incubated at 25$^0$ C for 48 hrs. A steel grid was inserted to stop further diffusion and divide the agar plate into 49 small agar (0.9 x 0.9 cm$^2$) squares. After being inoculated with spores on each small agar square, the plate was incubated at 25$^0$ C. This
was used as the control (see Section 3.2.81). Radial growth was measured at 1 day intervals using 40-10 image analyser.

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</table>

Figure 39. Two-dimensional gradient diffusion plate.

Results for radial growth of *A.niger* on the agar plate containing 4 %/4 % and 4.5%/4.5 % sodium sorbate are presented in Figure 40 a and b, respectively. Colonies for the 4 %/4 % sodium sorbate system were not visible on rows I-IV and col.1-3, after 3 days incubation. The numbers of rows inhibited (4 cm from the well) was exactly same as for the one-dimensional gradient diffusion plates for sodium sorbate. After 4 days incubation, the size of the incubation zone decreased to rows I-II and col.1-3. When the concentration was increased to 4.5 %/4.5 %, growth was not visible on rows I-IV and cols.1-3 after 3 days, but the size of inhibition zone was wider than that for the one-dimensional diffusion of sodium sorbate, under the same
conditions. After 5 days of incubation, the size of the inhibition zones decreased to rows I-II and cols.1-2 (Figure 40).

Another two-dimensional gradient diffusion system was obtained by adding 4 cm$^3$ sodium sorbate in well 1 of the plate and 4 cm$^3$ sodium benzoate in the well 2.

Results for 4 % sodium sorbate/4 % sodium benzoate and 4.5 % sodium sorbate/4.5 % sodium benzoate are presented in Figure 40 c and d, respectively. When 4 % sodium sorbate and 4 % sodium benzoate were used in well 1 and in well 2, respectively, the size of inhibition zones were 2 cm from wells 1 and 2. This was retained until the end of incubation (day 5). Compared to the control (ie sodium sorbate/sodium sorbate system), radial growth was less inhibited.

When the concentration was increased to 4.5 % sodium sorbate and 4.5 % sodium benzoate (Figure 40 d), growth in cols.1-2 (2 cm from well 2) and along rows I-III (3 cm from well 1) was not visible. Compared to the control, radial growth was also less inhibited. Compared to results for one-dimensional diffusion plates using sodium benzoate at the same level, the numbers of rows inhibited was larger, under the same conditions. However, this inhibition zone was narrower, compared to one-dimensional gradient diffusion agar plates for sodium sorbate, under the same conditions. This indicates that the presence of sodium sorbate strengthened the efficacy of sodium benzoate. However, the presence of sodium benzoate weakened the efficacy of sodium
sorbate. Scardavi (1965) indicated that the efficacy of a mixture does not always correspond to the additive effects of the single substances, for it may be sometimes superior and sometimes inferior to it. It is too soon to conclude that there is any synergistic effect between sodium sorbate and sodium benzoate, since the actual residues in each small square agar were not quantified. Gadd et al. (1985) postulated that the synergism between food preservatives could only doubtfully be determined by means of this system, since the superior food preservative would dominate.
Figure 40. Effect of Sodium sorbate and sodium benzoate on Radial Growth of *A. niger* Assessed by Gradient Diffusion Agar Plate Systems

a. 4% sodium sorbate (A) and 4% sodium sorbate (A1).

A-------------------------→ A-------------------------→

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Values in mm; day 2

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Values in mm; day 3

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Values in mm; day 4

values in mm; day 5

171
b. 4.5 % sodium sorbate (A) and 4.5 % sodium sorbate (A1).

Values in mm; day 2

Values in mm; day 3

Values in mm; day 4

Values in mm; day 5.
c. 4% sodium sorbate (A) and 4% sodium benzoate (B).

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<th>Day 5</th>
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d. 4.5% sodium sorbate (A) and 4.5% sodium benzoate (B).

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5. CONCLUSIONS.

1. Against *A. niger* and *A. penicillioides*, within the permitted levels, the effectiveness of the preservatives tested are ranked in decreasing order of effectiveness as follows: sodium sorbate, sodium benzoate, sodium propionate and sodium bisulphite, as far as the maximum permitted levels were concerned.

2. Against *H. salinarium*, the decreasing order of effectiveness was sodium benzoate, sodium sorbate, sodium propionate and sodium bisulphite.

3. Against *Staphylococcus* sp, the decreasing order of effectiveness was sodium sorbate, sodium bisulphite, sodium benzoate, and sodium propionate.

4. Sodium bisulphite.

   The maximum level of sodium bisulphite needed to inhibit *A. niger* grown in both CYA and CYB was 0.02%. *A. penicillioides* was apparently more resistant than *A. niger* to the presence of sodium bisulphite which at a level of 0.04% was not significantly inhibited when grown on CYA. However, total dry weight was significantly reduced when used at the same level. At the highest level tested, of 0.03%, sodium bisulphite was not able to inhibit growth of *H. salinarium* in complex
halophilic medium (CHM). SDF previously dipped in the highest level of 0.6 % had a significantly reduced microbial load, but reddening in SDF was not prevented. Therefore, this preservative cannot be recommended for preventing reddening in salted dried fish. Staphylococcus sp was completely inhibited at a level of 0.03 %.

5. Sodium propionate.

Sodium propionate at the highest level tested of 0.3 % did not prevent growth of A. niger in either CYA, CYB, 10 % salt-CYA or 10 % salt-CYB, although growth was partly inhibited at a minimum level of 0.05 %. A. penicillioides was more resistant than A. niger against this preservative. At the highest concentration of 0.4 %, growth was not totally inhibited. The use of sodium propionate at the highest level of 0.3 % was not capable of inhibiting growth of H. salinarium in CHM, but could significantly inhibit the growth in SDFH. However, reddening in SDFH was not prevented. At the highest level of 0.3 %, sodium propionate did not significantly inhibit growth of Staphylococcus sp.

6. Sodium benzoate.

Although sodium benzoate at a level of 0.2 % still allowed growth of A. niger in CYA and CYB, it delayed the initiation of growth for 3 days, when this preservative was used in conjunction with 10 % salt in CYA. In 10 %
salt-CYB, the use of 0.15 % of sodium benzoate completely inhibited growth. With *A. penicillioides*, sodium benzoate was not capable of totally inhibiting growth at the highest tested levels of 0.2 %. However, radial growth and total dry weight were significantly reduced at the minimum level of 0.1 %. This preservative was most effective against *H. salinarium*. The use of 0.1 % and 0.15 % in CHM prolonged the lag phase for 5 and 6 days, respectively. In SDFH, the use of 0.1 % was lethal to this bacterium which did not appear until the end of the incubation period (day 7). SDF previously dipped into a 1 % solution and inoculated with $10^4$ cells/cm$^3$ was free from this bacterium. Sodium benzoate was also effective against *Staphylococcus* sp and at a level of 0.2 %, complete growth inhibition was achieved.

7. Sodium sorbate.

The use of sodium sorbate at the lowest level of 0.1 % was not completely inhibitory to *A. niger*, but this preservative did delay visible growth in CYA for 3 days and growth was totally inhibited when CYA and CYB were supplemented with 10 % salt. With *A. penicillioides*, growth was completely inhibited when sodium sorbate was used at a level of 0.1 % both in 10 % salt-MEA and in 10 % salt-CYB. Sodium sorbate was also active against *H. salinarium*. The use of 0.1 % significantly inhibited
growth in CHM, but did not delay the lag phase, at the highest level tested of 0.2%. In contrast, the use of 0.1% in SDF prolonged the lag phase for 3 days and growth was still in the lag phase until the end of incubation when the concentration was increased to 0.15%. With SDF previously dipped in 1% of the preservative solution, this bacterium was completely inhibited, hence reddening in SDF was prevented. *Staphylococcus sp* were very sensitive to sodium sorbate and at a level of 0.05%, growth in 10% salt-NB was totally inhibited.

8. Using the two-dimensional diffusion gradient system, the comparative effectiveness of food preservatives could be determined. Sodium sorbate was clearly more effective than sodium benzoate against *A.niger* and the effectiveness of the latter was increased in the presence of the former.

9. The use of sodium sorbate in conjunction with sodium benzoate might have the widest action against fungal and bacterial spoilage in SDF. As a result, the use of these two preservative in salted dried fish may reduce losses. Smoked fish which is more susceptible to mould attack should be treated with these two preservatives to extend shelf life and reduce losses.
10. Application of food preservatives by dipping is very practical, since the preservatives coat only the fish product surfaces on which moulds and halophilic bacteria normally grow.
6. FURTHER WORK.

6.1 Analysis of residues.

Food preservatives are mostly used in homogeneous foods or drinks in which the concentration of a preservative can easily be assessed. However, application of food preservatives to fish products, in particular salted dried fish, smoked fish and boiled fish is normally achieved by dipping or spraying. To conform to permitted levels, analysis of residues in fish flesh are essential. The effects of dip concentration, dipping time and chemical composition of the fish on preservative residue levels need to be determined under field and laboratory levels. The effect of storage time on preservative residues should also be assessed.

6.2 Further assessment on other moulds and bacteria.

Results obtained showed that the organisms used (moulds and bacteria) responded differently to the food preservatives tested. These four preservatives should be further tested against other moulds and bacteria isolated from fish products, in particular salted dried fish. Examples of such organisms include the moulds, Polypaecilum pisce, A. candidus, A. wentii and the bacteria, Staphylococcus spp, Micrococcus spp, Pseudomonas spp and Halococcus spp. It is important that these experiments are conducted under field conditions.
6.3 The combined effect of sodium sorbate and sodium benzoate.

Sodium sorbate and sodium benzoate proved most effective against the moulds and the bacteria tested, with sodium sorbate being most effective of the four preservatives tested. However, the price of sorbic acid and its salts is c. 4 times that of benzoic acid and its salts. Hence the use of a combination of these two preservatives should be further investigated by means of radial growth and dry weight assessments and any other appropriate techniques.

6.4 Investigation on toxin producing moulds.

Some investigators have isolated toxin-producing moulds from fish products. Analysis of the effects of food preservatives on toxin producing moulds isolated from fish products may be important and should be further investigated.

6.5 Field work.

Application of sodium sorbate and sodium benzoate should be assessed for other cured fish products, in particular during storage and distribution. Field trial data is necessary to support laboratory work. Financial evaluation is also essential in small scale fish processing in developing countries.
7. REFERENCES.


Dussault, H.P. 1957. The salt tolerance of bacteria. In the microbiology of Fish and meat curing brines edited by Eddy, B.P. Her Majesty's Stationary Office, pp 61-68.


TDRI. 1982. Fish handling, preservation and processing in tropics: Part 2, pp 4-12.


8. APPENDICES

Appendix 1. Formulation of Media used.

Media used for cultivating moulds included the following:

1. Czapek Yeast Medium (CYM) (formulated by Pitt and, King, 1985).

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<tr>
<td>Czapek Concentrate</td>
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<tr>
<td>Yeast Extract</td>
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<tr>
<td>Agar (or without for broth)</td>
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</tr>
<tr>
<td>Water to</td>
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</table>

Czapek concentrate consisted of

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2. Defined medium.

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3. Malt Extract medium (MEM)

This medium was basically formulated by Oxoid which was supplemented with 5 % sucrose.

Malt extract : 30.0 g
Bacteriological pepton : 5.0 g
Sucrose : 50.0 g
Agar (or without for broth) : 15.0 g
Water to : 1.0 l

4. 10 % salt-Czapec Yeast Medium and 10 % salt- Malt Extract Medium.

These media were formulated similar to points 1 and 3 with the addition of 100 g salt/l solution (w/v).

5. Complex Halophilic medium (CHM).

This medium was basically designed by Sehgals Gibbons (1960) and Eimhjellen (1965):

Yeast extract : 10.0 g
Casamino acids : 7.5 g
Trisodium citrate : 3.0 g
KCl : 2.0 g
MgSO$_4$7H$_2$O : 10.0 g
FeSO$_4$7H$_2$O : 0.05 g
NaCl (sea salt) : 225.0 g
Agar (or without for broth) : 10.0 g
Water to : 1000.0 cm$^3$
6. 15 % salt-Nutrient Agar.

Nutrient agar formulated by Oxoid was supplemented with 100 g of NaCl/litre medium (w/v). It was dissolved in water and filtered through Whatman no.1 paper before being used.
Appendix 2  Standard curve of Staphylococcus sp

\[ Y = 0.6735X - 2.5355 \]
\[ r = 0.9812 \quad t = 8.8821 \quad P\% = 0.31\% \]
Appendix 3  Standard curve of *H. salinarium*

\[ Y = 0.7236X - 2.2462 \]

\[ r = 0.9807 \]

\[ t = 18.8338 \]

\[ P\% = 6E^{-2}\% \]