STUDIES ON GLUTAMIC ACID PRODUCTION BY BACILLUS ISOLATES INVOLVED IN 'DADDAWA' FERMENTATION.

A DISSERTATION

BY

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DEPARTMENT: MICROBIOLOGY

DECLARATION

I hereby declare that the content of this thesis is purely a record of my research findings. No part of this work has been submitted or accepted in any previous application for a degree or qualification elsewhere. All quotations and literature cited from other sources are duly acknowledged.

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CERTIFICATION

THESIS APPROVAL

This thesis by LUCY JUMEYI OGBADU meets the regulations governing the Degree of Doctor of Philosophy of Ahmadu Bello University and is approved for acceptance in partial fulfilment of the requirements for the degree.

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To God be the glory for everything.

DEDICATION

Through the grace of God

To the memories of
my late Dad - Egbunu,
grandparents - Ejiye, Ocheja and Asana;
and my Mum, for their love and unsurpassed
legacy of purity of heart.

To my husband - Goddy
Our children - Egbi, Attah, Agwuye and Odoma;
my sisters and brothers,
all for their love.

ABSTRACT

The fermentation of African locust bean (Parkia biglobosa) and soya bean (Glycine max) seeds for daddawa production are carried out by Bacillus species. The species which appeared to be responsible for the fermentation of locust bean seeds were found to be variable. In six separate fermentations, B._ subtilis, B. pumilus and B. licheniformis were identified. Bacillus subtilis predominated in four of the six fermentations. In soya bean fermentation, on the other hand, B._ subtilis and B._ pumilus were consistently isolated in all the three fermentations examined. The growth of the B._ subtilis was favoured by low oxygen tension. A rise in pH and temperature were observed in the fermentations of both African locust bean and soya bean.

A comparative analysis of glutamic acid contents of both locust bean and soya bean daddawa showed higher value in the latter. Release and increase in glutamic acid in fermenting locust bean daddawa by B. subtilis- DU_2 was observed to correspond with the trend of glutamic acid synthesis by the same isolate in a basal medium.

A total of twenty-five Bacillus strains comprising of eight species were isolated from five Nigerian fermented vegetable proteins and fermenting daddawa. The Bacillus isolates were screened for glutamic acid producing ability in the basal medium. They were all found to produce glutamic acid in varying concentrations and are thus confirmed to contribute to levels of glutamic acid in the fermented vegetable proteins.

Bacillus subtilis - DU_2 involved in daddawa fermentation was selected for studies in which the effects of various nutrients and growth parameters on glutamic acid production were investigated. It was observed that glucose, sucrose, galactose and fructose, all of which are found in locust bean seeds supported high yields of glutamic acid. Some intermediates of the Kreb's cycle namely succinate and citrate supported yields of glutamic acid which were generally lower than those of the sugars. Biotin was essential for glutamic acid production and the optimal concentration was 4-6ug/l. Among the metal ions investigated, manganese and magnesium were the best while zinc was the least in supporting glutamic acid synthesis. Cobalt was found inhibitory to both growth and glutamic acid production of the isolate. The best pH and temperature range for optimum glutamic acid production were 7.5 and 35°C respectively. Urea, ammonium chloride and ammonium sulphate were found to support good glutamic acid production as nitrogen sources.

When the basal medium was supplemented with different flours of leguminous seeds, there were no appreciable difference in the yield of glutamic acid over that of basal medium alone. Glutamic acid yield was highest in the soya bean supplemented medium followed by medium supplemented with locust bean, cowpea, peanuts and bambara nuts.

The surface active agents, tween-80 and triton-X were incorporated into the media at the beginning and at 66^{th} hour of the fermentation. Both surfactants caused increased glutamic acid yields by the organism and in about equal

concentrations for optimal yields. Addition of the surfactants at the beginning of the fermentation had a more enhancing effect on glutamic acid release than their addition at 66^{th} hour. Viability of the fermenting organisim did not appear to be affected at the concentrations of the surfactants used.

Exposure of the wild B._ subtilis - DU_2 to ultraviolet light and X-ray resulted in producing some strains which at 32°C produced levels of glutamic acid comparable with that of the wild strain but at 40°C, they (mutants) produced higher levels of the amino acid. There was no appreciable observable influence on the wild isolate exposed to acridine orange in glutamic acid synthesis.

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CHAPTER 1

INTRODUCTION

The art of fermenting foods for the purposes of improving taste and prolonging shelf life of the food is ancient in most cultures of the world including Africa. Fermented foods of African origin include varieties of beverages from cereals, starchy foods from tubers and condiments from leguminous plant proteins. African indigenous condiments are used for taste or flavour enhancement. They are added to dishes directly, used as thickening agents in soups, moulded and used as 'meat' or added to meat in small quantities.

The countless varieties of fermented leguminous plant protein foods found across West Africa may have their origin in the abundant existence of tropical tree legumes supported by the climatic conditions in the region (Campbell-Platt, 1980). The warm tropical temperatures of the region also favour the development of mesophiles generally associated with these fermentations. Among the different fermented leguminous proteins of West Africa are 'daddawa', varieties of 'ogiri', 'ugba' (Odunfa, 1985a) and 'okpihe' to name a few. 'Daddawa' appears to be most widely utilised among the different ethnic groups of West Africa, as shown by the consumption areas which span the whole breadth of the Savannah region (Campbell-Platt, 1980).

Until very recently, microbiological information on many African indigenous food fermentations, including those

of leguminous plant seeds, have been scarce or unavailable (Okafor, 1980). The recently published microbiological information on Nigerian plant protein fermentations include those of Odunfa (1981a) for locust bean 'daddawa' fermentations, Obeta (1983) for 'ugba' fermentations, Popoola and Akueshi (1985) for Soybean-'daddawa' fermentation and Odunfa (1981b) for 'ogiri' fermentation. In all the reports, the involvement of Bacillus species has been established. The importance of the genus Bacillus in fermentations of leguminous plant protein foods therefore cannot be overemphasised. The report of Antai and Ibrahim (1985) which implicated Leuconostoc species in locust bean-'daddawa' fermentation conflicts with the earlier publication of Odunfa (1981a) on the same fermentation. Microbiological studies of uncontrolled traditional fermentations often produce conflicting results since mere contaminants of the system can create dissimilarity in the reports of scientists if adequate care is not taken. Such was the case with reports on 'tempeh' the popular Indonesian soybean fermented product and 'garri', the equally popular West African fermented cassava gratings. While 'tempeh' was at one time reportedly fermented by Aspergillus oryzae (Burkill, 1935) it was later thought to be fermented by Rhizopus oligosporus (Djien and Hesseltine 1961). Later on, Hesseltine (1965) confirmed that, although other fungi could be isolated, only strains of Rhizopus could produce tempeh in pure culture. Collard and Levi (1959) reported Corynebacterium species as the

major fermenting organism in cassava fermentation for garri production, but Okafor (1976) reported Leuconostoc species as the major fermenting organism since Corynebacterium species were found in low numbers. On the other hand, Ngaba and Lee (1979) reported Lactobacillus and Streptococcus as the fermenting organisms.

Changes in fermenting systems often arise from a combination of some or all of the following activities; namely, microbially liberated enzyme activities, activities of enzymes indigenous in the food, microbial metabolites, nutrient uptake by the micro-organism and oxidation of food components. Odunfa (1985b) while monitoring the biochemical changes in fermenting locust beans reported that the most significant activity during the fermentation was the rapid and steady increase in the quantity of free amino acids. The number of amino acids, namely glutamic acid, valine, aspartic acid and alanine, increased rapidly and several fold. Similarly, Anosike and Egwuatu (1980) reported detection of liberated glutamic acid among other free amino acids in fermented castor oil (Ricinus communis) seeds for ogiri production and speculated on possible contribution of glutamine by the fermenting organism. Taste enhancement in soysauce has been attributed mainly to the soluble nitrogenous compound like amino acids, although about 300 flavour compounds have been reported by various workers (Yokotsuka, 1981).

Glutamic acid is now well established as a taste enhan-

cing compound. The monosodium salt of this amino acid is produced in tonnes in Asia and U.S.A and is exported in various forms to other countries including Nigeria. Unfortunately, there is a dearth of microbiological information on glutamic acid production in English language; most of the available literature is found largely in oriental languages, especially Japanese. There are reports of rapid release of glutamic acid in the fermenting indigenous condiments (Odunfa, 1985b; Anosike and Egwuatu,1980) but no information exists on glutamic acid release by the fermenting isolates from these condiments.

With the introduction of taste enhancing preparations like bouillon cubes in Nigeria, the indigenous fermented condiments suffered neglect particularly amongst the enlightened urban dwellers. However, it was soon realised that the bouillon cubes could not fill the place of the indigenous taste enhancers in traditional dishes where the latter's typically characteristic aroma form an inseparable part of the taste. Consequently, the indigenous condiments now feature prominently along with imported ones in nationwide television programmes in which recipes are advertised. Apart from the strong and typical aroma of the native condiments, comparative analyses have shown that the nutritive value of the daddawa is much greater than that of bouillon cubes (Okeoma, 1984).

Although it is generally known that micro-organisms

liberate certain metabolites during metabolism, it was the discovery of efficient glutamic acid producing bacteria that heightened interest in production of amino acids by micro-organisms (Kinoshita et al., 1957). Soon after the awareness of the significance of accumulating various amino acids it became apparent that wild type strains obtained from nature could not produce industrially significant amounts of amino acid. It is now well known that certain mechanisms exist which regulate cellular metabolism, thereby controlling over-synthesis of metabolites in a cell. The regulatory effector or co-repressor is usually either the end products or derivatives of the end product. Proof of this is seen in auxotrophic mutants which tend to oversynthesize precursors or related metabolites of a blocked reaction as a result of not being able to produce the co-repressor. This discovery paved the way for the search or selection of mutants with improved production abilities.

Apart from improving flavour of foods, glutamic acid plays an important role in the body in that it serves as a common provider of amino groups for the process of transaminations which is necessary for synthesis of most of the non-essential amino acids (Holum, 1969). This role is clearly illustrated in Fig.2.

In view of the afore-mentioned observations concerning conflicting or unavailable microbiological information on daddawa and glutamic acid fermentations, this project was designed to:

1. re-investigate the microbiology of the fermentation and to confirm the kinds of micro-organisms involved.
2. examine the role of fermenting micro-organisms in glutamic acid release during the fermentation.
3. explore the potential utility of selected isolates in commercial production of glutamic acid by studying the effect of fermentation parameters on their performance.
4. investigate the influence of surfactants on glutamic acid release in culture.
5. induce genetic improvement of glutamic acid producing strains by exposing them to some mutagens.

CHAPTER 2

LITERATURE REVIEW

2.1 Food Fermentations

The art of fermenting foods in various parts of the world is believed to be ancient, dating back to thousands of years. Van Veen (1957) defines fermented food as any food that has one or more of its constituents acted upon by micro-organisms, namely bacteria, yeasts or fungi for a period, so that the final products have often undergone very considerable changes in chemical composition and other aspects. He goes on to say "sometimes these changes are due not only to microbial action but also to autolytic processes brought about by the enzymes of the product itself". Undoubtedly, the origin and true nature of the changes or interactions occurring in a food fermentation process can only be elucidated by research. Thus in advanced countries, considerable research has been done on the common fermented foods. This has led to better control of the fermentations by means of starter cultures and optimum process conditions and consequent improvement in the quality of the foods. In addition production of the foods has become factory- rather than village-level operations. In contrast, most African fermented foods have not been developed to any appreciable extent. Okafor (1980) has proposed a scheme for a research-oriented improvement of African fermented foods.

2.1.1 Desirable Attributes of Food Fermentation

In general, fermentation preserves and detoxifies foods, improves nutritional, flavour and other organoleptic properties, and reduces the cost of subsequent processing or cooking of foods. Various aspects of these advantages will be treated in greater detail.

2.1.2 Preservative Value

The preservative attribute of food fermentation is now well accepted. Tanner (1944) opined that preservation, among others, is the main reason for fermenting foods. Fabian (1951) reported the preservation of foods by use of micro-organisms acting on such foods, converting their constituents into products with preservative properties which result from changes in acidity, alkalinity or the presence of specific organic compounds. Organic acids generally have preservative property, depending on the concentrations employed. Cell-free filtrates and cell suspensions of six strains of lactic acid bacteria were found inhibitory to growth of Pseudomonas on agar lawns while Lactobacillus casei was said to have had an inhibitory effect on the growth rate of Vibrio parahaemolyticus on iced shrimp (Moon, et al., 1982.) Lactic acid, in addition to other organic products in minor amounts, also played a preservative role in sausage production where they were reported to be the main product of carbohydrate fermentation in sausage ripening (Halvarson, 1973). In another report on sausages, the ratio of lactic acid to acetic acid was said to be seven

after fermentation, and went up to 12 after drying with a recovery level of 80-103 percent of carbohydrate in the form of these two acids if oxygen was excluded from the sausages by vacuum packaging (Deketelaere, et al., 1974). Other organic products detected in fermenting sausages which were known to have antimicrobial properties are ethanol, acetoin, 2,3-butanediol and diacetyl, all of which are liberated by the microbial flora of the sausage (Jay, 1982; Lücke, 1985). Most sausages fermented in Belgium and Germany have pH values of 4.8-5.0 which correspond to lactic acid concentrations of approximately 29g/kg dry weight (Deketelaere, et al., 1974). The preservative effect of lactic acid is to be seen also in dairy fermentations. The acidity produced by Lactobacillus acidophilus in acidophilus milk reaches one percent which discourages proliferation of other bacteria that are found in most dairy fermentations (Oberman, 1985). Other lactic acid fermentations include 'burukutu', 'ogi', 'garri', etc. (Wood and Hodge, 1985). Another organic acid with strong preservative property is acetic acid which is produced by acetic acid bacteria in vinegar fermentation. Vinegar is described as the strongest acid known in antiquity, while pickling foods in vinegar is said to be one of the traditional technologies of food preservation (Adams, 1985). Apart from acidity, alkalinity as caused by micro-organisms in the food environment also checks proliferation of other micro-organisms. The pH in fermenting vegetable proteins often rises as a result of ammonia release (Hesseltine, 1965). Daddawa fermentation is characterised by a

rise in pH above 8 (Odunfa, 1981a).

2.1.3 Enhancement of the Food and Nutritional Value of Some Seeds

A number of foods which would otherwise have been wasted as a result of customs and traditions which do not favour use of such are, after fermentation, used as foods (Stanton, 1985). The unfermented substrates (seeds) of a number of indigenous Nigerian condiments fall in this category, namely locust bean (*Parkia* spp), castor oil (*Ricinus communis*) and oil bean (*Pentaclethra macrophylla*) seeds. In addition to utilising otherwise non-utilizable foods, the nutritional value of the food is enhanced. Platt (1964) gave a review of this contribution by fermentation which he referred to as "biological ennoblements" showing increase in nutrients in fermented foods over the unfermented. The level of riboflavin is reported to increase during the fermentation of locust bean seeds for 'daddawa' to about 0.2-0.8 mg/g (Platt, 1962). Also on the same 'daddawa', it was reported that both protein and fat content of the fermented product are higher than in the unfermented substrate (Eka, 1980). Nutrient enhancement in fermented foods was also reported in an Indian cereal-protein fermented food known as 'Idli' obtained from black gram (beans) and rice where the content of methionine increased to about 20 percent over that in the unfermented cereal-protein substrate (Platt, 1964).

2.1.4 Detoxifying Effect

Certain foods known to contain toxic substances lose them in the process of fermentation. The toxic substances in castor oil seeds (Ricinus communis) ricin, ricinine and ricinoleic acid are reportedly eliminated during fermentation for ogiri production (Odunfa, 1985a). During 'garri; fermentation, the level of linamarin, the cyanogenic glucoside in cassava, is reportedly broken down either as a result of acid developed which created a favourable environment for the detoxification (Collard and Levi, 1959) or by the action of the endogenous enzyme in the cassava (Okafor, 1977). Also in 'daddawa' preparation , oxalic and phytic acids, both of which are known to form insoluble salts with mineral elements thus preventing their utilization, are significantly reduced (Eka, 1980). In addition to reduction of toxic substances, development of the fermenting organisms in the system, coupled with production of certain metabolites, creates steep competition for nutrients and antibiosis respectively thus preventing other contaminants from developing (Fabian, 1951).

2.1.5 Flavour Enhancement and Organoleptic Qualities of Foods

Although Tanner (1944) suggested improvement of flavour as one of the main reason for fermenting foods he did not hesitate to stress that desirability of flavour is a matter of opinion and what one is used to. According to him, "although flavour is changed, differences may exist as to whether the flavour is improved". It is generally viewed,

however, that food fermentation improves flavour and texture, and makes the food more enjoyable (Stanton, 1985).

Many flavour compounds are produced during food fermentations. For example, diacetyl and acetoin produced by lactic acid bacteria, particularly Streptococcus lactis subsp. diacetylactis during fermentations of dairy products (Oberman, 1985). Esters and aldelydes produced during garri fermentation are believed to impart flavour to the finished product (Collard and Levi, 1959). Glutamic acid produced in daddawa fermentation contributes to the flavour enhancing attributes of the product (Odunfa, 1985b).

2.1.6 Other Benefits of Food Fermentation

Since fermentation is a method of processing, fermented foods are at least semi-processed foods. Thus cooking time for fermented foods is often shortened, with consequent reduction in fuel or energy demand (Stanton, 1985). Another value derived from food fermentation is that it provides variety. Thus milk, an edible substance is fermented into a wide variety of products like cheese, yoghurt, butter, etc.

2.2 An Overview of Sources of Fermented Foods

The lack of most of the present preservative facilities in ancient times must have meant that all perishable animal and plant foods that were available in excess must have been left to undergo fermentation; such that the kinds and types of fermented foods all over the world as are currently known

can be said to be those that have been selected over the time as having desirable attributes.

The fermented products of animal origin which are derived from either the animal flesh or secretion from the animals are limited, compared with the numerous variety obtained from plant products. In Asia, fish fermentation is common (Beddows, 1985) while it is not so common in Africa. However in Senegal excess fresh fish are piled together to ferment, giving a product called 'guedj' (Toury et al., 1970) while in Ghana, a similar product is obtained, sun-dried and is called 'bonome' (Odunfa, 1985a). Numerous varieties of sausages on the other hand which are prepared from ground meat are more commonly found in Europe, particularly Central and Southern Europe (Lücke, 1985). Among the other fermented products of animal origin are varieties of fermented milk (Oberman, 1985) and 'tej', a sweet alcoholic wine fermented from honey commonly produced in Ethiopia (Stainkraus, 1979).

Fermented products of plant origin can be considered as those obtained from plant tubers, cereals and leguminous seeds. In Africa, cassava serves as the major source of fermented products from plant tubers. The variety of fermented cassava products in Nigeria include garri, lafun and fufu all of which are also known in other parts of West and Central Africa (Okafor 1980). Plant cereals on the other hand form an important source of fermented alcoholic drinks and non-alcoholic puddings. Fermented products of

cereals include 'ogi' (Akinrele, 1970) 'mahewu', 'pito', 'kishk', 'busasa', 'keffir', 'merissa', etc. (Odunfa 1985a). The sap or juice from palm trees, namely oil palm (Elaeis guineense) and raphia palm (Raphia spp) is fermented to give palm wine commonly found in West Africa (Okafor, 1975). The seeds of leguminous plants also serve as a major source of substrate for food fermentation, giving a variety of condiments. Since the current review is concerned with vegetable protein fermentations, a more detailed review will be made on that aspect.

2.2.2 Fermented Vegetable Protein Foods - Oriental and Africa

Most fermented proteins of the world have their origin in Africa and Asia. Those in common use in Africa include, among others, condiments, seasoning agents or 'meat' in soups. In Asia, the seeds of the popular soya beans (Glycine max) are fermented with or without other cereals to produce a variety of products with their history dating back hundreds of years (Yokotsuka, 1985). One of the most popular of the soya bean fermented products, tempeh, is the staple food of Indonesia. Tempeh is fermented from whole cooked dehulled soya bean or soya bean and wheat cultured with Rhizopus oryzae (Van Veen, 1957). The fermented tempeh is then eaten fresh or deep fried like meat (Wood, 1985).

Another popular fermented soya bean product is soy-sauce with its history dating back a thousand years in the

Orient. It is known as Shoyu in Japan and is fermented from soya bean with or without barley and rice. A variety of soy-sauce exist in the oriental countries (Platt, 1964). Fermentation of soy-sauce occurs in stages and is carried out first by Aspergillus oryzae or A. sojae followed by lactic acid fermentation which lowers the pH of the fermenting moromi for development of the yeast at the final stage (Yong and Wood, 1976: Yokotsuka, 1985). Ontjom is another popular Indonesian fermented soya bean product. It is fermented from a mixture of soya beans, okara (solid residue from soy milk production) and peanut press-cake (residue after oil has been expressed from Arachis hypogoea). Fermentation of ontjom is reportedly carried out by species of Neurospora and Rhizopus (Wood, 1985). Other soya bean products of the orient are miso, natto and shi-tou-shi. Miso, a flavouring agent, is the fermented soya bean paste of the orient (Yokotsuka, 1985). The fermenting micro-oganisms are Aspergillus oryzae, A. sojae or A. tamari and lactic acid bacteria such as Lactobacillus and Pediococcus species which are introduced along with rice, barley or beans and salt. Like tempeh and soy-sauce, miso is now produced on an industrial scale (Wood, 1985). Natto is fermented by Bacillus subtilis (natto). It is a side dish which is served with either cooked rice or soy-sauce. During natto fermentation the Bacillus produces a viscous polymer consisting of polyglutamic acid polypeptide and fructose (Hara, et al., 1982; Yokotsuka, 1985). Shi-tou-shi

is similar to natto and is reportedly fermented by species of Bacillus (Yokotsuka, 1985).

Until recently, the uses of soya bean in Africa have been very limited despite the nutritious value of the product. It is fermented in the middle belt region of Nigeria to give 'daddawan-soya', a condiment. Fermentation is carried out by species of Bacillus (Popoola and Akueshi, 1985). Other fermented legume products used as seasoning agents in West Africa include 'daddawa' which is known as 'iru' in the south western parts of Nigeria. It is produced by fermenting the seeds of the African locust bean, Parkia biglobosa welw. ex. oliv. (syn. P. clappertoniana). Daddawa, though a Hausa name, has been widely adopted by most tribes in Northern Nigeria and has been erroneously used as dawadawa by many non-Hausa speaking peoples (Pederson, 1971; Odunfa, 1985a). 'Ogiri - igbo' and 'ogiri-nwan' are condiments mainly used by the Igbo speaking people of Eastern Nigeria. 'Ogiri-igbo' is fermented from castor oil (Ricinus communis schrad) seeds (Odunfa, 1985a) while 'Ogiri-nwan' is obtained from fermented pumpkin beans (Telfairia occidentale Hook).

Another 'ogiri', common among the Ijebus and Ondo tribes in the forest zone of South-western Nigeria is fermented from melon seeds (Citrullus vulgaris schrad). The micro-organism fermenting this 'ogiri' is a Bacillus spp found consistently in the fermenting mash (Odunfa, 1981b). 'Ogiri-saro', on the other hand, is fermented from the seeds

of sesame plant (Sesamum indicum).

2.3 Biochemical and Nutritional Studies on Fermented and Unfermented Legumes

Oyenuga (1968) analysed the nutrient contents of raw locust bean seeds and compared them with those of decorticated groundnut seeds. He reported that Parkia seeds contain higher crude protein and ash but lower crude fibre and other extract than the decorticated groundnut. Fetuga et al. (1974) analysed the amino acid composition of Parkia seeds and compared them with those of soyabean meal, groundnut meal and whole hen's eggs. Lysine level in Parkia seeds was found to be comparable with that of hen's egg while the sulphur amino acid content was higher than that of groundnut meal but less than that of soya bean meal. Parkia beans like other legumes is defficient in the sulphur amino acids, methionine and cysteine. Campbell-Platt (1980) reported that the major constituent of daddawa is crude protein which is approximately 40 percent while fat (ether extract) is approximately 35 percent and carbohydrate 15 percent. Eka (1980) reported increase in amount of protein with varied influence in the different amino acids in the fermented product as compared with the unfermented locust bean seeds. Amount of fat increased in the fermented beans similarly. The fat content of Parkia seeds is reported to be approximately 60 percent unsaturated fatty acid; a high proportion--40 percent of the total fatty acids--is linoleic acid, an essential fatty acid (Busson, 1965). Lysine has been

reported higher in Parkia beans than in most other legumes (FAO, 1970). Odunfa (1985b) analysed the nutrient content of the fermented seeds of Parkia biglobosa and reported rapid and steady increase in the quantity of free amino acids throughout the fermentation. Glutamic acid, valine, aspartic acid and alanine were most rapidly liberated. In the same study it was observed that the most marked enzyme activity was that of proteinase, followed by lipase. Amylase activity was not detected. In another study, Odunfa (1983) observed flatus-forming oligosaccharides such as stachyose, raffinose and sucrose as the main carbohydrate constituents in the fermenting 'daddawa' while reducing sugars such as galactose, glucose and fructose were detected both in the fermented and unfermented locust beans. A host of other workers have reported proximate composition of this popular condiment--daddawa (table 1).

Analysis of the fermented castor oil (Citrullus vulgaris) seeds by thin layer chromatography showed presence of amino acids like phenylalanine, tryptophan, tryrosine, serine, glutamic acid, cysteine and glutamine which were not detected in the unfermented seeds (Anosike and Egwuatu, 1980). Analysis of soya bean shows the crude protein and true protein content to be about 44 and 40 percent, respectively, of the dry matter (Oyenuga, 1968). The taste and quality of Shoyu, the oriental fermented soy bean sauce have been attributed to the soluble nitrogenous constituents of the fermented product (Yokotsuka, 1981). A more detailed

study of the shoyu revealed about seven flavour compounds isolated from the weakly acidic fraction of the shoyu. Analysis of the ogiri (Citrillus vulgaris) showed increased activity of proteolytic enzymes and consequently increase in amounts of amino acids and a rise in pH to alkaline region (Odunfa, 1982). Analysis of the seeds of the African oil bean tree, Pentaclethra macrophylla (Benth) shows that the seeds contain 17 per cent carbohydrate and 44 - 47 percent oil which is rich in oleic acid (Irvine, 1961). Analysis of the oil content carried out by Nwokedi (1973) confirmed the results of Irvine (1961). Mba et al. (1974) assayed for the protein content of the oil bean tree seeds and reported 43.89 per cent crude protein. Though the observed levels of amino acids were generally high, the contents of sulphur amino acids were lower than in other plant proteins.

Table 1: Proximate composition of daddawa per 100 gram dry matter, according to various sources (Number of tested samples in parentheses).

Property	Platt (1982)	Toury et al. (1967)	Leung et al. (1968)	Watson (1971)	Eyeson & Ankah, (1975)	Eka (1980)
Moisture (%)	22	14.8	13.4 (10) 6.0-22.4	25.5 (3) 24.0-26.7	28.4 (6) 21.0-37.7	13.75 (6) ±1.11
Crude protein (g)	47.8	41.1	39.7 (10) 30.7-49.2	40.7 (2) 36.5-43.5	40.5 (6) 37.6-45.3	38.5 (6) ±1.16
Fat (ether extract)	32.7	34.0	31.4 (9) 11.1-39.8	40.8 (3) 37.1-47.2	37.1 (6) 12.4-52.7	31.20 (6) ±1.35
Carbohydrate (g) by difference	15.4	12.2	16.0	11.7	14.7	
Crude fibre (g)	4.5	7.1	7.7 (6) 7.4-12.6	3.5 (3)	3.1 (5) 2.1-3.6	6.23 (6) ±0.55
Ash (g)		5.6	5.2 (9) 3.1-6.1	3.3 (3) 3.2-3.5	4.6 (6) 3.2-8.7	6.75 (6) ±0.95

2.4 Patterns of Consumption of Various Condiments by Indigenous Communities

The importance of indigenous condiments especially among the rural populace cannot be over emphasised as they are used to replace taste effect of meat or fish in many dishes. African locust bean is said to contribute the second cheapest source of protein after groundnuts in northern Nigeria while, in the west, it provides the third cheapest after groundnut and dried fish (Ladipo and Adegboye, 1976). The consumption rate of 'daddawa', though varying from one locality to another, is reported to range between one and seven grams per person per day (Nicol, 1949) in northern Nigeria. Another report offers different consumption rates for moslems and non-moslems, with moslems consuming about 17g and non-moslems four grams of daddawa per person per day. Generally, consumption of Nigerian condiments tends to be localised as a result of the distribution in different vegetation zones of the plants which produce the appropriate fermentable seeds. Among the Igbo tribe of south eastern Nigeria, 'daddawa' which is not commonly found carries a consumption rate of about 1g per person per day (Dema, 1965) as opposed to 'Ugba' which has been described as an important and cheap source of protein for the Ibos (Obeta, 1983). The Yorubas of the south western Nigeria and other parts of West Africa, like the tribes in Northern Nigeria, consume a lot of 'daddawa' which they call "iru". The consumption rate of 'daddawa' is reported to be about 10g per person per day among the Yorubas (Dema, 1965).

The importance of condiments, especially 'daddawa' in the culinary economy of most West African countries has been compared with that of cheese among the Western nations, especially Europe. Traditional fermented leguminous seeds commonly used among nations in West Africa have peculiar flavour and are either generously introduced into soups or are taken whole (Campell-Platt, 1980). In Sudan, locust bean seeds are reportedly roasted and used as coffee substitute known as 'cafe du Sudan' in addition to the use of the fermented bean (Dalziel, 1937). In Indonesia and Malaysia on the other hand, the whole pods of Parkia speciosa are eaten raw or cooked as a vegetable referred to as 'petai' which is preserved by salt pickling (Douglas, 1972).

2.5 Microbiology of Fermented Foods

Microbiological studies aimed at isolating and identifying organisms relevant to food fermentations are potentially useful for development of appropriate fermented foods and for improving the scale of production from domestic to factory level. This fact is recognised by Okafor (1980) in his scheme for developing African fermented foods. In the past three decades considerable research interest has been devoted to the microbiology of food fermentations in Nigeria. The publications of Okafor (1980) and Odunfa (1985a) have reviewed broadly the available literature. It must be noted that information on microorganisms responsible for certain food fermentations is often conflicting. However, what seems to be relevant to this thesis is that the

genus Bacillus is considered to be important in fermentations of indigenous vegetable protein products such as 'daddawa', 'ogiri', 'ugba', etc. which are widely used in Nigeria. It is therefore pertinent to review some aspects of the biology of the genus.

2.5.1 The Genus Bacillus

General Characteristics

The genus Bacillus, according to Bergey's "Manual of Determinative Bacteriology" (1964) consists of rod-shaped cells, sometimes occurring in chains and capable of producing endospores which may or may not give the cells spindle shaped or drum-stick shaped appearance. Bacillus species are either non-motile or motile by means of peritrichous flagella. They are mostly Gram-positive, while some are Gram variable or Gram-negative. Some species produce rough colonies, form pellicle on broth whereas others produce smooth colonies. The organisms are mostly saprophytes commonly found in soil whereas a few are animal parasites.

2.5.2 Taxonomy and Classification

Species of Bacillus have been classified using different methods by different workers. Numerical taxonomy which has been used quite a lot in the classification of many bacterial taxa (Goodfellow, 1971) is only recently being used in a systematic way to establish the relationship between species in the genus Bacillus (Logan and Berkeley, 1981). Strains and species of Bacillus isolated from

natural habitats have been classified by various workers (Sneath, 1962; Bonde, 1973, 1975 and Priest *et al.*, 1981) using numerical taxonomy, thus confirming the importance of numerical methods in *Bacillus* taxonomy. Very recently Gil *et al.*, (1986) classified and established the strains of *Bacillus* species isolated from orally administered drugs using numerical taxonomy.

Smith *et al.*, (1946) used morphology of the *Bacillus* to classify them into species. Another classification of *Bacillus* species developed has been based on the guanine and cytosine content of the deoxyribonucleic acid (Hill, 1966). Cowan and Steel (1965) on the other hand observed that there are no sharp divisions between organisms differentiated into species but only slight and subtle difference in characters that make them blend into each other resembling a spectrum. This spectrum resemblance as arranged by Gordon *et al.*, (1973) also utilises morphology of the *Bacillus* to place them into three groups on the basis of their spore shape, swelling of the sporangium by the spore and diameter of the rod with appearance of its protoplasm (Smith *et al.*, 1946; Gordon *et al.*, 1973). According to Gordon *et al.* (1973) group 1 consists of species with ellipsoidal or cylinderical spores which are centrally to terminally located within cells that have bigger diameter than the spore. The species of *Bacillus* belonging to this group are *B. cereus* and its related species (table 2) *B. megaterium*, *B. licheniformis*, *B. subtilis*, *B. pumilus* and *B. firmus* all progressively

smaller in diameter. B. firmus is placed last in the group because it has physiological properties that differ from those of members in group 1. Group 2 comprises of species with swollen sporangia as a result of oval spores which are centrally to terminally located within cells smaller than the spore diameter. Their Gram reaction is positive to negative or variable. The group begins with B. polymyxa followed by B. macerans, B. circulans, B. stearothermophilus, B. alvei, B. laterosporus, B. brevis all of which are catalase positive. The other species belonging to this group are B. larvae, B. popiliae and B. lentimorbus whose strains are catalase negative or weakly catalase positive and fastidious. Bacillus coagulans is the link between group 1 and group 2 because certain strains of B coagulans are found with sporangia of the type in group 1 and others with the type in group 2.

Group 3 consists of species with swollen sporagia which bear spherical spores that are terminal or subterminal. Their Gram reaction is positive to negative or variable. They include B. sphaericus and B. pasteurii.

Table 2. The three major groups of *Bacillus* species and their distinguishing characteristics.

Characteristics	Group 1	Group 2	Group 3
Gram reaction	Gram +	Gram +, - or ±	Gram +, - or ±
Spore shape	Oval or cylinderical	Oval	Spherical
Spore location in cell	Central to terminal	Central to terminal	Terminal to subterminal
Spore size	Sporangia not distended	Sporangia distended	Sporangia distended
Species in the group	*B. megaterium*	*B. polymyxa*	*B. sphaericus*
	B. cereus	*B. macerans*	*B. pasteurii*
	B. thuringiensis		
	B. mycoides	*B. circulans*	
	B. anthracis	*B. stearothermophilus*	
	B. licheniformis		
	B. subtilis	*B. alvei*	
	B. pumilus	*B. laterosporus*	
	B. firmus	*B. brevis*	
		B. coagulans	
		B. pulvifaciens	
		B. popillae	
		B. lentimorbus	

Source - Gordon *et al.*, 1973

Table 3: Matrix for Identification of Typical Species of *Bacillus*

Physical and Biochemical Tests	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Gram reaction	+	+	+	+	+	+	+	+	+	#	#	±	±	±	±	±	+	+	+	±
Motility	±	+	±	-	+	+	+	+	+	+	+	+	+	+	+	+	±	±	-	+
Spore shape	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	S
Spore location	C	C	C	C	C	C	C	C	X	X	T	X	T	X	T	X	X	X	X	T
Distention of sporangia	-	-	-	-	-	-	-	-	±	+	+	+	+	+	+	+	+	+	+	+
Catalase reaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	+
Growth at 65°C	-	-	-	-	-	-	-	-	±	+	-	-	+	-	-	-	-	-	-	-
Growth at pH 5.7	+	+	+	+	+	+	+	-	-	+	+	±	±	-	-	±	-	-	-	+
Growth in 7% NaCl	+	±	+	+	+	+	+	+	-	-	-	±	-	-	-	-	-	-	-	±
Anaerobic growth in glucose broth	-	+	+	+	+	-	-	-	+	+	+	±	-	+	+	-	+	+	+	-
Starch hydrolysis	+	+	+	+	+	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-
Acid from Glucose	+	+	+	+	+	+	+	+	+	$	$	+	+	+	+	+	+	+	+	-
Acid from Arabinose	±	-	-	-	+	+	+	-	±	$	$	+	-	-	-	-	-	-	-	-
Acid from Xylose	±	-	-	-	+	+	+	-	±	$	$	+	-	-	-	-	-	-	-	-
Acid from Mannitol	±	-	-	-	+	+	+	+	±	$	$	+	-	-	+	±	-	-	-	-
Voges Proskaur test	-	+	+	+	+	+	+	-	±	+	-	-	-	+	-	-	-	-	-	-
Citrate utilization	+	+	+	+	+	+	+	+	±	-	-	-	-	-	-	±	-	-	-	±
Nitrate reduction	±	+	+	+	+	+	-	+	±	+	+	+	+	-	+	±	-	-	-	-
Indole test	-	-	-	-	-	-	-	-	-	-	-	-	-	+	±	-	-	-	-	-
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+	+	+	±	+	+	+	-	-	-	+
Casein hydrolysis	+	+	+	+	+	+	+	+	±	+	+	+	+	+	+	+	-	-	-	±
Urease reaction	±	±	-	-	±	±	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Egg-yolk reaction	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

1. *B. megaterium*
2. *B. cereus*
3. *B. thuringiensis*
4. *B. anthracis*
5. *B. licheniformis*
6. *B. subtilis*
7. *B. pumilus*
8. *B. firmus*
9. *B. coagulans*
10. *B. polymyxa*
11. *B. macerans*
12. *B. circulans*
13. *B. stearothermophilus*
14. *B. alvei*
15. *B. laterosporus*
16. *B. brevis*
17. *B. larvae*
18. *B. popillae*
19. *B. lentimorbus*
20. *B. sphaericus*

T Terminal
Positive in young cultures
$ Positive with gas production
O Oval Shape
C Central
X Central, Subterminal or terminal

2.5.3 Distribution of *Bacillus* species in Nature and in Foods.

Although *Bacillus* species are mainly found in the soil, they are widely distributed in nature. Owing to their saprophytic nature they are found contaminating a wide variety of materials. Some species are pathogenic. In this review, a brief account of the occurence of *Bacillus* species in some specialised natural and artificial habitats will be offered.

Wakisaka and *Koizumi* (1982) grouped *Bacillus* species which they isolated from the soil into major and minor soil populations with *B. cereus, B. megaterium B. sphaericus* and *B. subtilis* considered as the major soil *Bacillus* population. Investigations into rhizosphere microflora of some plants in Libya revealed *B. subtilis* as the predominant bacteria (ElDein Selim and Khalil, 1979). *Bacillus* species have also been found in lakes. In a bacteriological study carried out on a mesophilic lake in the USSR, *Bacillus* species ranked highest in number among all the bacteria isolated with a count of 10 *Bacillus* species out of a total of 32 bacteria species belonging to nine genera obtained (Koleshko *et al.*,1982). Another lake study in Japan revealed *Bacillus* as the predominant isolates among the Gram positive bacteria, constituting 55.4 percent (Konda and Tezuka, 1979). *Bacillus* species are also found in the atmosphere, as revealed by microbiological studies of the air in grain storing and processing plants, tobacco plants

and in poultry houses (Dutkiewicz, 1980). In that study it was observed that bacteria including Bacillus were more numerous than actinomycetes and fungi. In a study carried out on a commercial manure innoculant preparation which allows the use of the latter as feed, it was reported that B. subtilis, B. pumilus, B. circulans and B. coagulans were the predominant bacteria obtained above the fungal population (Siopongco and Aspiras, 1981). Bacillus species have been isolated even from seeds of plants (Lindblow-kull and Gherna, 1982).

Various foods have been found contaminated with species of Bacillus. Out of a large number of milk samples tested, Johnston and Bruce (1982) observed a high percentage of thermoduric psychrotrophs in 27.2 percent of the samples. Even ultrahigh temperature-processed milk has shown presence of B. subtilis, B. cereus and some atypical strains of Bacillus species (Westhoff and Dougherty, 1981). They reported that B. cereus occurred most frequently while B. licheniformis and B. coagulans also occurred in significant numbers. Contamination of milk-cereal and pure cereal diets have also been reported. The milk-cereal alcoholic beverage, 'kefir', common in Russia is reported liable to contamination and spoilage by Bacillus species (Oberman, 1985). Alvarez (1982) reported Bacillus species and Penicillium species as the major aerobic microorganisms isolated from the final arepas (the national bread of Venezuela prepared from corn dough). A similar result was reported by Koburger

et al. (1981) while conducting a microbiological study on laboratory-prepared samples of 'tostones' (fried plantain). Bacteriological determination of the coating crumbs of 60 raw frozen foods for frying also showed Bacillus species to be among the major bacteria found (Ueda et al., 1981). Out of the 151 isolates of Bacillus species obtained, B. subtilis and B. licheniformis made up 55 percent and 25 percent, respectively. Other Bacillus species obtained were B. cereus, B. sphaericus and B. megaterium. Even after frying at 170°C, Bacillus species constituted the only bacterial flora in the fried foods which increased from 10^2 to 10^5/g in 24 hours at 25°C.

Bacillus species have also been isolated from meat and meat products. In a bacteriological examination of beef extracts used for making soup, B. subtilis, B. macroides, B. sphaericus B. megaterium, B. pumilus, B. circulans and B. coagulans were observed predominantly occurring among some clostridium species (Konuma et al., 1980). Thermophilic Bacillus species such as B. stearothermophilus have been reported to cause spoilage of improperly cooked meat (Reiman, 1963) and flat sour spoilage in under-processed canned foods (Fields, 1970). Bacillus species have been isolated from poultry products (Kraft, 1971) and from different animal products (Sulzbacher and McLean, 1951) and sausages (Steinke and Foster, 1951).

Bacillus species have also been isolated from drinks

and spices. Bacillus species have been isolated from Mexican indigenous alcoholic and non-alcoholic beverages (Ulloa, 1981). Also, Bacillus species have been reported among some water-borne organisms frequently contaminating thawing frozen blood products in a water bath (Dietz and Burnie, 1982). Seenapa and Kempton (1981) on the other hand reported that four unprocessed Indian spices sampled at the point of export were highly contaminated with bacteria among which B. cereus was predominantly seen in more of the spices than any other spore-forming aerobes. Spices have also been reported as the source of Bacillus species isolated from spiced sausages (Lücke, 1985). Fruits are also frequently contaminated by Bacillus species. Soft stem end spoilage in olives has been attributed to Bacillus polymyxa and B. macerans often found on the olives (Vaughn, 1985). Bacillus nigrificans has been isolated from pickles where they were reported to cause blackening as a result of a water-soluble pigment they produced (Fabian and Nienhuis, 1934).

Although Bacillus species are generally saprophytic, a few species are pathogenic to humans and animals. Bacillus anthracis is pathogenic to sheep, cattle and sometimes human beings, causing anthrax, a severe pneumonia aggravated by the toxins liberated by these bacilli (Nestler et al., 1978). Certain serotypes of Bacillus cereus and B. licheniformis have been implicated in food-borne disease outbreaks (Gilbert, 1979) while B. cereus is reported to have caused serious infections of the eye through use of contaminated

eye drugs (Nestler et al., 1978). A complementary bacteriological examination of 56 abbatoir carcases (46 cattle and 10 horses) with neoplastic diseases revealed bacteremia or septicaemia in 30.3 percent of the cases (Imreh et al., 1979) with the genus Bacillus and coliforms making up the saprophytic isolates among the bacteria obtained. Some Bacillus species namely, B. thuringiensis, B. sphaericus, B. larvae, B. popillae and B. lentimorbus are pathogenic to insects (Gordon et al., 1973). The pathogenicity of B. thuringiensis and B. sphaericus to insects is now so well understood that they are cultivated for their protein products which are used as insecticides (Lysenko and Kucera, 1970).

2.5.4 The Genus Bacillus in Food Fermentation

The involvement of the genus Bacillus in a lot of fermentations can be attributed to the array of enzymes possessed by Bacillus species (Fogarty, 1971). As a result of this, a number of Bacillus species are used for industrial production of enzymes. The array of enzymes now produced on industrial scale by Bacillus species include amylases, β-galactosidases, β-glucanases, glucose isomerases, lipases and proteases (Godfrey, 1985). It is not surprising therefore that Bacillus species have been associated with the fermentations of protein, carbohydrates, lipid-rich and even vegetable based foods.

The earliest available microbiological information records Bacillus species among the bacteria that play signi-

ficant roles in milk fermentation (Oberman, 1985). A species of Bacillus named B. acidilacti, among other bacteria, was reported to be the fermenting organism in the production of 'taette', a Scandinavian fermented viscous, acid milk product (Olsen-Sopp, 1912). Bacillus subtilis, together with Lactobacillus and yeast, are reported as the dominant agents in the fermentation of 'kishk' (a wheat - milk fermented product which is popular in Egypt and other Arab countries).

Involvement of Bacillus in the fermentation or curing of certain fruits used in beverage production is also known. For example, in production of coffee, Bacillus species are reported to play a significant role at the initial stage of the fermentation of the cherries (Vaughn et al., 1958). In a microbiological study on cocoa fermentation it was observed that B. subtilis, B. cereus, B. licheniformis and B. megaterium were commonly encountered particularly in the later stages of the cocoa fermentation (Ostover and Keeney, 1973).

Other fermentations in which Bacillus are involved include those of vegetables. Bacillus subtilis and B. pumilus are reportedly involved in softening of olives (Olea spp). The blackening observed in black pickles fermentation was said to result from the production of black water soluble pigment by Bacillus nigrificans. In silage fermentation it was reported that after the crops have been ensiled there

was a transformation of the bacterial flora from aerobic Gram negative organisms to coliforms and other genera of bacteria which include Bacillus (Woolford, 1985).

Bacillus are also involved in the fermentation of carbohydrate-based substrates. Bacillus species are reported to be involved in the fermentation of rice in Ecuador (Van Veen et al, 1968). 'Fufu', a popular cassava (Manihot esculenta) fermented food in Eastern Nigeria is reportedly fermented by Bacillus along with four other bacteria (Okafor et al., 1984).

Bacillus species are also involved in the fermentation of protein-based substrates. Although Bacillus species had earlier been reported as spoilage organisms in sausages (Lücke, 1985), they failed to cause spoilage when innoculated into raw sausage mixtures, even if faulty ripening conditions were employed (Neumayr et al., 1983). Fermentations of African locust bean (Parkia biglobosa) seeds for daddawa production is reported to be carried out by B. subtilis (Odunfa, 1981a). Another study of the same fermentation confirmed the involvement of B. subtilis although other bacteria (Leuconostoc species) were implicated along with the Bacillus (Antai and Ibrahim, 1986). In the fermentation of soya bean daddawa production, Popoola and Akueshi (1985) reported involvement of Bacillus species. A similar soya bean fermented product, a popular food in the orient called 'shi-tou-shi' is also fermented by Bacillus (Yokotsuka, 1985). Another oriental soya bean

fermented product 'natto' is also fermented by Bacillus (Hesseltine, 1965). Other vegetable protein fermentations include that of African oil bean plant (Pentaclethra macrophylla) seeds for 'ugba' production in which Bacillus species have been implicated as the fermenting organisms (Obeta, 1983). In the fermentations of the various types of ogiri, Bacillus species are found consistently along with some genera of Enterobacteriaceae (Odunfa, 1981b).

2.6 Microbial Production of Amino Acids

Industrial production of amino acids is now commonly carried out by microbial fermentation, chemical syntheses and protein extraction. Microbial fermentation methods have the advantage of producing mainly L-forms of the amino acid which happen to be the form which the living cells utilise. The L-amino acids are also the most naturally occurring amino acids (Holum, 1969).

Most amino acids are produced by direct fermentation of cheap carbon sources such as carbohydrates. Glutamic acid and lysine were the first amino acids produced microbiologically on a large scale. Using Corynebacterium glutamicum as a high-yielding agent (Kinoshita et al., 1957) glutamic acid is now fermented by bacteria in a basal medium directly from carbohydrates and non-carbohydrates. Lysine production on the other hand is now achieved via a two stage fermentation whereby diaminopimelate is produced by a lysine auxotroph from carbohydrate, followed by a decarboxylation of the

diamino pimelate by Aerobacter aerogenes or a wild strain of E. coli which is rich in diaminopimelic decarboxylase (Dulaney, 1967). A one-stage direct fermentation from carbohydrate uses a homoserine or threonine plus methionine auxotroph of Corynebacterium glutamicum or Brevibacterium flavum. The basal medium required for lysine production contains amino acid sources in the form of protein, like soyabean meal hydrolysate, and the biotin level must be above 30µg. Lysine is also produced from -aminocaprolactam by fungi (Fukumura, 1976).

Threonine, an essential amino acid, is produced microbiologically from carbohydrate (Huang, 1961) using a diaminopimelate (DAP) or DAP plus methionine auxotroph of E. coli or by conversion of L-homoserine to threonine (Shimura, 1972). A variant of Bacillus subtilis was found most promising among a number of other bacteria capable of this conversion (Dulaney, 1967). Microbial fermentation of serine is done in a medium containing glycine which is now known to induce one of the key enzymes, serine hydroxymethyl transferase (Kubota et al., 1975). Threonine, homoserine and glycine are important in the synthesis and direct production of serine from cheap carbon sources (Nakayama, 1982).

Until recently, it has not been possible to carry out microbial synthesis of branched amino acids from carbohydrate sources in the absence of precursors. Microbial fermentation of isoleucine, for instance, requires incorporation of α-aminobutyrate, D-threonine, α-hydroxybutyric acid

or bromobutyric acid (Matsushima et al., 1974). In addition to its role as a precursor, α-aminobutyrate acts as a regulatory factor on acetohydroxy acid synthetase, thus stimulating synthesis of acetohydroxy butyrate, an isoleucine precursor and also releasing the feedback inhibition of some metabolites (Nakayama, 1982). Leucine, on the other hand, is produced by leaky-type isoleucine auxotrophs of Serratia marcescens in media lacking isoleucine (Kisumi et al., 1973). Valine produced by wild bacteria is mainly copiously released by members of the family Enterobacteriaceae, particularly the genera Escherichia and Enterobacter all of which possess α-acetolactate synthetases (Nakayama, 1982).

Microbial production of aromatic amino acids is also known. Tryptophan is produced by conversion of anthranilic acid (Arima et al., 1971). Phenylalanine is produced by means of regulatory mutants. Using a hyperproducing mutant of E. coli, phenylalanine production went up to 8.7g/l (Tribe and Choi, 1982). Tyrosine production was reportedly high when mutants of phenylalanine auxotroph with high tyrosinase activity was used (Tanaka et al., 1974). Unlike the other amino acids, alanine produced by certain bacteria is the racemic form. Efficient production of L-arginine was obtained by using regulatory mutants of some bacterial species and Bacillus subtilis (Kisumi et al., 1971). Production of histidine has been obtained using mutants of Corynebacterium glutamicum from basal medium containing meat, thiamin and biotin (Araki et al., 1974).

Proline is fermented by an isoleucine auxotroph and a histidine auxotroph of Brevibacterium flavum which has been made resistant to sulfaguanidine by mutation (Tsuchida et al., 1974). Proline production required high concentrations of biotin, magnesium and ammonium ions and is commonly produced by members of the Enterobacteriaceae. Aspartic acid and cysteine production have been reported in members of the Enterobacteriaceae with high activity of the key enzymes, aspartase and L-cystein desulfhydrase, respectively (Nakayama, 1982). L-methionine is produced by a strain of a mutant of Corynebacterium glutamicum which was capable of yielding 2g/l in a medium containing 10 percent glucose (Kase and Nakayama, 1975).

2.6.1 **Glutamic Acid Biosynthesis**

The discovery by Kinoshita et al. (1957) of an efficient glutamic acid-producing bacterium Corynebacterium glutamicum (synonym, Micrococcus glutamicus) paved the way for the present industrial-scale production of the amino acids. The pathway of glutamic acid production is illustrated in Fig. 1. The final stage involves the transamination and reductive amination of a-ketoglutaric acid by glutamic acid dehydrogenase (Nakayama, 1982). A wide variety of microorganisms capable of producing α-ketoglutaric acid are able to produce glutamic acid when they possess glutamic acid dehydrogenase. Dulaney (1967) and Nakayama (1982) have presented a catalogue of microorganisms which produce gluta-

mic acid and the yields of the acid from such organisms.

Glutamic acid-producing bacteria are said to make use of the Emden-Meyerhof glycolytic pathway as well as the hexose monophosphate shunt to produce acetyl Co-A, a key intermediate compound in the metabolism of a variety of substrates (Nakayama, 1982). The pathway of glutamic acid biosynthesis comprises essentially the citric acid cycle and reductive amination reaction of α-ketoglutaric acid. Glutamic acid synthesizing microorganisms utilise the citric acid cycle or the glyoxylate pathway (Nakayama, 1982). Conversion of oxalosuccinic acid to a-ketoglutaric acid, a decarboxylating process, requires manganese ions as the cofactor (Holum, 1969). Possession of the NADP-specific glutamic acid dehydrogenase becomes a limiting factor after overcoming the initial steps. The presence of this enzyme is essential for glutamic acid formation (Holum, 1969). The efficiency of glutamic acid dehydrogenase in the formation of glutamic acid has been investigated. While a low content of the enzyme is said to favour glutamic acid production, glutamate dehydrogenase which is influenced by carbon source rather than nitrogen source serves a catabolite function, and is induced by glutamate (Kane et al., 1981) . The enzyme is also subject to catabolite repression. Kane and Deshpande (1979), on the other hand, reported that glutamate dehydrogenase level was influenced by the stage of bacterial growth, source of nitrogen, and rate of tryptophan biosynthesis. They suggested that the enzyme was probably serving

an anabolic function; and that this might be the route of incorporation of inorganic nitrogen into an organic form. The reductive amination process whereby α-ketoglutaric acid is converted to glutamic acid via the catalytic action of glutamic acid dehydrogenase is represented by the following equation:

$$\underset{\text{α-Ketoglutaric acid}}{HO\text{-}OC\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}CO\text{-}OH} + NH_3 + NADH + H^+ \longrightarrow \underset{\text{glutamic acid}}{HO\text{-}OC\text{-}CH_2\text{-}CH_2\text{-}CH(NH_2)\text{-}COOH} + NAD^+ + H_2O$$

Only the L-enantiomer is formed by the catalytic action of this enzyme (Holum, 1969).

Apart from synthesising glutamic acid from simple carbohydrates via the TCA cycle and reductively aminating the α-ketoglutaric acid, glutamic acid can also be synthesised by transamination of other amino acids. By a process of transamination and amination of alanine, aspartic acid or α-ketoglutaric acid, glutamic acid was produced by B. subtilis which synthesised group B vitamins, including biotin (Kolchinska et al., 1970).

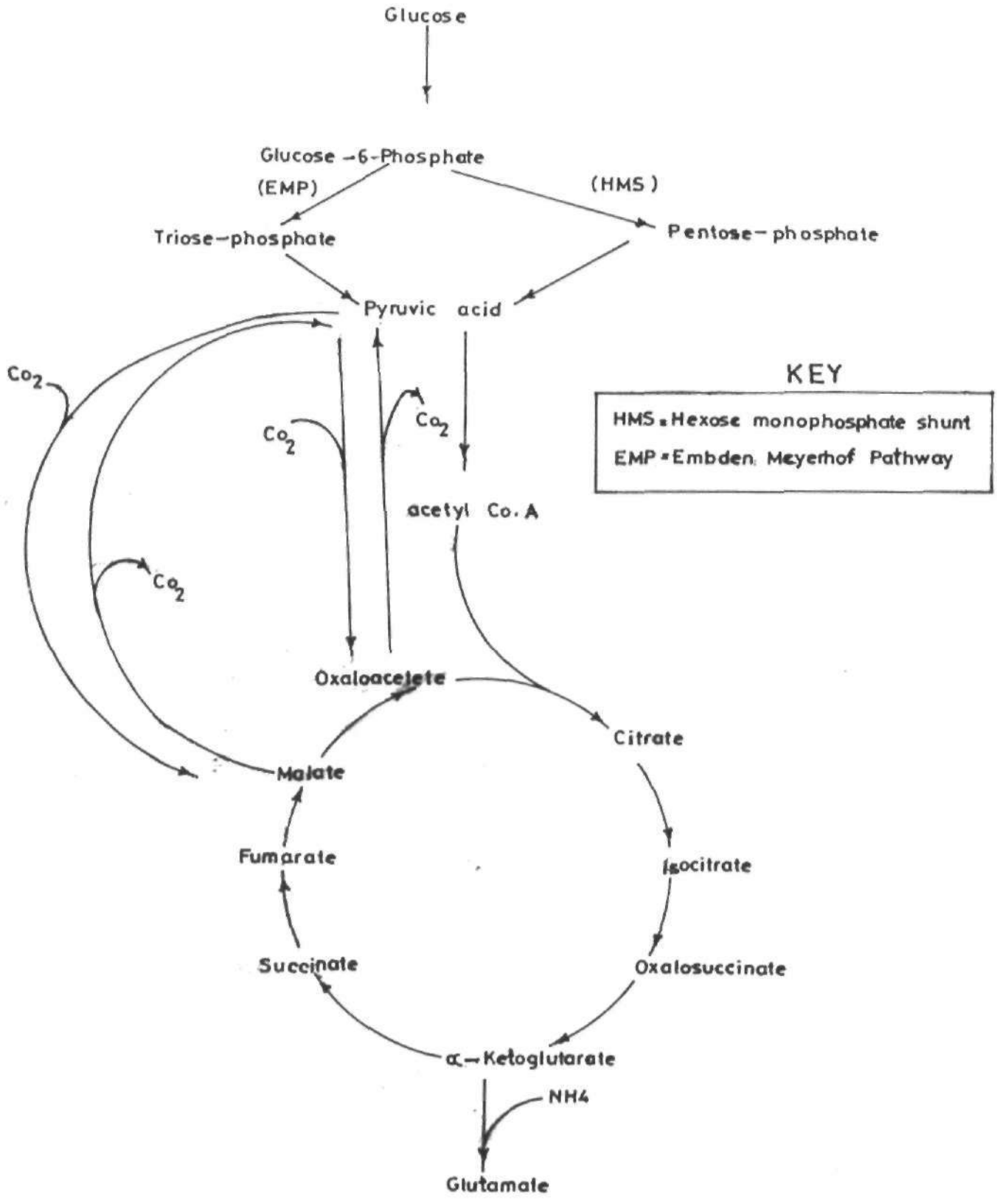


Fig. 1: PATHWAYS OF GLUTAMIC ACID SYNTHESIS FROM GLUCOSE (ADOPTED FROM NAKAYAMA, 1982)

2.6.2 Cultural Conditions for Industrial Production of Glutamic Acid

Glucose and some non-carbohydrate compounds such as acetic acid have been used for glutamic acid production (Tanaka et al., 1971). Molasses from different sources have been shown to give high yields of glutamic acid when a biotin-resistant glutamic acid producer of Corynebacterium glutamicum was employed in the fermentation (Terryshny et al., 1981). However, a decrease in yield of glutamic acid upon replacement of glucose with other sugars has been reported (Cetin et al., 1973). Other substrates of non-carbohydrate origin which have been used include ethanol and n-paraffins. Chalumeau and Rapport (1971) reported that L-glutamate constituted a substantial part (up to 30-40 percent of total incorporated radioactivity) of products accumulated in B. subtilis grown in sucrose, glucose, fructose or glycerol labelled with ^{14}C.

Biotin requirement has been described as the major factor controlling the fermentation of glutamic acid the vitamin being an essential growth factor for glutamic acid bacteria. A suboptimal concentration of biotin is required for good glutamic acid yield as optimal growth leads to a decrease in glutamic acid production. The influence of biotin is believed to be in limiting growth and allowing carbon and nitrogen to go to fermentation products rather than to cells and CO_2 (Dulaney, 1967). A number of other postulates have been advanced for the biotin effect. One

observes that succinic oxidation in biotin-deficient cells is low, suggesting a partial block of the flow of carbon to fumarate and aspartate and shunting back to glutamic acid production (Dulaney, 1967). Findings of other workers disagree with these reports, making uncertain the exact mode of biotin influence. It is now held that biotin deficiency either causes an aberation in the normal synthesis and distribution of cellular fatty acids leading to an abnormal ratio of saturated and unsaturated fatty acids. In E. coli, a ratio of 22.1: 63.9 has been observed at 10°C for the major saturated and unsaturated fatty acids (Marr and Ingraham, 1962). Any factor which alters the normal ratio, be it a mutation, or inclusion in the culture medium of growth factors or detergents which affect synthesis or stability of membrane components, influences the permeability of the membrane and the release of metabolites. Such factors affect the membrane which then lets out the glutamic acid; the biotin also influences the phospholipid content of the membrane (Kikuchi and Nakao, 1973).

Among other cultural conditions important for high glutamic acid accumulation is pH of the medium. Chattopadhyay and Banerjee (1973) reported a higher yield of glutamic acid when the pH of the medium was regulated. The most suitable pH for optimal glutamic acid accumulation is reported to be between 7 and 8 (Nakayama, 1982). Other cultural conditions required are the overall coefficient of oxygen transfer which is facilitated by aeration rate and availabi-

lity of inorganic ions (Nakayama, 1982). Optimal temperature required for glutamic acid accumulation is reported to be in the mesophilic range, and specifically around 30°C (Dulaney, 1967).

2.6.3 Permeability of Microbial cell membrane and release of Glutamic acid and Other Metabolites

Release of many microbial metabolites into the environment depends on the permeability of the cell membrane of the organisms. Demain and Birnbaum (1968) gave a review on the release of glutamic acid from microbial cells. The importance of cell permeability as a requirement for high glutamic acid accumulation was reported in a study utilising antibiotics where high levels of glumatic acid production was recorded even in the presence of high biotin concentration (Dulaney, 1967).

The high yield of glutamic acid obtained in biotin-containing media has been attributed to a disruption of normal synthesis of fatty acids, thus leading to an abnormal ratio of saturated to unsaturated fatty acids (Nakayama, 1982). In a study with glycerol auxotroph, it was reported that the phospholipid content of the cell membrane, rather than cellular content of unsaturated fatty acid residues, controls the membrane permeability for glutamic acid production (Kikuchi and Nakao, 1973). Glutamate and succinate binding activities were reportedly solubilised from membrane vesicles by non-ionic detergents particularly Lubrol Wx (Kay, 1981). Some other non-ionic surfactants such as

tween-80 and sucrose monopalmitate were reported to have increased permeability of membrane in Aspergillus niger causing release of enzymes (Reese and Maguire, 1969). Apart from the enzymes, pigments and enterotoxins were reported to have been released from the cells in higher yield. Paunescu et al., (1964) reported that treatment of mycobacterial cell growth with tween-80 showed increased cell permeability, thus allowing triphenyl tetrazolium entry into the cells. Higher amylase production by cells of Lactobacillus cellobiosus was reported when they were treated with tween-80. Treatment of B. subtilis with a bacteriostatic concentration of clofoctol caused alteration of the cell envelope and strongly inhibited uptake of ^{14}C-glutamate while preloaded glutamate leaked out from the bacteria (Yablonsky, 1983). Dimethyl sulfoxide has been used to permeabilise yeast cells for higher yield of enzyme, thus leading to a rise in enzyme activity (Denenu, 1985). Incorporation of certain antibiotics which act on the cell wall or cell membrane has proved successful in increasing the yield of glutamic acid. Such antibiotics include penicillin and cephalosporin (Nakayama, 1985).

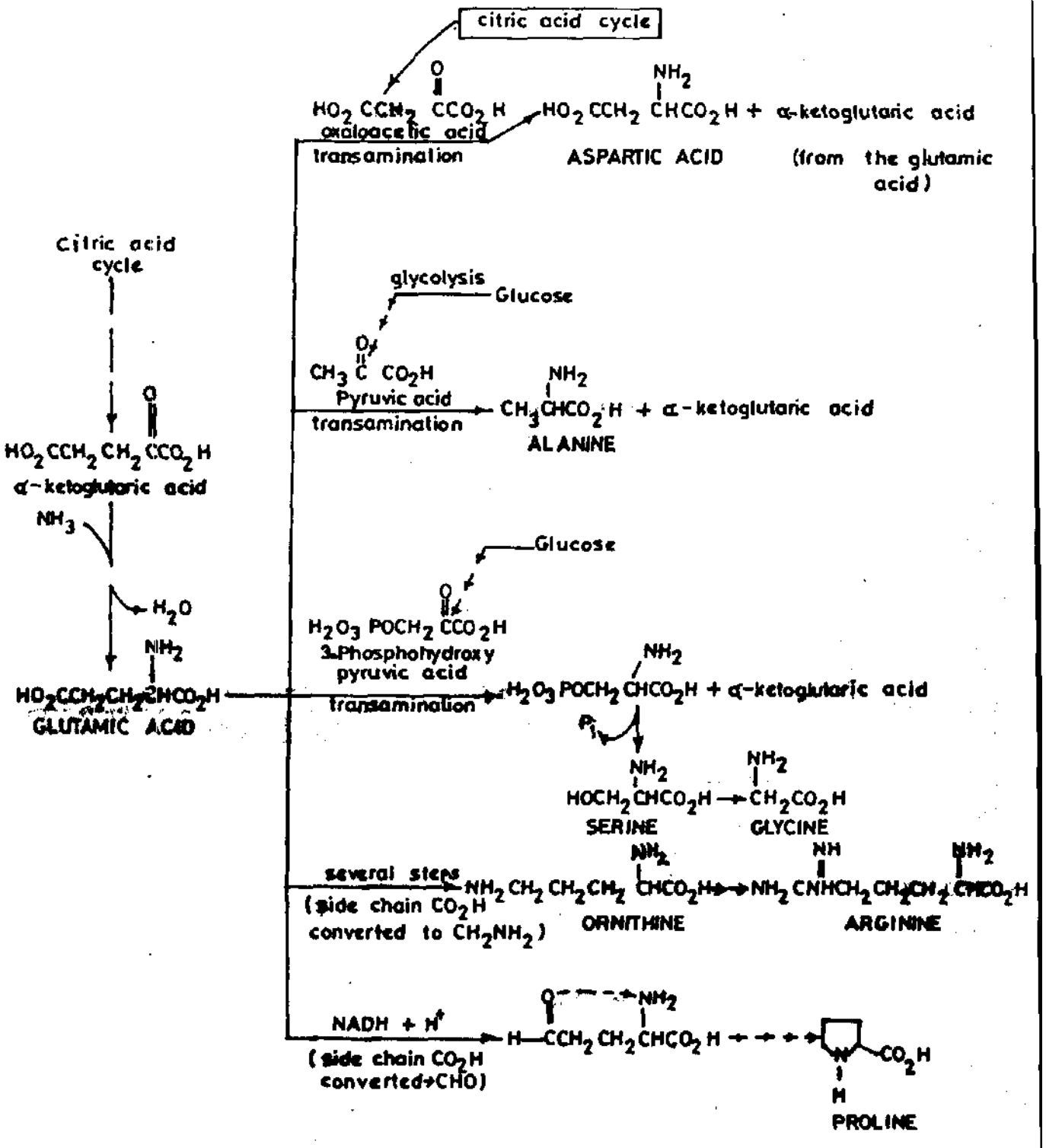


Fig. 2: THE BIOSYNTHESIS OF SOME NONESSENTIAL AMINO ACIDS FROM GLUTAMIC ACID (ADOPTED FROM HOLUM, 1969)

2.7 Genetic Improvement of Industrial Microorganisms

Even among bacteria, spontaneous mutation which is known to occur randomly also occurs relatively rarely. This necessitates pressing for induced mutation. Microorganisms, particularly bacteria have been considered ideally suitable for mutagenic studies because they have short generation time, give rise to enormous population which can easily be obtained and manipulated under controlled conditions while the few mutants arising in the population can be detected by selective methods. The overall goal in improving or selecting strains of microorganism for their products is to optimise the particular trait necessary for production of that product (Johnston, 1985). Often the process of screening large numbers of isolates for a promising variant is a trial and error exercise even where programmes of mutation and hybridisation have been followed. An organism with an improved and more efficient production trait of a particular metabolite over the parent strain can be obtained by subjecting that parent strain to mutagenic substances or/and recombination processes. Mutations have been classified on the basis of mode of generation.

2.7.1 Mutagenesis

A change in the genetic make up of microorganisms can be achieved through many ways. The processes by which these changes may occur naturally and biologically are via transduction conjugation and transformation. For the process of transduction a culture of an organism was treated with a

cell-free extract of another in the presence of a filterable agent-virus which mediated the process of transduction (Lederberg et al., 1951). On the other hand, the recombination of characters by two mating cells (conjugation) was discovered in E. coli K-12 by Lederberg and Tatum (1946) who were able to select rare prototrophic recombinants from mixtures of auxotrophic mutant strains on unsupplemented minimal media. Lactic acid bacteria with improved performance and resistance to phage in dairy fermentations have been developed via the processes of transduction and conjugation (Gasson, 1983). The discovery of the process of transformation whereby genetic material from dead pneumococci were incorporated into the genetic make up of living pneumococci in the test tube (Dawson and Sia, 1931) and even from cell-free extracts (Alloway, 1933) threw light on another method of effecting development of an organism with changed characters. Hybrids or recombinant strains with improved product yield are also obtained from recombination of different strains which combine their genes. By the process of protoplast fusion, genes have also been transferred to effect improvement in the new strain (Johnston, 1985).

2.7.2 **Agents of Mutagenesis**

The induction of mutation by physical and chemical agents is also a method which has been extensively used in strain improvement programmes. Such mutations have been classified on the basis of their mode of generation. The

base analogue class whereby the base analogues replace the normal bases of DNA during replication is exemplified by 5-bromo deoxyuridine (BD) or 5-bromouracil. The ease with which these base analogues cause mutation is explained to be due to destabilisation of their hydrogen atoms by the bromine atom which has a higher electronegativity than the substituted methyl group (Freese, 1959a).

Other mutagens such as nitrous acid act on the nucleic acid bases of resting DNA in such a way that the specific pairing with other bases is changed. Watson and Crick (1953) explained that spontaneous mutation could arise from a tautomeric shift in the position of a hydrogen atom on adenine, thereby leading to improper pairing of adenine with cytosine instead of with thymine. Nitrous acid is highly mutagenic, acting on the nucleic acids by oxidatively deaminating their bases. For example, adenine is deaminated to hypoxanthine which now pairs with cytosine instead of thymine. The action of nitrous acid is predominantly on adenine and cytosine which can also be deaminated to uracil (Hayes, 1964). Hydroxylamine is said to have a mutagenic property similar to that of nitrous acid (Hayes, 1964).

Another category of mutagens are the alkylating agents which remove bases from DNA. Alkylating agents like ethyl ethane sulphonate and ethyl methane sulphonate act specifically on guanine at the N-7 position. This labilises the deoxyriboside linkage so that 7-alkyl guanine is released from the DNA even at 37^{o}C and neutral pH (Brookes and

Lawley, 1960). N-nitroso-N-methylurea has been used to induce development of a phage-resistant mutant of Streptococcus cremoris for use as cheese starters (Odegov and Kagan, 1980). Similarly, mutants of Lactobacillus bulgaricus and L. casei with increased acidity and protease activity in milk fermentations have been produced by exposure to nitrosoguanidine, one of the most potent mutagens (Singh and Choppa, 1982).

2.7.3 **Dyes - Acridines**

Other mutagenic agents are well known dyes of the acridine series such as proflavin and 5-aminoacridine. These were among the first to be reported as mutagens (DeMars, 1953) for bacteriophages although they are less significant as mutagens for bacteria. Acridine-induced mutations were at first said to form an exclusive class as the exact mode of action was not clear. It was however observed that there is a similarity between the characteristics of spontaneous mutation and those of acridine-induced mutations. Both types cannot be reverted by base analogue (Freese, 1959b). An alternative explanation was given by Brenner et al., (1961) to the earlier one of transversion. It explains that acridine mutations are caused by deletion or addition of a base pair. Further investigations of the possible mode of action of acridine on DNA, using X-ray diffraction analysis and other physical methods, led to confirmation of this deletion or insertion hypothesis. Lerman (1961) reported that, at high concentrations, acridines are bound to the

phosphate groups of the DNA backbone on the outside of the double helix while, at relatively low concentrations, evidence strongly suggested that acridine molecules become inserted or intercalated between adjacent base pairs in the DNA (fig.3), thus extending and unwinding the phosphodiester backbone (Lerman 1963). This has the effect of stretching the distance between them to 6.8 Å as opposed to 3.4 Å, the normal base pair distance on the DNA (Hayes, 1964). Treatment of a strain of Bacillus subtilis natto with acridine orange resulted in the conversion of approximately 64.2 percent of the population to polyglutamate non-producing colonies (Hara et al., 1982).

2.7.4 Radiations

Artificial induction of mutation was first accomplished by means of X-rays in Drosophila (Muller, 1928) after which it was discovered that ionising radiations in general which include X-rays, neutrons and ultraviolet rays are also mutagenic. Promptov (1932) reported inducing mutation by use of ultraviolet light in Drosophila. It was not until about a decade later that a detailed study of the induction of mutations in bacteria using radiation (X-rays and ultraviolet light) was reported (Demerec and Latarjet, 1946). Since then the advantages of short generation time, haploid DNA and large population have led to increasing use of bacteria for both irradiation and mutational research.

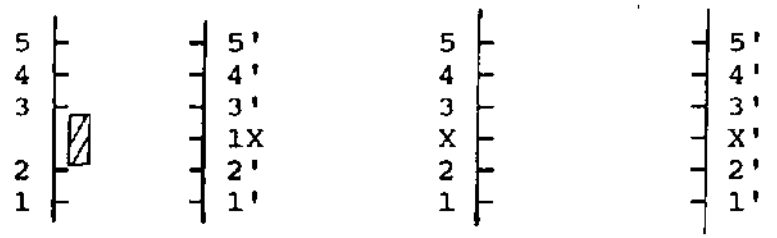
Nucleic acid is said to display a specific absorption

spectrum for ultraviolet light of wavelength about 254mu. Among the bases of the DNA the pyrimidines are more easily altered by becoming hydrated at their 4,5-bond than the purines. Cytosine nucleotides are even more susceptible to the photolytic action of ultraviolet light than thymine nucleotides (Shugar, 1960). It was however later observed that the actual effect of the ultraviolet light on the DNA is really a dimerisation of the thymine nucleotides, resulting in formation of thymine dimers (Beukers and Berends, 1960) leading to a weakening of the bonds between them and a distortion of the architecture of the double helix. This distortion then later gives rise to dimerisation of thymines on opposite strands causing crosslnkages of the strands and preventing their separation, thus interferring with replication (Kelner, 1953). Mutation has been achieved in parent strains of Brevibacterium or Corynebacterium spp. that produce L-glutamic acid in a high yield by exposing them to ultraviolet light or X-ray irradiation and other mutagens (Chome, 1981).

Intercalation mutation by acridines

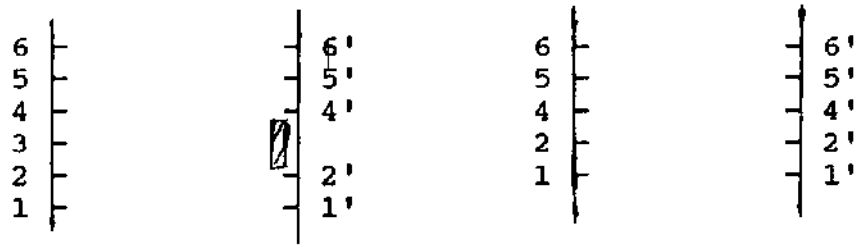
The acridine compound ▭ inserts between bases increasing the distance from the normal 3.4Å to 6.8Å gap.

i: By isertion



During replication, a base inserts in the gap on the opposite new strand labelled X. This new base will be perpetuated in the double-stranded DNA during subsequent replication.

ii: By Deletion



During replication, the interposed acridine masks the base on the template strand so that the complementary partner cannot pair with it. This gives rise to a complementary strand deficient in base 3', and subsequent replication will perpetuate the deficiency in that missing base.

CHAPTER 3

MATERIALS AND METHODS

3.1 SECTION A

Collection and Distribution of Legume Seeds for Daddawa Fermentation

Locust bean (Parkia biglobosa) and soya bean (Glycine max) seeds were purchased in bulk from the market in Zaria, Kaduna State, Nigeria. The two types of legume seeds were submitted separately to two traditional experts, Mallama Iya Tasallah (locust bean) and Mrs. Deborah Dada (soya beans) for the fermentations of the two types of daddawa.

3.1.1 Daddawa Fermentations

a). Locust bean - daddawa fermentation

The locust bean seeds were boiled for 12 hours after which the seed testa became soft and removable from the cotyledons. The seeds were then pounded lightly in a wooden mortar. The testa were removed by hand and the cotyledons were washed and boiled again for two hours with some potash added to soften it. The soft seeds were then drained and spread on a raffia mat to cool to about 35^{o}C, after which they were packed in bamboo baskets which had been plastered on the outside with garden soil. Each basket was covered with three sheets of polythene kept in place by a weight and the seeds were left to ferment in a warm environment (35^{o}C) for about 48 hours. Six separate fermentations were carried out in the fermentation hut of Mallama Iya Tasallah at Bomo

village, eight kilometers away from the laboratory in the Department of Microbiology at Ahmadu Bello University, Zaria.

b) Soya bean - daddawa fermentation

The soya bean seeds were roasted in a frying pan for three minutes and were then soaked in water for one hour. Subsequently they were pounded lightly in a wooden mortar, dehulled and washed. The dehulled seeds were boiled for about 45 minutes, placed in bamboo baskets which had been lined on the inside with washed banana leaves. The beans were covered with other washed banana leaves and left to ferment at ambient temperature (32 $\pm$ 2^{o}C) for two days. Three separate fermentations were carried out in the kitchen store of Deborah Dada (a trained Home Economist) at one of the university staff housing quarters.

3.1.3 **Sampling and Microbiological Analyses**

In the sampling process, 10g samples of the fermenting beans were taken aseptically at intervals with a sterile spatula from both types of daddawa fermentations. Each sample was shaken by hand in 90ml sterile saline to dislodge micro-organisms from the beans. The suspension was streaked for isolation on Nutrient agar (Oxoid), Tryptone soy agar (Oxoid) Sabouraud dextrose agar (BBL) and maltose agar for trial runs. Maltose and Sabouraud dextrose agar were incubated at room temperature (27 $\pm$ 1^{o}C) for one week. Others were incubated at 37^{o}C, half aerobically and half anaerobically. Thereafter only nutrient and Tryptone soy agars were

used for every sample as neither maltose agar nor Sabouraud dextrose agar showed evidence of fungal involvement in the fermentations.

3.1.4 Characterisation of Isolates

Representative colonies of the micro-organisms which developed on the aerobic plates of both nutrient and tryptone soy agars were Gram stained, suspended in saline, and heated in a water bath at 80°C for 15 minutes to assess their heat resistance. The suspensions were streaked on nutrient agar and growth (presumably resulting from heat-resistant spores) was checked after 24 hours. Sporulation was further observed by streaking the isolates on soil extract agar (Gordon et al., 1973). They were then subjected to biochemical tests according to the stepping stone identification scheme described by Gordon et al., (1973) for Bacillus spp.

3.1.5 Determination of Cell Counts

The surface viable count method of Miles and Misra (1938) was used as colonies trapped within the agar could not be differentiated from each other in the pour plate method. Serial dilutions of the suspension of each sample taken from the fermenter were prepared and 0.02 ml volume of each suitable dilution was dropped on each of the pre-dried media in Petri dishes. The plates were incubated at 37°C and plate counts were taken after 24 hours and were expressed as colony forming units per gram (cfu/g) of sample.

3.1.6 Growth of Predominant Isolates at Different Oxygen Levels

Three sets of nutrient agar plates which had been dried in the incubator for two hours at 37°C, were streaked and three sets of nutrient broth in test tubes were inoculated with the isolates. One of the three sets was incubated aerobically, another incubated semi-anaerobically in a candle jar, and the third set, anaerobically in a gas pak anaerobic system, all at 37°C. Average colony sizes were recorded by measuring diameters of a few colonies on plates incubated under each oxygen tension. Counts of the incubated nutrient broth in test tubes were determined using pour plate method.

3.1.7 Temperature and pH Measurement

Temperature and pH readings were taken at intervals during the fermentations. Ten grams of the fermenting sample was blended in 40 mls distilled water and the pH of the suspension determined using a pH meter (PYE UNICAM Model 292 MK2). The temperature was monitored by means of a thermometer inserted in the basket.

3.1.8 Determination of Glutamic Acid and Moisture Contents of Daddawa

Both locust bean and soya bean seeds were prepared for fermentation and half of each was fermented in the traditional uncontrolled manner as in section 3.1.1a. Out of the other half, 300g of each was weighed into separate 500ml

Erlenmeyer flasks, plugged with cotton wool and sterilised in the autoclave at 15 lbs/sq" pressure for 15 minutes. After cooling, they were inoculated with 1ml stock culture of the Bacillus subtilis DU_2 and DS_3 respectively (section 3.3.2). The inoculated beans were incubated for fermentation at 37°C for 48 hours. The fermentations were then terminated by autoclaving as previously described. Five gram samples were taken from each of the laboratory-fermented and uncontrolled-fermented daddawa, mixed separately with 10ml distilled water, shaken vigorously and filtered through Whatman #1 paper. The filtrates were assayed for glutamic acid as in section 3.3.4b. Another set of 5g samples was taken in glass Petri dishes without cover and oven-dried at 70°C for 4 hours until constant weight was attained after which the moisture content was calculated.

3.1.9. **Glutamic Acid Release in Fermenting Locust Bean Seeds**

Locust bean seeds were prepared for laboratory fermentation of daddawa as above (Section 3.1.8). Before fermentation, a sample of 5g was taken and during fermentation samples of 5g were taken at 12 hourly intervals; half of these samples were assayed directly for glutamic acid while the other half were hydrolysed and assayed for glutamic acid using Technico Sequential Multisample (TSM) autoanalyser as described by Spackman et al. (1958).

3.2 SECTION B

3.2.1. Isolation of *Bacillus* species from Fermented Legume Condiments.

The following Nigerian condiments were purchased from the local market: locust bean daddawa, soyabean daddawa, ophihe, ogiri and ugba. They were examined as follows for isolation of *Bacillus* species: 10g of each sample was transferred into 90mls sterile saline, shaken vigorously, allowed to stand for 10 minutes and shaken again to dislodge micro-organisms. The suspension was heated in a water bath (Gallenkamp) at 80°C for 15 minutes and was thereafter plated on nutrient agar for isolation of heat resistant micro-organisms. The isolates obtained were Gram-stained, streaked on soil extract agar for sporulation and subjected to tests in the stepping stone identification key of Gordon *et al.*, (1973). Those that gave typical reactions and the isolates from section A were stored on nutrient agar slants at 5°C, subcultured monthly and used for the next experiment.

3.2.2 Preservation of Stock Cultures

Heavy inocula of 24 hour-old nutrient agar cultures of the *Bacillus* isolates (section B 3.2.1) were transferred into sterile distilled water in medical bottles. They were diluted with sterile distilled water to give an absorbance reading of 1.8 on the spectrophotometer (WPA S101) at 540 nm wavelength. Counts were determined by pour plate method on nutrient agar. Subsequently, the suspensions were dispensed aseptically in volumes of 2ml into tubes and were freeze-

dried using Edwards high vacuum (PIRANI II) freeze drier. These were stored as stock cultures and were reconstituted when needed.

3.3 SECTION C

3.3.1 Screening and Selection of *Bacillus* Isolates for Glutamic Acid Production

The strains of *Bacillus* maintained previously as stock cultures, were grown separately in a basal medium as described below (section 3.3.3) and examined for ability to secrete glutamic acid into the medium.

3.3.2. Inoculum Preparation for Glutamic Acid Fermentation

Each stock culture was reconstituted with 1.0ml sterile distilled water and shaken. The contents were transferred into a test tube containing 10ml sterile distilled water. One millilitre of the reconstituted stock culture was used as inoculum in the experiments.

3.3.3. Preparation of Basal Medium

The basal medium used for glutamic acid fermentation was a modification of that recommended by Nakayama (1982). It was constituted with the components listed in Table 4. Ammonium chloride was used as the source of ammonium ions for the fermentations in section 3.2 while urea was employed for the fermentations using *B. subtilis* DU_2 because it was found to be urease positive.

After constituting the medium, the pH was adjusted to 7.4 using sodium hydroxide. It was then filter-sterilized using a membrane filter (Millipore) with 0.3μ pore size and dispensed aseptically in 200ml amounts into 500ml Erlenmeyer flasks in duplicates. Each flask was then inoculated with 1ml of the reconstituted stock culture and incubated at 32^{o}C for 72 hours on a shaker (Lab-line orbit Environ-shaker 18) at 200 rpm. Samples were taken from the shaken flasks for analysis at 12 hourly intervals where necessary; otherwise they were taken at the 72nd hour (end) of the fermentation.

Table 4 **Composition of Basal Medium used for Glutamic Acid Production**

Components	Percentage
Glucose	10.0
Urea/ammonium chloride	0.5
KH_2PO_4	0.1
K_2HPO_4	0.1
$MgSO_4.7H_2O$	0.05
$FeSO_4.7H_2O$	0.005
$MnSO_4.4H_2O$	0.005
Biotin	0.3 μg

3.3.4 Analyses

a. Determination of Cell Growth during the Fermentation

The growth of the Bacillus in each fermentation flask was estimated on the basis of changes in absorbance at 540nm using spectrophotometer (WPA S101). Where media composition altered the absorbance significantly before the fermentation began, the spore count of the organism was taken by pour plate technique and recorded as colony forming unit per ml (cfu/ml).

b. Detection and Estimation of Glutamic Acid

Glutamic acid in fermentation broth was detected and estimated qualitatively and quantitatively. The qualitative assay involved chromatographic comparison of samples of the fermentation broth with a pure glutamic acid standard. Whatman No.1 chromatography papers were used. The chromatography papers were prepared, ruled and labelled using pencil. They were spotted with about 2.0µl of L-glutamic acid standard prepared by acidifying 10ml of 0.1M solution of the standard with 10 drops of 19 percent hydrochloric acid, and heating to dissolve. Twenty microlitre of each test sample was spotted on the labelled chromatogram after which it was allowed to air-dry and was developed in 80 percent aqueous phenol in a descending chamber for 4 hours. The developed chromatogram was again air dried and sprayed uniformly with 0.2 percent ninhydrin (w/v in ethanol) in a chamber. After drying, the chromatogram was placed in an oven at 110^{o}C for about 3 minutes to develop purple spots. The width and

intensity of the purple spots (which have the same R_f values with the standard) were scored + or ++ on comparative basis.

The quantitative assay was carried out according to the method of Rangaswamy and Yathirajan (1985). Five millilitres of the fermented sample was taken after filtering through Whatman No.1 filter paper and added to 2.5ml of acetate buffer (41.0ml of 0.2M acetic acid and 9.0ml of 0.2M sodium acetate pH4) in 250ml Erlenmeyer flask. Twenty-five millilitres of 0.05M bromamine-T solution was added and allowed to stand for five minutes with occasional shaking. Then 15ml of 1M sulphuric acid was added, followed by 15ml of 10 percent potassium iodide solution. The iodine liberated was titrated with 0.1M solution of sodium thiosulphate. A blank titration was similarly carried out with bromamine-T but without glutamic acid containing sample. Glutamic acid content (per 5ml sample) was calculated using the following formula:

$$\text{Glutamic acid (Xmg)} = \frac{M \times Y \times (V2 - V1)}{4}$$

where M = Molecular mass of glutamic acid

Y = Molarity of the thiosulphate solution

V_1 = Volume of thiosulphate for the test titration

V_2 = Volume of thiosulphate for the blank titration

3.4 SECTION D

3.4.1 Effect of Cultural Conditions on Glutamic Acid Production by *Bacillus subtilis* DU_2

The *Bacillus* isolate from section A which was identified as *B. subtilis* and was observed to be one of the highest glutamic acid yielding isolate was selected for subsequent studies. After preparing the media and varying the different cultural parameters described below, they were inoculated, incubated for 72 hours and their glutamic acid contents were determined as in section 3.3.4b quantitatively.

3.4.2. Effect of Different Carbon Sources

Different carbon sources, namely sucrose, galactose, fructose, maltose, lactose, mannitol, xylose, starch, hydrolysed starch, molasses (sugarcane) glycerol, succinate, citrate and paraffin were substituted separately for glucose in the basal medium (Section C 3.3.3). The non-carbohydrate carbon sources were incorporated in 5 percent concentrations. They were filter-sterilised, inoculated and fermented in duplicates as in the preceeding sections. The fermented samples were analysed for glutamic acid yield and growth of the micro-organism.

3.4.3 Effect of Biotin

The final concentrations of biotin in the basal medium in duplicate flasks were varied from 0 (no biotin) to 10µg/litre. Suitable volumes of a filter-sterilized stock

solution of biotin were introduced into the basal media to give the desired final concentrations.

3.4.4 Effect of Different Leguminous Seeds Flour Supplements

The dry milled flour of selected leguminous vegetable seeds - soya bean (Glycine max), locust bean (Parkia biglobosa) bambara nuts (Voandzeia subterranea), cow pea (Vigna unguiculata L. Walp.) and peanuts (Arachis hypogea) were sterilised by autoclaving at 121°C for 15 minutes and each was incorporated into the filter sterilised basal medium to give a concentration of 0.5 percent w/v.

3.4.5 Effect of Different Nitrogen Sources

The following different nitrogen salts: ammonium sulphate, ammonium nitrate, sodium nitrate and ammonium chloride were each incorporated at 0.5 percent concentrations into the basal medium in place of the urea.

3.4.6. Effect of Metal Ions

The chlorides of Mn^{2+}, Ca^{2+}, Mg^{2+}, Zn^{2+}, Na^{+} and Co^{2+} were incorporated into the basal medium at concentrations varying from 0.2 to 2.0 mg/ml. Where the particular metal ion is a component of the basal medium it was made to replace such in the formula.

3.4.7. Effect of Initial pH

The pH of the basal medium was adjusted using 0.1N sodium hydroxide or 0.1N sulphuric acid to obtain a range

varying from 6.0 to 8.5. A pH meter (PYE UNICAM model 292 MK2) was used.

3.5 SECTION E

3.5.1 Effect of Surfactants

Alkyl phenyl polyethylene glycol (Triton-x) and polyoxy-ethylene sorbital monooleate (Tween-80) were used as surface-active agents in this section, to determine their effects on glutamic acid production.

Each of the surfactants was incorporated into the filter-sterilised, basal medium to give concentrations of 0, 0.025, 0.05, 0.1 (v/v) at zero hour and at the 66th hour of fermentation. Where the surfactants were not added initially before filter-sterilizing, they were autoclaved separately and added to the filter-sterilised basal medium. Glutamic acid determinations were carried out at the 66^{th} and 72^{nd} hour (end) of the fermentations.

3.6 SECTION F

3.6.1 Irradiation

A lyophilised culture of B. subtilis was grown on nutrient agar. From the nutrient agar it was inoculated into nutrient broth and incubated overnight.

The overnight broth culture was centrifuged in a bench centrifuge (B & T - Searle Co.) at 2000 rpm for 10 minutes. The pellet formed was resuspended in 25ml of sterile irra-

diation buffer (Clowes and Hayes, 1968) containing 7g Na_2HPO_4, 3g KH_2PO_4, 4g NaCl, 0.2g $MgSO_4.7H_2O$ all dissolved in the order listed in 1000ml of distilled water and sterilised by autoclaving. The total cell population was adjusted with the sterile buffer to approximately 2.3×10^5 per ml.

Ten millilitre aliquots of the buffer suspension were transferred into flat-bottomed glass petri dishes. The culture in each plate was then exposed to irradiation sources (UV light and X-rays) by removing the lid for the required exposure period.

3.6.1a **Ultraviolet light**

A germicidal ultraviolet fluorescent tube mounted 9cm above the laboratory bench was used as the source of ultraviolet light (UV). The fluorescent UV light was switched on for one hour to stabilise the emission. After this the sample plates were exposed to it for 5, 10 and 15 minutes. Survivor counts were determined by the agar overlay method. The samples with 90 percent or more reduction in cell counts were randomly selected as possible mutants for fermentation studies and were tested biochemically.

3.6.1b **X-ray**

An X-ray emission machine (Roentgen V) was placed 24 inches above the working surface. The sample plates were exposed to varying doses (70, 100, 110, 120, 130 KVP) of X-ray radiation for 3, 3, 2, 2 and 1 sec respectively. After

irradiation, counts were determined by the agar overlay method and irradiated isolates picked randomly for use as inocula for glutamic acid fermentation studies and were tested biochemically.

3.6.2 Acridine Orange Treatment

Nutrient broth was prepared and acridine orange was incorporated at three concentrations (5, 10 and 15µg/ml) in separate 250ml Erlenmeyer flasks. They were inoculated with an overnight culture of the test organism. The inoculated flasks containing the acridine orange-broth were incubated on the shaker (Lab-line orbit Environ shaker 18) with shaking at 200 rpm for 36 hours. Thereafter, the cultures were inoculated into nutrient agar-acridine orange plates. The colonies that developed after incubation were randomly selected for glutamic acid fermentation studies and were tested biochemically.

CHAPTER 4

RESULTS

In all the six separate fermentations for locust bean daddawa production, only bacteria, namely Bacillus, Staphylococcus and Micrococcus species were isolated at the initial stages (Table 4.1). The Bacillus species predominated from about the sixth hour up till the end of the fermentations, as shown by the counts of the bacteria obtained in the fermentations. Varied species of Bacillus (Table 4.2) were obtained, namely B. pumilus, B. licheniformis and B. subtilis which predominated in four of the fermentations.

Observation of these fermentations showed that adequate covering of the system, to exclude as much air as possible, was essential for obtaining a good fermentation, free of bacterial contaminants. Under different oxygen tensions, the predominant Bacillus thrived differently. The counts obtained under aerobic and reduced oxygen tension conditions were 3.6×10^8 and 4.2×10^8, respectively; giving virtually the same growth. Under reduced oxygen tension the organism spread better and showed larger colony sizes (Plate 4.1).

Table 4.4 shows the characteristics of the two species of bacteria found involved in the soya - daddawa fermentation. They were identified as Bacillus pumilus and B. subtilis. The succession of these organisms (table 4.5) shows that both species of the Bacillus were found up till the end of the fermentation with the B. subtilis predomi-

nating. The rhizoidal appearance of the B. pumilus colonies often obtained at the initial stage, changed as the fermentation progressed to a pale yellowish translucent colonial morphology.

Physical changes in both locust bean and Soya bean - daddawa fermentations showed steady rise in both temperature and pH (Fig. 3 and Table 4.5). The temperature rise was from 28°C at the start to 42°C at the end of the fermentations in both cases while the pH rise was from 6.0 (locust bean-daddawa) and 5.5 (Soya bean - daddawa) to 8.3 and 8.6 respectively.

The glutamic acid and moisture contents of both locust bean and soya bean - daddawa are shown in Table 4.6. The moisture content for laboratory fermented locust bean - daddawa was 66.8 per cent while that for soya bean - daddawa was 65.4 per cent. These values were hihger than those recorded in traditionally fermented products. The glutamic acid content for the laboratory fermented types of daddawa was more than that of the latter. Analyses of samples of fermenting locust bean seeds (hydrolysed and unhydrolysed) at time intervals for glutamic acid showed progressive release of glutamic acid with time (Fig. 3). The level of glutamic acid in unfermented hydrolysed seeds (3.7mg/g) formed the differential baseline between the two curves (Fig 3) whereas there was no detectable level in the unhydrolysed unfermented seeds. The pH changes in the fermentation fol-

lowed a similar curve with those of glutamic acid release.

A total of twenty-five Bacillus strains which were identified as eight different species were isolated from the five indigenous condiments (Appendix A). All the isolates showed evidence of glutamic acid production (Table 4.7 and Plates 2-4). The yields of glutamic acid in the basal medium by the isolates in this study ranged from 5.32 to 12.31 mg/ml and were detectable only after 24 hours of fermentations. The time course of glutamic acid production on glucose basal salts medium by the two selected strains of B. subtilis DU_2 and DS_3 involved in the fermentations of the condiments, showed a rather prolonged lag period of over 12 hours (Fig. 4). This period was followed by a period of rapid release of glutamic acid reaching a plateau after 60 hour.

Table 4.8 shows the yields of glutamic acid by the B. subtilis-DU_2 when supplied with different carbon sources. Various sugars differed in their ability to support glutamic acid production. Sucrose, glucose and galactose appeared most suitable for high glutamic acid yield followed by fructose, hydrolysed starch, mannitol, xylose, maltose, glycerol, citrate and n-paraffin. Low yields were obtained with molasses, starch, succinate and lactose. Drop in pH were more in the media with those carbon sources that supported more yields of glutamic acid.

Varying the concentrations of biotin gave varying

yields of glutamic acid (Fig. 5). The maximal glutamic acid yield was obtained with 4 - 6 µg/l of biotin. Below or above these concentrations the yields of glutamic acid dropped.

Fig. 6 shows the influence of initial pH of the basal medium on the yield of glutamic acid Maximum yields were obtained with initial pH of 7.5 followed by pH 7.0, 8.0, 6.5 and 6.0. The final pH after fermentation were 6.2, 6.8, 7.0, 6.6 and 6.0 respectively. Fig. 7 on the other hand shows maximal yields of glutamic acid at temperatures between 30 and 40^{o}C. Below and above this range the yields decreased.

Table 4.9 shows the influence of various nitrogen sources on the yield of glutamic acid. The maximum yields were obtained with urea followed by ammonium chloride, ammonium sulphate ammonium nitrate and sodium nitrate.

Table 4.10 shows the influence of various metal ions on glutamic acid production by the B. subtilis DU_2. Growth of the organism in the absence of magnesium and manganese ions produced remarkably low yields of glutamic acid. In contrast, conspicuously high yields (12.0mg/ml) of glutamic acid were achieved at 0.2 mg/ml concentrations of the metal ions. Yields decreased at hihger level of the ions. Cobalt, zinc and copper ions appeared inhibitory giving reduced growth or no growth at all and low yields or no yield at all of glutamic acid. Calcium and sodium ions did

not seem to make any difference.

Different leguminous protein supplements of the basal medium gave yields of glutamic acid that are comparable with the yield in the basal medium alone (Table 4.11). This yield was highest with soya bean followed by locust bean, peanuts and bambara nuts in that order.

The influences of the two surfactants (Triton-X and Tween - 80) on the level of glutamic acid produced by the Bacillus species were similar. The presence of certain concentrations of both surfactants in the medium enhanced glutamic acid production (Tables 4.12 and 4.13). Incorporation of the surfactants into the medium at the beginning of the fermentation was more favourable for increased glutamic acid production than surfactant addition towards the end (66th hour).

Glutamic acid production at two different temperatures by mutants of B. subtilis DU_2 are shown in Tables 4.14 - 4.16. The lethal effect of ultraviolet light on the population of the wild strain which although shows a kill of about 99 percent (Appendix B) did not alter the biochemical characteristics (Appendix D) of the organisms. The X-ray irradiation on the other hand did not give a noticeable kill (Appendix C). At 32°C, three out of the nine ultraviolet-derived mutants showed marked reduction in glutamic acid yields over the yield in the wild strain while the rest gave comparable yields. Most of the mutants (Six) at 40°C gave

higher yields of glutamic acid than the levels obtained at 32°C and about half the number of isolates (five) gave higher yields over the wild strain at the same temperature. The yields of glutamic acid from X-ray derived mutants fell within a close range at both temperatures except for two isolates which gave higher yields at 40°C while those from acridine orange mutants all fell within a close range at the two temperatures.

Table 4.1: Development of Micro-organisms in fermenting locu beans in six fermentations

	Bacteria counts (cfu/g) in six fermentations					
Time (Hrs)	1,2,4 & 6		3		5	
	(Average counts in the 4 fermentation)					
	Bacillus subtilis	Staphylococcus & Micrococcus Species	Bacillus pumilus	Staphylococcus & Micrococcus Species	Bacillus licheniformis	Staphylococcus & Micrococcus species
0	$6.5x10^5$	$6.2x10^2$	$3.0x10^4$	$1.5x10^2$	$2.1x10^3$	$1.8x10^2$
6	$9.6x10^6$	$5.4x10^2$	$4.3x10^5$	$3.3x10^2$	$5.2x10^4$	$2.3x10^3$
12	$5.3x10^8$	-	$5.6x10^7$	-	$4.7x10^6$	-
24	$5.7x10^9$	-	$7.5x10^9$	-	$4.7x10^6$	-
36	$3.5x10^{10}$	-	$1.6x10^{10}$	-	$4.1x10^{10}$	-
48	$3.8x10^{11}$	-	$8.3x10^{10}$	-	$8.8x10^{10}$	

- = Not detected

Table 4.2 Morphological and Biochemical Characteristics of *Bacillus* spp isolated in six fermentations.

Tests	Separate fermentations		
	1,2,4 & 6	3	5
Gram reaction	+	+	+
Catalase activity	+	+	+
Voges Proskauer	+	+	+
Growth in			
anaerobic agar	-	-	+
Starch hydrolysis	+	-	+
Acid from;			
Mannitol	+	+	+
Glucose	+	+	+
Xylose	+	+	+
Citrate utilization	+	+	+
Propionate utilization			
Indole production	-	-	-
Urease activity	-	-	-
Nitrate reduction	+	-	+
Gelatin Liquefaction	+	+	+
Litmus milk reaction	ALD	ALD	ALD
Lipolytic activity	+	+	+
Growth at 65^{o}C	-	-	-
Growth at pH 5.7	+	+	+
Bacillus spp identified	*B. subtilis*	*B. pumilus*	*B. licheniformis*

AL = Alkaline reaction of litmus

D = Digestion of casein with reduction of litmus

Table 4.3 Characteristics of bacterial isolates from soya beans fermentation during 'daddawa' production.

Physical and Biochemical Characteristics	Strain No. 1	Strain No. 2
Colony morphology at;	Pale Yellowish	White
0-6 hours	rhizoidal colonies	colonies
	(6-8mm)	(4-7mm)
12-48 hours	Creamy translucent	"
	colonies	
	(4-6mm)	
Cell characteristics	Gram positive	Gram positive
	Sporeforming	Sporeforming
	rods	rods
Catalase activity	+	+
Voges Proskaur Test	+	+
Growth in anaerobic agar	+	-
Starch hydrolysis	-	+
Acid from;		+
Mannitol	+	+
Glucose	+	+
Xylose	+	+
Citrate utilization	+	+
Propionate utilization	-	-
Nitrate reduction	-	+
Litmis milk reaction	ALD	ALD
Galatin liquefaction	+	+
Urease activity	+	+
Bacillus spp identified	*B. pumilus*	*B. subtilis*

ALD = Alkaline digestion of casein

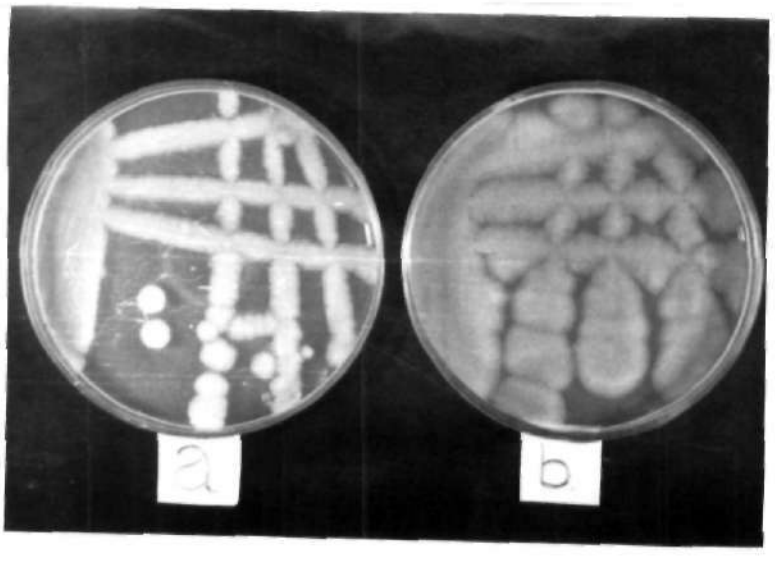


Plate 4.1: Colonies of B. subtilis (isolated from fermenting locust bean seeds) grown (a) under aerobic condition (average diameter 5 - 8mm); (b) under reduced oxygen tension in a candle jar (average diameter 12 - 17mm).

Table 4.4: Development of *Bacillus* species in fermenting soya beans during 'daddawa' production.

Time (hr)	Counts in colony forming unit per gram (cfu/g) of fermenting beans for three fermentations			
	B. pumilus		*B. subtilis*	
	Range	Mean	Range	Mean
0	$3.0\text{-}3.5\times10^{1}$	3.2×10^{1}	$3.9\text{-}4.8\times10^{2}$	4.3×10^{2}
12	$2.9\text{-}3.3\times10^{2}$	3.1×10^{2}	$3.1\text{-}3.4\times10^{4}$	3.3×10^{4}
24	$5.0\text{-}5.2\times10^{2}$	5.1×10^{2}	$4.8\text{-}5.5\times10^{6}$	5.1×10^{6}
36	$2.3\text{-}5.4\times10^{4}$	3.3×10^{4}	$3.1\text{-}9.7\times10^{8}$	6.8×10^{8}
48	$4.6\text{-}6.3\times10^{4}$	5.2×10^{4}	$2.3\text{-}10.8\times10^{9}$	6.4×10^{9}

Table 4.5: Changes in Temperature & pH during the fermentation of soya beans

Time (h)	Average values for the three fermentations	
	Temperature (^{o}C)	pH
0	28	5.5
12	32	6.8
24	38	7.6
36	41	8.4
48	42	8.6

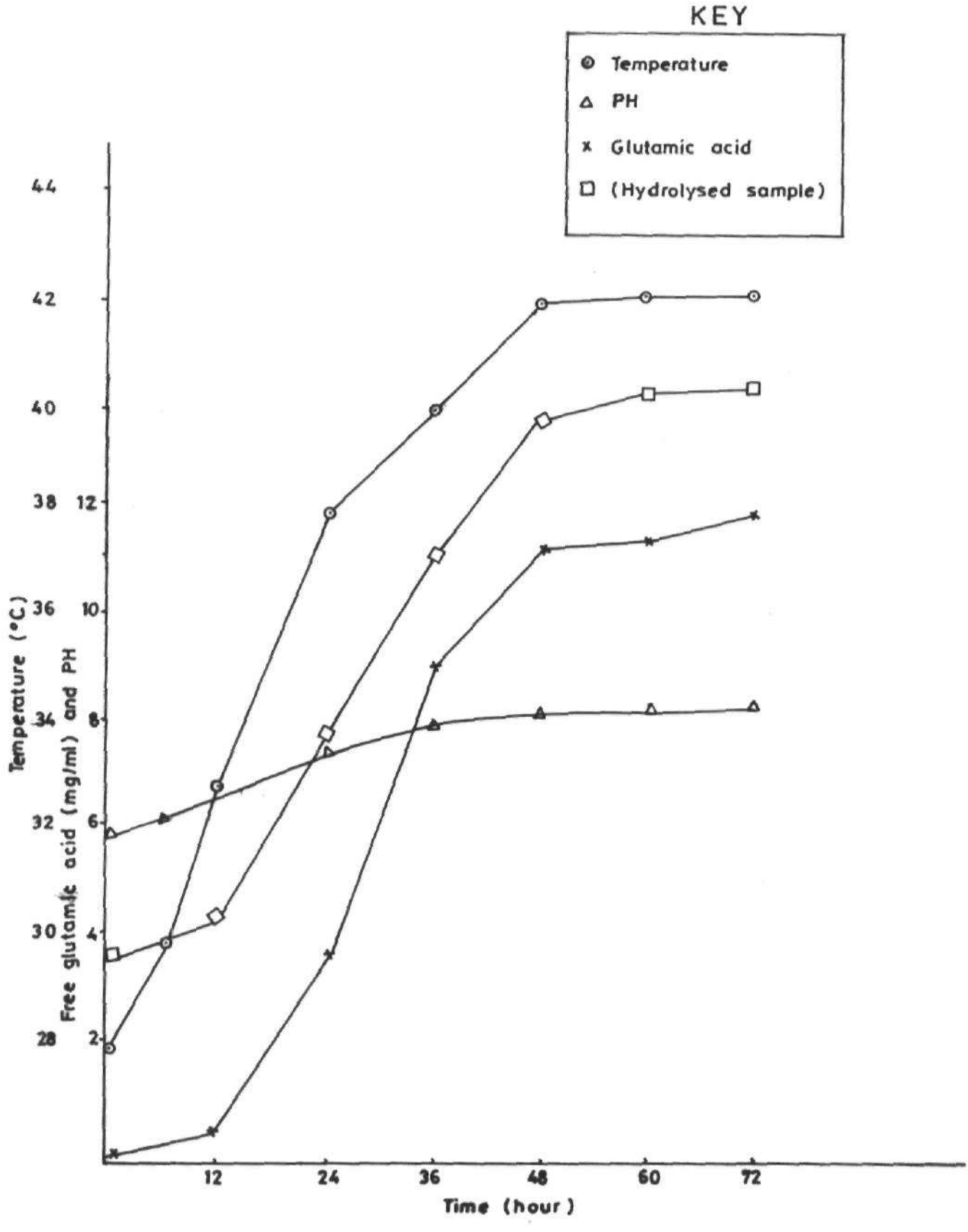


Fig. 3: GLUTAMIC ACID RELEASE AND PHYSICAL CHANGES (TEMPERATURE AND PH) DURING LOCUST BEANS-DADDAWA FERMENTATIONS.

Table 4.6: Free glutamic acid contents of dried and fresh locust bean and soya bean daddawa

Parameters	Locust bean - daddawa		Soya bean - daddawa	
	Laboratory fermented	Uncontrolled fermented	Laboratory fermented	Uncontrolled fermented
Wet weight (g)	5	5	5	5
Dry weight (g)	1.66	1.79	1.73	1.81
Percentage moisture in fermented seeds	66.8	64.2	65.4	63.8
Glutamic acid (mg/g wet weight)	11.72	9.8	12.64	9.87
Glutamic acid (mg/g dry weight)	35.31	27.37	36.55	27.27

Data - means of duplicate determinations.

Table 4.7: Screening of *Bacillus* isolates from fermented condiments for Glutamic Acid Production

Identity of *Bacillus*** Isolates	Qualitative: Time interval (h) 0 Ab	0 G	24 Ab	24 G	48 Ab	48 G	72 Ab	72 G	Quantitative: Glutamic acid (mg/ml)*
DS_1	0.09	-	0.18	-	1.1	+	1.6	+	10.50
DS_2	0.05	-	0.23	-	1.3	+	1.8	+	6.21
DS_3	0.05	-	0.19	-	1.1	+	1.6	++	10.60
DS_4	0.09	-	0.15	-	1.3	+	1.8	+	6.73
DS_5	0.13	-	0.22	-	1.3	+	1.8	++	10.54
DS_6	0.07	-	0.19	-	1.2	+	1.6	++	9.37
DU_1	0.09	-	0.17	-	1.3	+	1.7	++	8.51
DU_2	0.10	-	0.30	-	1.4	+	1.6	++	12.31
DU_3	0.08	-	0.23	-	1.3	+	1.5	++	8.35
DU_4	0.09	-	0.18	-	1.3	+	1.6	++	9.15
DU_5	0.08	-	0.16	-	1.3	+	1.7	++	12.22
DU_9	0.08	-	0.20	-	1.3	+	1.6	++	11.50
DO_1	0.09	-	0.20	-	1.4	+	1.7	+	7.15
DO_2	0.08	-	0.23	-	1.3	+	1.7	+	5.32
DO_3	0.05	-	0.20	-	1.4	+	1.8	++	11.31
DO_4	0.09	-	0.22	-	1.2	+	1.7	++	11.31
DO_5	0.10	-	0.30	-	1.3	+	1.8	++	10.25
DO_6	0.08	-	0.22	-	1.2	+	1.7	+	7.36
DO_7	0.08	-	0.13	-	1.1	+	1.8	++	10.75
OG_1	0.12	-	0.26	-	1.2	+	1.8	+	6.83
OG_2	0.09	-	0.28	-	1.2	+	1.7	++	10.72
OG_3	0.08	-	0.30	-	1.3	+	1.6	++	10.20
DP_1	0.07	-	0.18	-	1.1	+	1.6	+	7.14
DP_2	0.08	-	0.18	-	1.1	+	1.7	+	6.47
DP_3	0.09	-	0.28	-	1.3	+	1.7	+	5.84

** = Identity of *Bacillus* isolates codes (Appendix A); Ab = Absorbance of the culture at 540 nm

G, +, ++ = Glutamic acid assayed qualitatively; * = Glutamic acid estimated after 72 hours

- = No glutamic acid detected. Data - means of duplicate determinations.

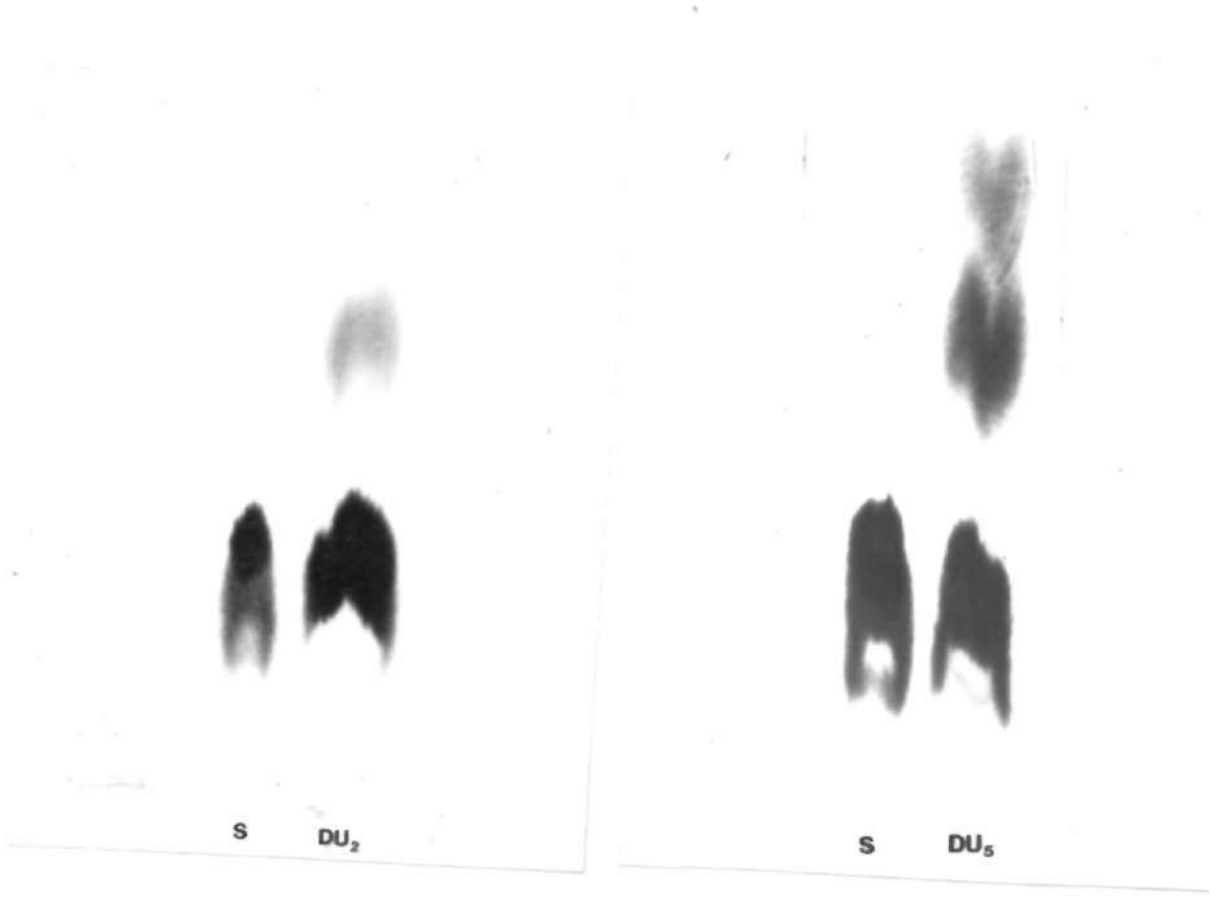


Plate 4.2: Paper chromatograms of glutamic acid production in culture medium by Bacillus subtilis DU_2 and B. subtilis DU_5. {S = Standard (glutamic acid).}

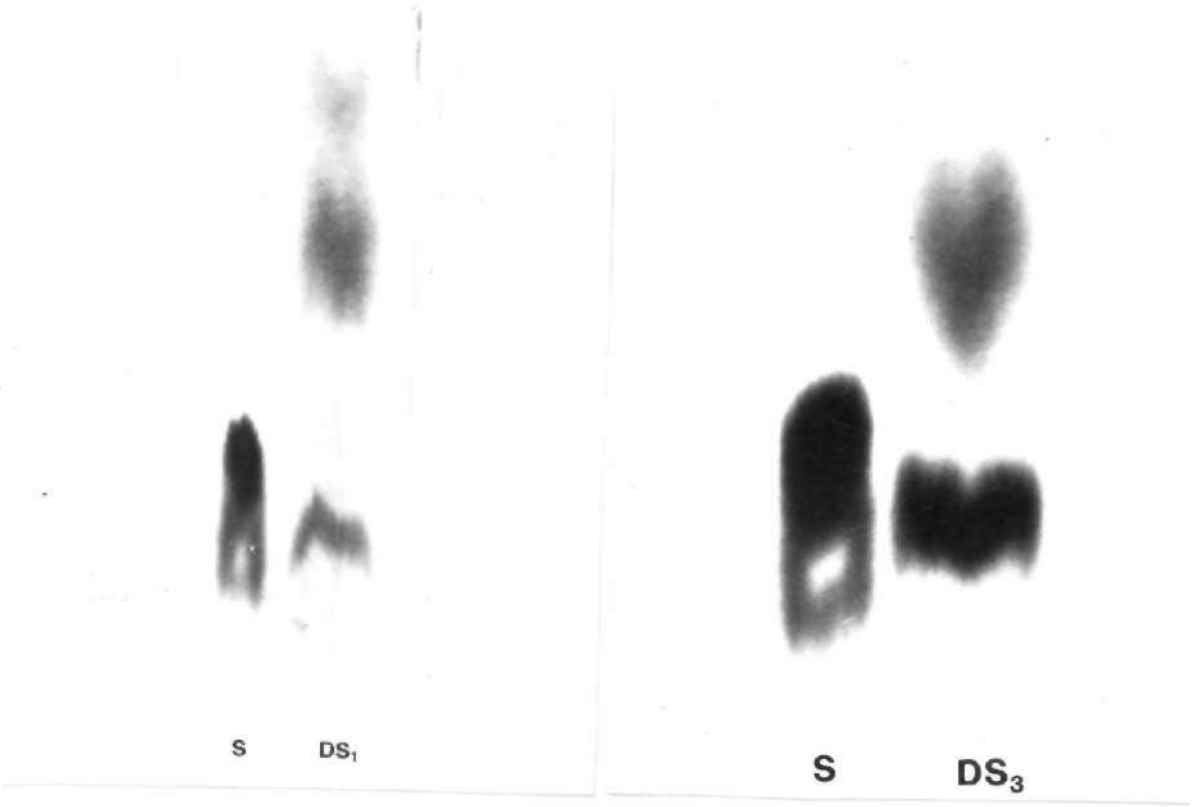


Plate 4.3: Paper chromatograms of glutamic acid production in culture medium by Bacillus subtilis DS_1 and B. subtilis DS_3. {S = Standard (glutamic acid)}

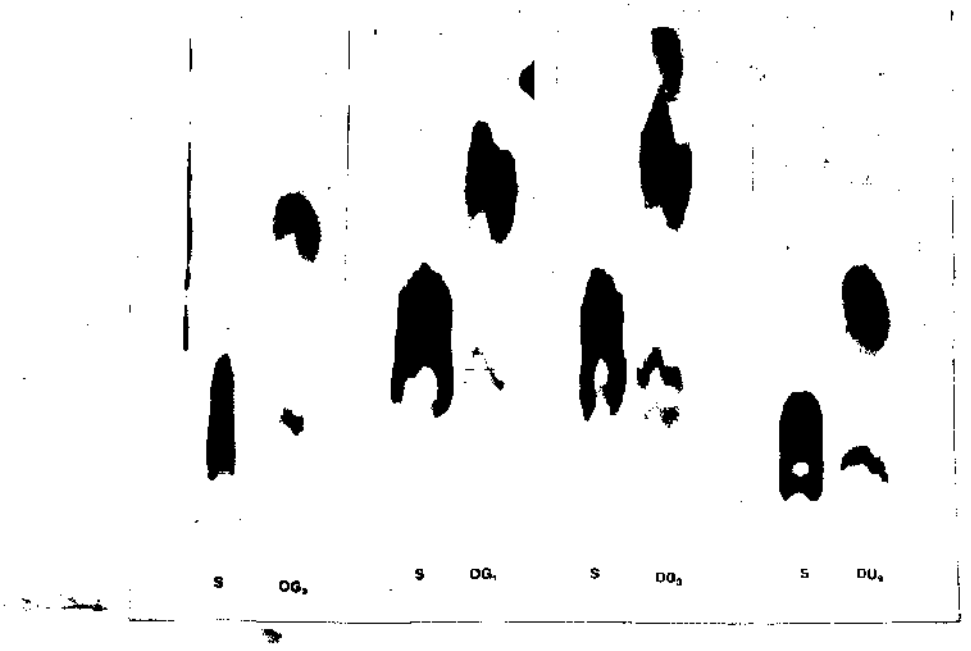


Plate 4.4 Paper chromatograms of glutamic acid production in fermentation broth by Bacillus isolates from ogiri (OG_3 & OG_1) Okpehe (DO_3) and daddawa (DU_9). (S = Standard (glutamic acid))

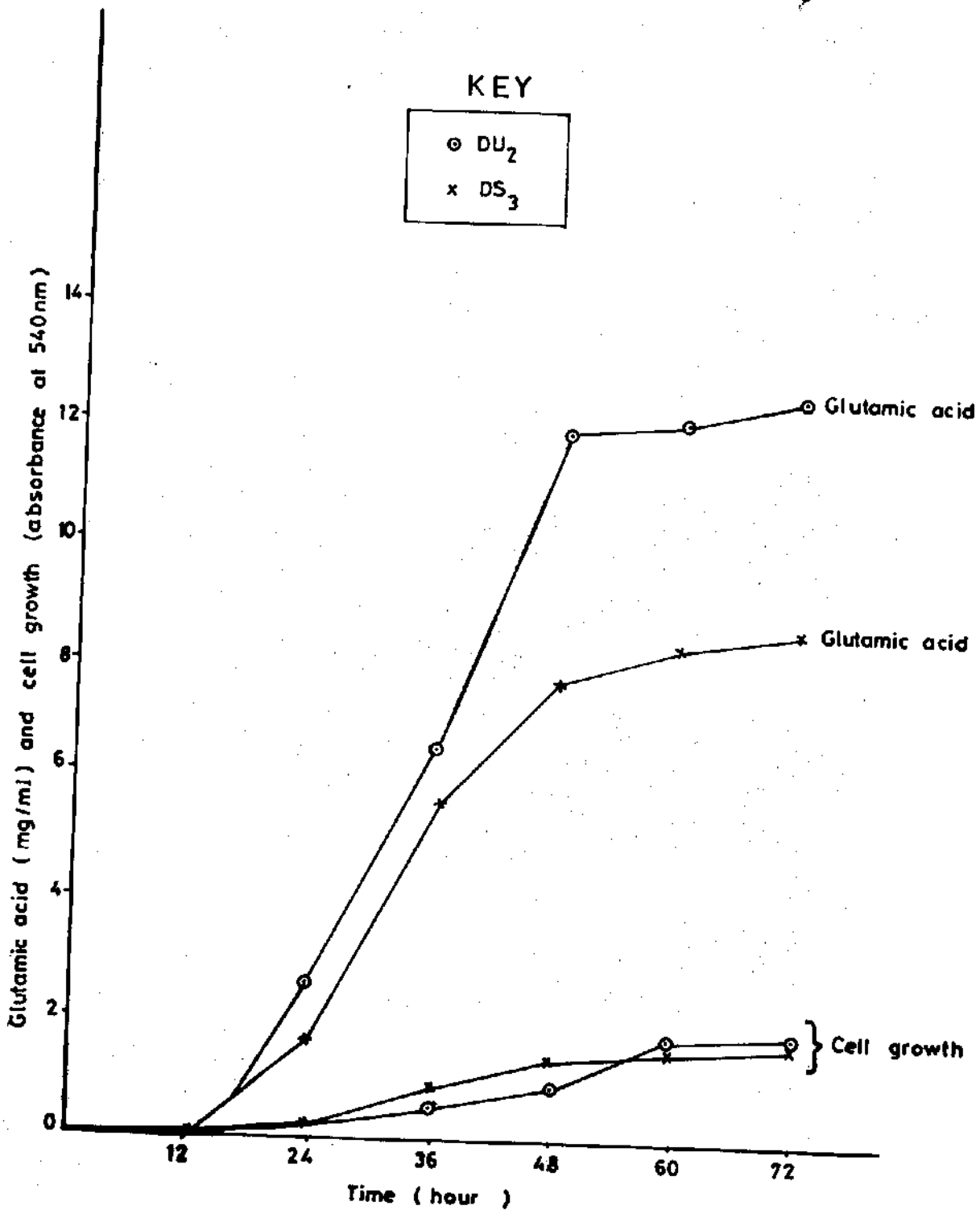


Fig. 4: TIME COURSE OF GLUTAMIC ACID PRODUCTION BY B. SUBTILIS DU_2 AND DS_3 IN GLUCOSE BASAL SALTS MEDIUM.

Table 4.8: Influence of Different Carbon Sources on Glutamic Acid Production by *B. subtilis* - DU_2 in Glucose Basal Salts Medium.

Carbohydrate	Growth (Absorbance*)	Glutamic acid (mg/ml)	Final pH
Sucrose	1.84	12.40	6.0
Glucose	1.70	12.22	6.0
Galactose	1.70	11.74	6.1
Fructose	1.24	6.77	6.3
Hydrolysed starch	1.90	6.22	6.3
Mannitol	1.80	5.58	6.3
Xylose	1.72	5.08	6.4
Maltose	1.84	4.64	6.7
Glycerol	1.20	4.03	6.7
Citrate	1.20	3.22	6.8
Molasses	1.70	2.82	6.8
n-paraffin	1.00	2.72	6.8
Starch	ND	0.87	6.9
Lactose	1.08	0.36	7.0
Succinate	1.00	0.25	7.0

ND - Not determined Data - means of duplicate determinations.

* - measured at 540 nm

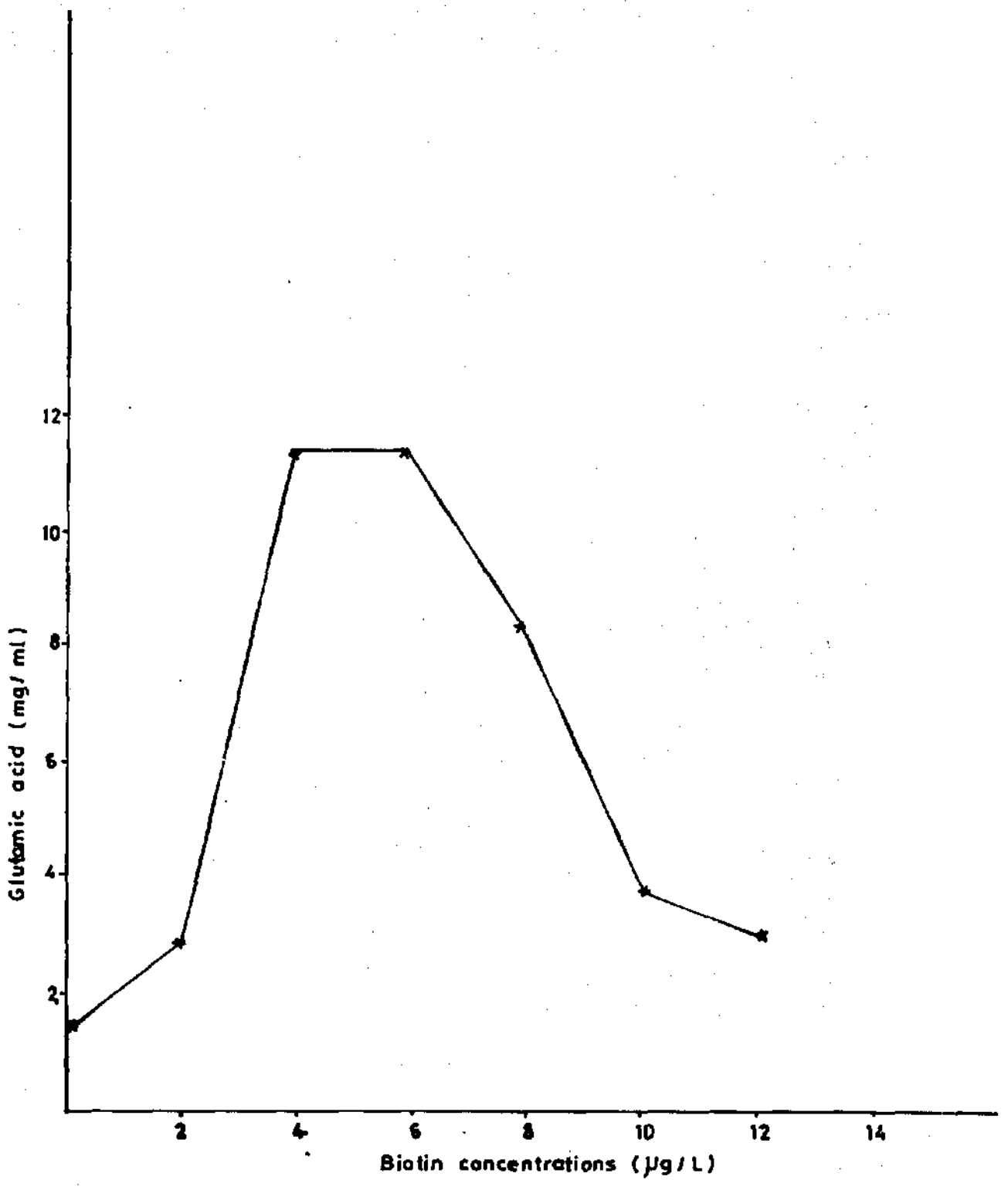


Fig. 5: INFLUENCE OF BIOTIN CONCENTRATIONS ON GLUTAMIC ACID PRODUCTION BY B. SUBTILIS DU_2 IN GLUCOSE BASAL SALTS MEDIUM DURING FERMENTATION.

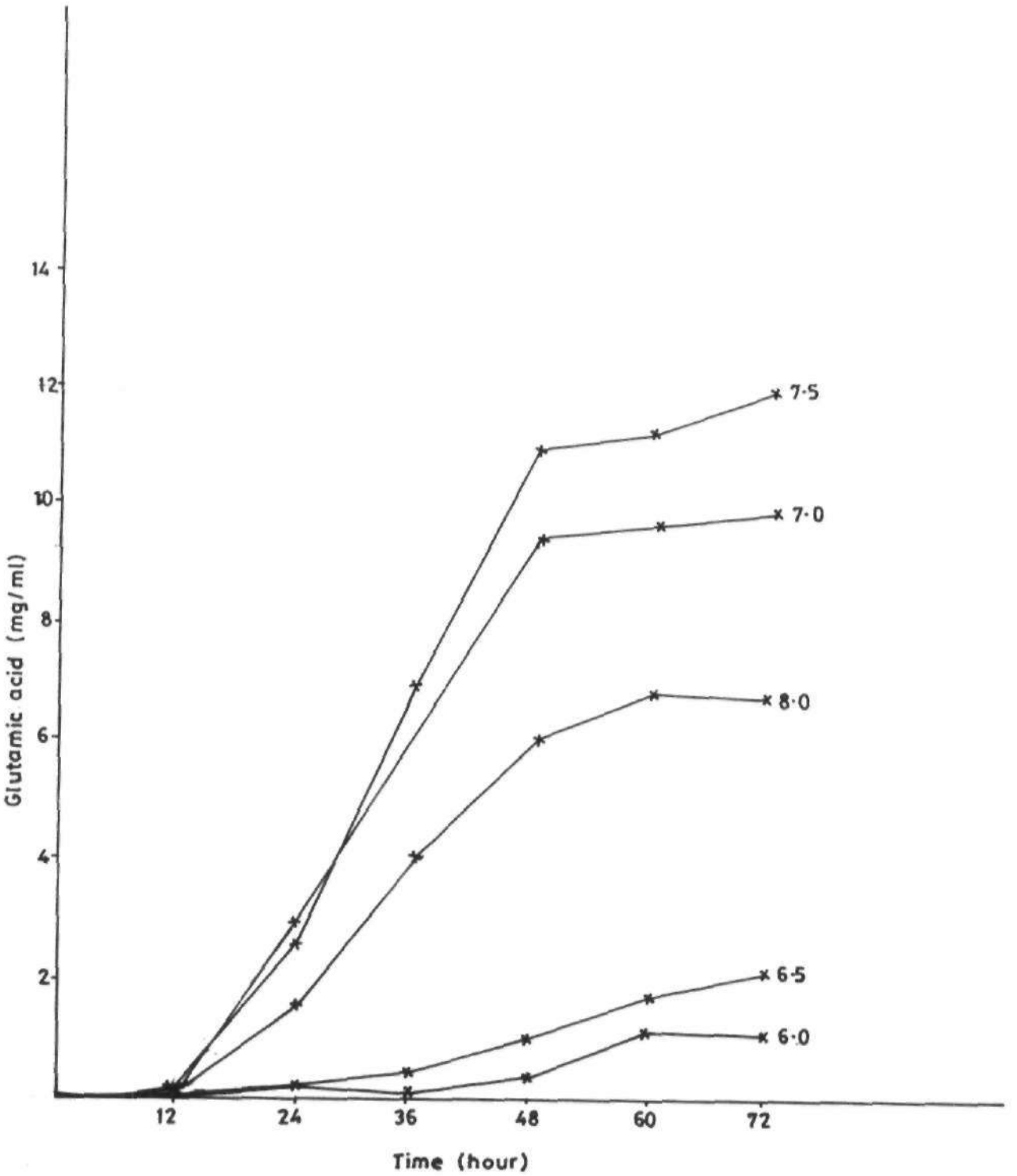


Fig. 6: INFLUENCE OF INITIAL P^H OF GLUCOSE BASAL SALTS MEDIUM ON GLUTAMIC ACID PRODUCTION BY B. SUBTILIS DU_2 DURING FERMENTATION.

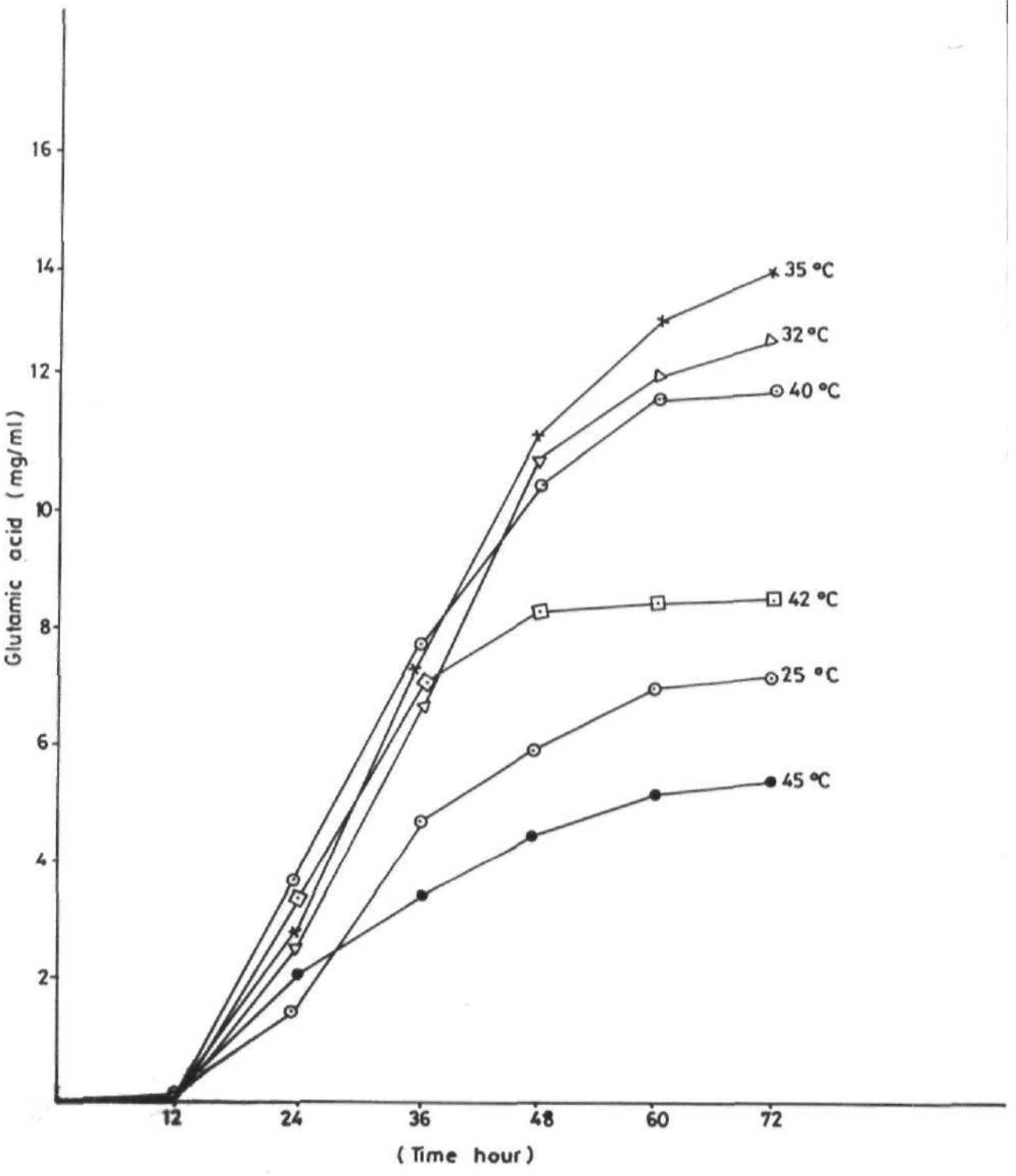


Fig. 7: INFLUENCE OF FERMENTATION TEMPERATURE ON GLUTAMIC ACID PRODUCTION BY B. SUBTILIS – DU_2 IN GLUCOSE BASAL SALTS MEDIUM DURING FERMENTATION.

Table 4.9: Influence of different nitrogen sources on glutamic acid production by B. subtilis - DU_2 in basal medium.

Nitrogen Source	Growth (Absorbance*)	Glutamic acid (mg/ml)	Final pH
Urea	1.79	12.51	6.1
Ammonium chloride	1.84	10.29	6.1
Ammonium sulphate	1.70	8.81	6.3
Ammonium nitrate	1.70	6.94	6.4
Sodium nitrate	1.48	0.66	6.8

* Measured at 540 nm Data - means of duplicate determinations.

Table 4.10: Influence of metal ions on glutamic acid production by B. subtilis - DU_2 in basal medium.

	Glutamic acid (mg/ml) produced with various concentrations of the metal ions (mg/ml)					
Metallic ions*	0.00	0.20	0.60	1.00	1.50	2.00
Manganese	0.54	12.52	8.02	4.60	2.20	2.23
Calcium	12.40	12.37	12.65	10.05	7.50	7.53
Magnesium	4.54	12.21	8.41	7.04	4.02	3.50
Sodium	12.20	8.45	8.08	5.84	5.08	5.10
Cobalt	12.11	0.00	0.00	0.00	0.00	0.00
Cupper	11.02	7.21	3.44	3.08	2.70	0.40
Zinc	12.01	7.40	4.11	1.41	0.41	0.00

* The metal ions were added as their chloride salts.

Data - means of duplicate determinations

Table 4.11: Influence of leguminous flour supplements on glutamic acid production by *B. subtilis* - DU_2

Leguminous flour* Supplement of basal medium	Growth (cfu/ml)	Glutamic acid (mg/ml)	Final pH
Basal medium	3.2×10^7	12.38	6.1
Soya bean	1.4×10^7	13.87	7.6
Locust bean	1.4×10^7	12.73	7.4
Cow pea	1.3×10^7	9.73	7.4
Peanuts	1.3×10^5	8.44	7.2
Bambara nuts	1.7×10^7	7.78	7.3

* Leguminous flour supplement of basal medium at 0.5 percent concentrations.

Data - means of duplicate determinations

Table 4.12: Influence of Triton-X on glutamic acid production by *B. subtilis* DU_2

		66** Hours		72 Hours	
Time of addition of triton-X (h)	Conc. of triton-X (%)	Growth (Absorbance*)	Glutamic acid (mg/ml)	Growth (Absorbance*)	Glutamic acid (mg/ml)
0	0.000	1.78	12.44	1.78	12.92
	0.025	1.78	12.81	1.8	13.72
	0.050	1.79	12.60	1.8	13.64
	0.100	1.8	10.75	1.78	11.04
66	0.050	1.74	9.51	1.76	10.86
	0.100	1.8	9.22	1.8	10.98

* Measured at 540 nm

** Sample for assay taken before addition of triton-X

Data - means of duplicate determinations.

Table 4.13: Influence of Tween-80 on glutamic acid production by *B. subtilis* DU_2

Time of addition of Tween-80 (h)	Concentration of Tween-80 (%)	66** Hours		72 Hours	
		Growth (Absorbance)	Glutamic acid (mg/ml)	Growth (Absorbance)	Glutamic acid (mg/ml)
0	0.000	1.80	11.97	1.80	12.9
	0.025	1.80	12.42	1.80	14.18
	0.050	1.82	13.02	1.79	14.66
	0.100	1.79	12.02	1.79	13.75
66	0.050	1.78	11.71	1.78	12.67
	0.100	1.80	11.68	1.78	12.07

* Measure at 540 nm

** Sample for assay taken before addition of Tween-80

Data - means of duplicate determinations

Table 4.14: Glutamic acid production by ultraviolet mutants of *B. subtilis* - DU_2 at two temperatures

Time of exposure of wild strain to UV-light (min).	Irradiated isolates	Cell growth and glutamic acid yields at 32°C: Growth (Absorbance)	32°C: Glutamic acid (mg/ml)	40°C: Growth (Absorbance)	40°C: Glutamic acid (mg/ml)
0	1	1.80	12.28	1.80	12.01
5	1	1.81	11.23	1.80	14.82
	2	1.82	12.58	1.80	15.36
	3	1.80	12.62	1.82	12.59
10	4	1.70	5.27	1.63	5.32
	5	1.80	10.44	1.78	12.08
	6	1.78	11.24	1.80	15.46
15	7	1.80	11.21	1.78	11.36
	8	1.70	8.42	1.76	9.12
	9	1.72	7.51	1.74	8.50

Data - means of duplicate determinations.

Table 4.15: Glutamic acid production by X-ray mutants of *B. subtilis* DU_2 at two temperatures

X-ray radiation KVP	Time (sec)	Irradiated isolates	Cell growth and glutamic acid yields: 32°C Growth (Absorbance)	32°C Glutamic acid (mg/ml)	40°C Growth (Absorbance)	40°C Glutamic acid (mg/ml)
70	3	1	1.82	10.41	1.8	9.32
		2	1.79	11.81	1.8	11.06
100	3	3	1.8	11.07	1.79	11.21
		4	1.8	12.38	1.82	12.06
110	2	5	1.82	12.05	1.8	14.12
		6	1.8	11.14	1.8	11.04
120	2	7	1.79	10.85	1.79	12.13
		8	1.8	11.51	1.79	11.61
130	1	9	1.8	12.01	1.8	11.69
		10	1.8	11.05	1.8	12.21

Data - means of duplicate determinations.

Table 4.16: Glutamic acid production by acridine orange (AO) mutants of B. subtilis - DU2 at two temperatures.

Concentration of AO (ug/ml)	AO treated isolates	Cell growth and glutamic acid yield at: 32°C Growth (Absorbance)	32°C Glutamic acid (mg/ml)	40°C Growth (Absorbance)	40°C Glutamic acid (mg/ml)
5	1	1.80	12.18	1.80	12.20
	2	1.78	12.31	1.80	12.48
	3	1.80	11.46	1.78	11.32
10	4	1.78	12.50	1.80	12.48
	5	1.76	11.74	1.78	11.68
	6	1.78	11.65	1.78	11.66
15	7	1.78	10.12	1.78	10.06
	8	1.80	11.82	1.80	11.80
	9	1.78	12.04	1.78	12.10

Data - means of duplicate determinations.

CHAPTER 5

DISCUSSION

The predominance of Bacillus isolates up till the end, of the locust bean fermentations over Staphylococcus and Micrococcus species which were isolated at the initial stages of the locust bean fermentations resemble the results of Odunfa (1981a) except that in the present study, species of the Bacillus varied considerably. Bacillus pumilus and B. licheniformis were identified in addition to B.subtilis which predominated in four of the fermentations. In a fermentation of this nature, the involvement of any of various species of an organism is known to be possible. Rhizopus oligosporus, R. arrhizus or R. oryzae is reported to be capable of fermenting soya bean for tempeh production (Hesseltine et al., 1963). Since only one species of Bacillus predominates in each fermentation examined in this study, Odunfa (1981a) may have identified only B. subtilis because he studied only one system.

Bacillus species have been found to be associated with fermentation of plant seeds for production of other condiments. Obeta (1983) isolated a number of Bacillus species from fermenting African oil bean tree (Pentaclethra macrophylla) for 'Ugba' production. In that study, it was reported that B. licheniformis produced the most satisfactory product when the different isolates were tested.

Gordon et al. (1973) has indicated that the three Bacillus species isolated in this study are closely related organisms; B. pumilus differs from B. subtilis in its (B. pumilus) inability to hydrolyse starch. This difference may be responsible for the failure of Odunfa (1985b) to detect amylase activity in fermenting locust beans as B. pumilus could have been the fermenting organism in that system.

The Leuconostoc species reported by Antai and Ibrahim (1986) were not isolated in this study. By the end of 24 hours the pH of the fermenting beans was always above neutral and rose above 8.0 in 48 hours (fig 3). The rise in pH together with the rise in temperature from 28 to 42^{o}C are similar to the observations of Odunfa (1981a), Antai and Ibrahim (1986). The system being a vegetable protein fermentation is accompanied by ammonia generation (Hesseltine, 1965) which would not encourage development of lactic acid bacteria of the magnitude reported by Antai and Ibrahim (1986). The difference in fermentation periods is inadequate to explain the development of their lactics. By 48 hours the lactics were already in 10^5/g (a population which could not have been missed) and continued to develop at a faster rate than the Bacillus, reaching almost equal proportion with the Bacillus by 72 hours with an unusual rise in pH.

This study has shown that adequate covering of the system to exclude as much air as possible was essential for obtaining a good fermentation free of bacterial contami-

nants. Contaminants often isolated from unsuccessful fermentations were Bacillus cereus var. mycoides accompanied with high counts of Staphylococcus and Micrococcus species.

Under different oxygen tensions, the predominant Bacillus thrived differently. While it did not grow under anaerobic condition, the counts obtained under aerobic and reduced oxygen tension conditions were 3.6×10^8 and 4.2×10^8 respectively, giving virtually the same growth. The organism however spread better under reduced oxygen tension showing larger colony sizes (Plate 1). The environment provided in the traditional process as already described would, in addition to providing a semi-anaerobic environment, also keep the seeds moist and thus aid the spread of the motile Bacillus (Gordon et al., 1973).

In general this study shows that B. subtilis, B. pumilus and B. licheniformis could be developed as starter cultures and that semi-anaerobic condition is required for locust bean fermentation.

The micro-organisms isolated from the soyabean-daddawa fermentations are similar to those obtained from the locust bean fermentations. In the preliminary trials only bacterial growth was obtained; there was no fungal growth on the selective media employed. Although most of the very many soya bean fermented products of Asia are fermented by moulds (Aspergillus and Mucor species) a few are also carried out by bacteria (Yokotsuka, 1985). However, it has been noted

that while fermented foods of south-East Asia are generally fermented by moulds, those of Africa are fermented by bacteria; a fact noted as an important difference between African fermented foods and those of South-East Asia (Odunfa 1985a). Again while starter cultures are used to initiate the soya fermentations in Asia, they are not used in 'daddawa' production. Fermentations that do not require conscious introduction of starters would depend on chance inoculation by the microbial flora of the fermenting environments.

On the basis of morphology of the colonies and cell characteristics, only two species of bacteria were obtained from the nutrient agar and the tryptone soy agar for the three fermentations. These were later identified as Bacillus pumilus and Bacillus subtilis. The roasting of the beans before fermentation may be responsible for eliminating the fungi and for restricting the number of bacterial species to the sporeformers. These two isolates which withstood the heat were observed to be sporeformers and were subsequently identified. Popoola and Akueshi (1985) reported two unidentified species of Bacillus in their fermentation. The involvement of Bacillus species in the fermentation of various seeds for production of condiments has been reported by other workers. For example, in the fermentation of African oil bean tree (Pentaclethra macrophylla) seeds for production of a local condiment, Obeta (1983) reported Bacillus species as the predominant organism. In another

fermentation Odunfa (1981a) reported B. subtilis (one of the species obtained in this fermentation) as the fermenting organism in locust bean - daddawa production. As was observed in the latter case there was a rise in pH and temperature. The pH of the soya - daddawa rose from 5 at the initial period to 8.6 while the temperature rose from 28° to 42°C at the end of the fermentation in this study.

Both Bacillus subtilis and B. pumilus isolated at the initial stages were found up till the end of the fermentation even though B. subtilis predominated. This observation differs from that of Popoola and Akueshi (1985) who reported disappearance of their second Bacillus isolated by the end of their fermentation but instead recorded the appearance of Staphylococcus species. Their failure to show the succession of their isolates by viable counts could be responsible for their missing out the other Bacillus which could be fewer in number. Moreover the rapid rise in pH to the alkaline region observed in this study, which usually accompanies vegetable protein fermentations (Hesseltine, 1965) would make the environment unsuitable for development of Staphylococcus species.

The rhizoidal appearance of the B. pumilus colonies, often obtained at the initial stage gave place, as the fermentation progressed, to a pale yellowish translucent colonial morphology. Some Bacillus species are known to change their primary colonial morphology during subculture.

Palumbo et al. (1975) reported loss of a volcano-like colony of Bacillus subtilis during subculture.

In view of the fact that the two Bacillus species, B. subtilis and B. pumilus increased in population up to the end of fermentation, it could be inferred that they are indeed involved in the fermentation.

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The distribution of twenty-five isolates of Bacillus which comprise of eight species obtained from five indigenous condiments shows that Bacillus species constitute part of the bioload of these condiments. Although some of the isolates were obtained from already fermented condiments found in market stalls, these isolations show the importance of Bacillus species in African vegetable protein fermentations since most of the Bacillus isolates were likely survivors of the fermenting organisms. The twenty-five Bacillus isolates screened for glutamic acid all showed evidence of glutamic acid production. Species of Bacillus are said to be superior glutamic acid accumulators. The pathway of glutamic acid synthesis follows either the glycolytic or Hexose monophosphate shunt(HMS) pathway and enters the kreb's cycle up to α-ketoglutaric acid, where the α-ketoglutaric acid is converted to glutamic acid by NAD-dependent glutamate dehydrogenase (fig 1; Dulaney, 1967). Bacillus subtilis and other Bacillus species possess enzymes needed to follow this pathway (Kane and Deshpande, 1979). Bacillus circulans grown under aerated conditions in a basal medium was reported to have produced 14.9g/1 of glutamic

acid after 96 hours of incubation (Dulaney 1967). Under varying pH and medium composition Bacillus cereus var mycoides 10B5 was observed to have yielded different amounts of glutamic acid ranging from 175 to 400µg/ml (Chattopadhyay and Banerjee, 1973). Other genera of bacteria are known to produce glutamic acid when grown in the appropriate media (Nakayama, 1982). The significance of detecting glutamic acid in all the cultures of the isolates screened, rests in the importance attached to monosodium salt of glutamic acid as a taste enhancing substance since all the isolates were obtained from traditional taste enhancing products. The similarity in the graph curve for the time course of glutamic acid content in hydrolysed fermenting locust bean seed, unhydrolysed fermenting seeds and that in basal medium suggest that the increase in the level of glutamic acid observed in fermented seeds over the unfermented seeds, is due to microbial synthesis of the amino acid in the fermentation. The range in value of the yield in glutamic acid in the basal medium from 5.32 to 12.31 mg/ml by the different Bacillus isolates may also be suggestive of the possible variance in contribution to the fermenting beans when each isolate is utilised as starter. Increase in glutamic acid level in fermented condiments have been reported as compared with the level in the unfermented substrates by other workers. For example, Odunfa (1985b) reported an increase of almost 6-fold in glutamic acid in fermented daddawa as compared with the unfermented locust bean. Simi-

larly, Anosike and Egwuatu (1980) reported an increase in glutamic acid level in Ogiri prepared from castor oil (Ricinus communis) as against the level in the unfermented substrate. Although the actual figures obtained in the current study differ from those in their reports, the general increase and the fact that the organisms involved in the fermentation of these condiments actually produce glutamic acid in culture media give agreeable and complementary results. The degree of sensitivity of the different methods used by different workers may account for the differences in the data obtained. In addition, while conditions for glutamic acid synthesis by bacteria are optimal in the basal medium, similar conditions do not exist in the fermenting condiments where the abundant organic nutrients could divert the metabolic activities of the organisms.

The time course for glutamic acid production in the basal medium by selected strain of the Bacillus known to be involved in the fermentation of the locust bean daddawa shows a rather prolonged lag period of up to 12 hours. This period was followed by a period of rapid release of glutamic acid reaching a plateau after 48 hours. The fermentation of glutamic acid is said to be divisible into two phases - a growth phase and an amino acid accumulating phase (Chattopadhyay and Banerjee 1973). Sugar utilisation in glutamic acid producing bacteria continues until about 30 hours followed by a growth period which is accompanied with glutamic acid production. A plot of the graph for growth and

glutamic acid release pattern (Nakayama 1982) confirms this. Results of other studies are also in agreement with the results of this study. For example, the study of the relationship between reduced nicotinamide adenine dinucleotide (NADH) and amino acid transport in membrane vesicle from B. subtilis (Bisschop et al., 1975) showed that the rate of NADH oxidation by the membrane vesicles increased 3-4 fold during logarithmic growth reaching maximum levels in early stationary phase and paralled the rate of L-glutamate transport energised by NADH. Microbial counts and pH changes which took place in the fermentation of daddawa appeared to follow a similar pattern with glutamic acid production in culture medium.

The differing yields of glutamic acid with various sugars observed in this study may be explained by the report that glutamate dehydrogenase level is influenced by the carbon source (Kane et al., 1981). The highest levels of glutamic acid in this study were observed with sucrose, glucose and galactose followed by fructose mannitol and xylose. Bacillus species are capable of utilising these sugars for L-glutamate synthesis. For example it was observed that L-glutamate contained 30-40 per cent of total incorporated radioactivity detected in products of B. subtilis grown in labelled sugars namely sucrose, glucose, fructose or glycerol (Fortnagel and Lopez, 1971). A study of the biochemical changes in fermenting locust bean (from which the Bacillus was isolated) showed that activities of α-

galactosidase and β-galactosidase reached maximum levels after 24 hours of fermentation (Odunfa, 1983). In the same study, the sugars namely glucose, galactose and fructose found in this study to support high levels of glutamic acid, were among the reducing sugars detected in both unfermented and fermented locust bean seeds. That study and the current one, show that the sugars can support glutamate production under natural and artificial conditions. The yields of glutamic acid from complex carbon sources studied here were comparatively low. Cetin et al. (1973) reported that the yield of glutamic acid decreased upon replacement of glucose and other sugars with citric acid and pyruvic acid among other compounds. Although Bacillus subtilis produces amylase, the very low yield of glutamic acid from starch may be a reflection of very low rate of starch hydrolysis by the Bacillus under the conditions of the fermentation study. Infact, Odunfa (1985b) was unable to detect amylase activity in fermenting locust bean. Glycerol and n-paraffin uptake may influence glutamic acid synthesis by their direct or indirect effect on the cell membrane composition since they affect the cellular phospholipid synthesis (Nakao et al., 1972) resulting in increased or decreased permeability of the cell membrane. Moderate yield of glutamic acid was obtained with citric acid as compared with succinate as substrates. These are intermediates of the Kreb cycle. Oxidation of succinate in biotin deficient cells is reported to be low thus blocking the flow of carbon to fumarate and aspartate and further shunting back the carbon to glutamic

acid production (Dulaney, 1967). The same explanation, probably holds for some of these Kreb's cycle intermediates resulting in glutamic acid production in biotin limited cells.

Biotin limitation was found to have a regulatory effect on glutamic acid yield in this study. Biotin is a vitamin and a coenzyme which is required for fatty acid synthesis (Holum, 1969). The regulatory effect of biotin in the light of its requirement for fatty acid synthesis explains the alteration of membrane permeability now associated with limited biotin. The concentration of biotin required for optimum yield of glutamic acid in this study is 4-6µg/l. As the biotin concentrations became higher the yield of glutamic acid reduced. Chao and Foster (1959) reported that limiting biotin level favoured glutamic acid production but the level allowing maximum growth shunted the carbon into cells and CO_2. The poor yield of glutamic acid from molasses may be attributable to comparatively high biotin content of natural carbon sources of agricultural origin.

Slightly alkaline pH (7-8) gave optimal favourable environment for glutamic acid production. Chattopadhyay and Banerjee (1973) investigated the influence of regulated and unregulated pH on glutamic acid accumulation by Bacillus cereus var mycoides in basal media with a neutral initial pH. Higher yields were obtained in media with regulated pH. The media were not regulated in this study as fermentation progressed so as to simulate what obtains in the tradi-

tional daddawa fermentation. Although the pH of the fermenting locust bean begins at 6.0, rapid biochemical changes are not observed until the pH rises above 7.0 (Table 4.3) on the second day of fermentation. Similarly the locust bean fermentation starts at temperature below 30°C and the exothermic nature of the fermentation causes a rise above 40°C through a period of rapid biochemical changes. The most favourable temperature zone for glutamic acid release in the basal medium was between 32 and 40°C in the present study. Bacillus species grow optimally within this temperature range which also afforded the Bacillus a favourable temperature range for release of glutamic acid.

Different nitrogen sources supplied in the medium gave different yields of glutamic acid with urea supporting the highest yield followed by ammonium chloride, ammonium sulphate and ammonium nitrate. The requirement for nitrogen as a growth factor by micro-organisms can be fulfilled by these compounds. However, in addition, the pathway of glutamic acid synthesis requires ammonia for the reductive amination of α-ketoglutaric acid (Holum, 1969). The fact that the least yield is obtained with sodium nitrate, the only source of non-ammonia nitrogen shows the importance of ammonia as an essential requirement for glutamic acid producing bacteria. The requirement for ammonia can easily be met in the traditional locust bean fermentaion by the ammonia generated during the fermentation. As a vegetable protein fermentation, the nitrogenous growth factor require-

ment will be adequately met.

The influence of leguminous protein supplementation of the basal medium on the glutamic acid release by the Bacillus subtilis is not much, as the yields are comparable with that in basal medium alone but the soya bean and the locust bean were more suitable supplements for the fermentation. The availability of the protein supplements probably provided the organism with nutrients for various metabolic activities thus diverting partially from the glutamic acid pathway. It was observed that the apparent lag phase in these media was averagely twelve hours as compared with the long lag phase observed in the basal medium. The higher glutamic acid obtained with soyabean supplemented medium over locust bean-supplemented medium (Table 4.11) was also obtained in soyabean-daddawa over locust bean daddawa (table 4.6). These leguminous proteins have their own levels of glutamic acid that are released by the proteolytic enzymes of the organism. The total glutamic acid level would probably be that due, in part, to microbial synthesis and that released as a result of microbial enzymatic activities. The latter contribution is probably more significant since soya bean which is known to have the highest amount of protein yielded the highest level of glutamic acid. The protein content of peanut is reported to be higher than that of bambara nuts (Anon, 1979). Data obtained in this study actually show the level of glutamic acid, released into the system during fermentation since the samples are not hydrol-

ysed in the process of quantitation. Although the yields in glutamic acid with the different legume flour supplements, did not rise as to encourage recommendation of these protein as supplements in daddawa fermentation, perhaps the increasing difficulties in obtaining chemicals and commercial media may necessitate use of these proteins with mineral supplements for commercial glutamic acid production.

The study on the effect of metal ions on the yield of glutamic acid which demonstrated relatively poor yields when manganese and magnesium were omitted from the growth medium showed that the two metal ions were essential in glutamic acid production. At 0.2mg/ml concentration all the metal ions except cobalt, supported good yields of glutamic acid. Trace ions of magnesium, ferrous and ferric ions have been observed to favour glutamic acid production (Dulaney, 1967). Manganese ions are required for conversion of oxalosuccinic acid to a-ketoglutaric acid (Holum, 1969). The presence of these metal ions in trace amount support glutamic acid synthesis. Cobalt ions inhibited glutamic acid synthesis, probably by inhibiting the growth of the organism. Sen and Chakrabarty (1984) reported that cobalt and zinc inhibited amylase production by Lactobacillus cellobiosus. The importance of copper ions in glutamic acid synthesis may probably be said to be in energy supply as a result of high efficiency of oxidative phosphorylation. Analysis of daddawa and bouillon cubes for their mineral contents (Okeoma, 1984) showed that most of these metal ions occur in both condi-

ments, though in higher amounts in daddawa.

The influence of the two surfactants (Triton-X and Tween -80), on the level of glutamic acid produced by the Bacillus species were similar. The presence of certain concentrations of both surfactants in the medium enhanced glutamic acid production. Glutamate and succinate binding activities were reportedly solubilized from membrane vesicles by non ionic detergents particularly Lubrol-WX (Kay, 1981). The most effective concentrations of the two surfactants were found to be 0.025 to 0.05 percent. Triton-X and Tween -80 have been used for increased amylase production (Sen and Chakrabarty, 1984). However, incorporation of the surfactants into the medium at the beginning of the fermentation was more favourable for glutamic acid production than surfactant addition at the 66th hour. By the 66^{th} hour, the enhancing effect on glutamic acid production, of the initially added surfactant was already obvious. It has been reported that glutamic acid in the cell tends to exert a feedback control on its synthesis while release of the acid leads to increased synthesis (Deshpande et al., 1980; Fortnagel, 1970). Thus early release of glutamic acid, caused by early application of surfactant is advantageous in glutamic acid fermentation. The surfactants acting on the cell membrane tend to keep the 'door' ajar for continuous out-pour of the glutamic acid thus releasing the feedback control. The permeabilising effect of these surfactants do not seem to interfere with the cells as viable entities as

they continued to metabolise (growth) and release glutamic acid. Konings (1975) reported that NADH oxidation in Bacillus subtilis occurs on the inside surface of the membrane but that components of the transport system for L-glutamate driven by NADH are on the outside and that no apparent difference could be observed in membrane bound functions between membrane vesicles and whole cells. This supports the contention that the vesicle membrane of Bacillus subtilis has the same orientation as the cytoplasmic membrane of whole cells. It has been shown that although addition of penicillin to a medium containing high concentrations of biotin reduces the viability of the cells, it leaves them unlysed and they continue to produce glutamic acid (Nakayama, 1982).

Glutamic acid - producing abilities, at two temperatures, of mutants derived from B. subtilis - DU_2 showed slightly different results with the three mutagens used. The acridine orange treated isolates and some of the irradiated isolates did not show any apparent difference in the yields of glutamic acid. This probably shows that the DNA bases (triplets) which code for glutamic acid were not affected by either the chemical nor the rays in these isolates. The doses of X-ray available for use in this study to which the wild strain was exposed did not show any marked lethal effect (Appendix C).

Most of the mutants derived from ultraviolet exposed wild strain and two derived from X-ray radiation showed

enhanced ability in glutamic acid production especially at 40°C. Enhanced production of secondary metabolities is known to occur with irradiated microbial cells over unirradiated ones. Irradiated Aspergillus flavus was reported to synthesise more aflatoxin than unirradiated germinated isolate (Applegate and Chipley, 1974). It has been shown in this study that a lower (than 40°C) temperature optimum is required by the wild type of Bacillus subtilis--DU_2. The mutants did not exhibit any changes in the biochemical characteristics used for the identification of the wild strain (appendix D) Thus the apparent temperature sensitive change which caused the mutants to produce higher levels of glutamic acid at 40°C is not clear. However it is known that the structure and function of the cell membrane can be changed by temperature control (Momose and Takagi, 1978) and this may cause a change in glutamic acid yield since cell membrane permeability is known to influence the yield of glutamic acid. Momose (1983) derived 20 temperature sensitive mutants from Brevibacterium lactofermentum which produced higher yields of glutamic acid even in the presence of high biotin concentrations.

Since in daddawa fermentation, temperature rises above 40°C which is considered to be optimum temperature for the fermentation (Odunfa and Adewuyi, 1985c) the possibility of using irradiation to develop temperature sensitive mutants with enhanced glutamic acid producing ability, for use as daddawa starter cultures deserves further investigation.

CONCLUSION

1. There are variable species of *Bacillus* in daddawa fermentations. *Bacillus subtilis*, *B. pumilus* and *B. lichemiformis* play important roles.

2. The *Bacillus species* involved in 'daddawa' fermentation contributed glutamate as a metabolite which enhances the level of glutamic acid in 'daddawa'.

3. *Bacillus subtilis* - DU_2 isolated from locust bean - 'daddawa' produces appreciable yields of glutamic acid under suitable cultural conditions and growth parameters which among others, are: -

   (a) 4 - 6 µg/ml biotin
   (b) Mn^{++} and Mg^{++}
   (c) 35°C and pH 7.5

4. Medium supplements consisting of flours of leguminous seeds serve well for glutamic acid fermentation by *B. subtilis* DU_2 and could be useful for industrial production.

5. Triton-X and tween-80 (surfactants) can be used to obtain higher yields of glutamic acid when incorporated into the medium at the early stage of fermentation.

6. Mutants with enhanced glutamic acid production ability at temperatures optimal for daddawa fermentation can be obtained from exposing wild strain of *Bacillus* to irradiation sources (X-ray and UV light)

REFERENCES

Adams, M.R. (1985). Vinegar. In: Wood, B.J.B. (Ed.) Microbiology of Fermented Foods. Vol. 2. Elsevier Pub. Co. pp. 1-48.

Akinrele, I. A. (1970). Fermentation studies on maize during the preparation of a traditional African starch - cake food. Journal of the Science, Food and Agriculture 21, 619-625.

Alloway, J. L. (1933). Further observations on the use of Pneumococcus extract in affecting transformation of type in vitro. Journal of Experimental Medicine 57, 265.

Alvarez, R. J. (1982). Microbiological safety of baked and fried arepas. Ecology of Food and Nutrition 11 (4) 207 - 210.

Anonymous (1979). Tropical legumes: Resources for the future. National Academy of sciences Washington, D.C.

Anosike, E.O. and Egwuatu, C.K. (1980). Biochemical changes during the fermentation of castor oil (Ricinus communis) seeds for use as seasoning agent. Qualitas plantarum, plant Foods for Human Nutrition 30 (3-4) 181-185.

Antai, S.P. and Ibrahim, M.H. (1986). Micro-organisms associated with African locust bean (Parkia filicoidea Welw) fermentation for dawadawa production. Journal of Applied Bacteriology 61, 145 - 148.

Applegate, K.L. and Chipley, J.R. (1974). Effects of ^{60}Co gamma irradiation on aflatoxin B_1 and B_2 production by Aspergillus flavus. Mycologia 66, 436-445.

Araki, K., Kato, F., Arai, Y and Nakayama K. (1974). Histidine production by auxotrophic histidine analog-resistant mutants of Corynebacterium glutamicum Agricultural and Biological Chemistry 38, 2209-2218.

Arima, K., Nogami, I. and Yoneda, M. (1971). Cited from Nakayama, K. 1982. Amino acids. In: Reed (Ed). Prescott and Dunn's Industrial Microbiology 4th Edition. Avi publ. Co. Inc. Westport connecticut pp. 774.

Beddows, C.G. 1985. Fermented fish and fish products. In: Wood, B.J.B. (Ed.) Microbiology of Fermented Foods. Vol.2 Elsevier Pub. Co. pp. 1-40.

Beukers, R. and Berends, W. (1960). Isolation and identification of the irradiation product of thymine. Biochemistry and Biophysics Acta 41, 550.

Bisschop, A., De Jong, L., Costa, M.E.L., Konings W.N., (1975). Relation between reduced nicotinamide adenine dinucleotide oxidation and amino acid transport in membrane vesicles from Bacillus subtilis. Journal of Bacteriology 121 (3) 807 - 813.

Bonde, G.J. (1973). The Genus Bacillus. An examination of 460 mesophilic strains. Copenhagen. International service system Research council series No. 2.

Bonde, G.J. (1975). The genus Bacillus. An experiment with cluster analysis. Danish Medical Bulletin 22, 41 - 61.

Breed, R.S., Murray, E.G.D. and Smith, N.R. (1964). Bergeys Manual of Determinative Bacteriology 7th Edition. The Williams and Wilkins Co.

Brenner, S., Barnett, L., Crick, F.H.C. and Orgel, A. (1961). The theory of mutagenesis. Journal of Molecular Biology 3, 121.

Brookes, P. and Lawley, P.D. (1960). The reaction of mustard gas with nucleic acids in vitro and in vivo. Biochemical Journal 77, 478.

Burkill, I. H. (1935). A dictionary of the economic products of the Malay Peninsula. Crown Agents for the colonies, London Vol. 1 pp. 1080 - 1086.

Busson, F. (1965). Cited from Campbell-platt, G. (1980). African locust bean (Parkia species) and its West African fermented food products, Dawadawa. Ecology of Food and Nutrition 9, 130.

Campbell-platt, G. (1980). African locust bean (Parkia species) and its West African fermented food products, Dawadawa. Ecology of Food and Nutrition 9, 123 - 132.

Cetin, E.T., Ocal, S. and Guven, K.C. (1973). Production of glutamic acid by fermentation. Eczacilik Bulletin, 16 (6) 87 - 96.

Chalumeau, H. and Rapoport, G. (1971). Identification of L-glutamate in the products of sugar accumulation in Bacillus subtilis. C.R. Academy of Science Series D. 272 (12) 1690 - 1693.

Chao, K.C. and Foster, J.W. (1959). Cited by Dulaney, E. L. (1967). Microbial production of amino acids. In: Peppler, H.T. (Ed.) Microbial Technology. Reinhold Publ. Corp. New York pp 311.

Chattopadhyay, S.P. and Banerjee, A.K. (1973). Effect of composition and pH of medium on extracellular glutamic acid accumulation by Bacillus cereus var mycoides 10B5. Indian

Journal of Microbiology. 13, 169-173.

Chome, K. (1981). Producing L-glutamic acid by fermentation and Novel mutants. Ajinomoto Co. Inc. No. 5-8. GB 2056 451.

Clowes, R.C. and Hayes W. 1968. Experiments in Microbial Genetics Blackwell scientific Publications Oxford.

Collard, P. and Levi, S. (1959). A two-stage fermentation of cassava. Nature, London 183, 620 - 621.

Cowan, S.T. and Steel, K.J. (1965). Manual for the identification of Medical Bacteria. Cambridge University Press. pp 217.

Dalziel, J.M. (1937). The useful plants of West Tropical Africa. Crown Agents, London pp 217-220.

Dawson, M.H. and Sia, R.H.P. (1931). A technique for inducing transformation of pneumococcal types in vitro. Journal of Experimental Medicine 54, 681.

Deketelaere, A., Demeyer, D., Vandekerchlove, P and Vervaeke, I. (1974). Stoichiometry of carbohydrate fermentation during dry sausage ripening. Journal of Food Science 39, 297 - 300

De Mars, R.I. (1953). Chemical mutagenesis in bacteriophage T2. Nature, London. 172, 964.

Dema, I.S. (1965). Nutrition in relation to Agricultural production. FAO Rome p. 123.

Demain, A.L. and Birnbaum, J. (1968). Alteration of permeability for the release of metabolites from the microbial cells. Current Topics in Microbial Immunology 46, 1-25.

Demerec, M. and Latarjet R. (1946). Mutations in bacteria induced by radiations. Cold spring Harber Symposium on quantitative Biology, 11, 38.

Denenu, E.O. (1985). Use of permeabilised Hansenula polymorpha cultures for anthranilate synthase assay. Nigerian Food Journal 2 & 3 (3) 177 - 180.

Deshpande, K.L., Katze, J.R. and Kane, J.F. (1980). Regulation of glutamate synthase of Bacillus subtilis by glutamine. Biochemical and Biophysics Research Communications 95 (1) 55 - 60.

Dietz, G. E. and Burnie, K. L. (1982). A simple method to inhibit the growth of bacteria in water baths used for thawing frozen blood products. Canadian Journal of Medicine

and Technology 44 (1) 33.

Djien, K.S. and Hesseltine, C.W. (1961). Indonesian fermented foods made from soybean. Soybean Digest 22, 14.

Douglas, S.J. (1972). Tree crops for food, forage and Cash. World Crops 24 (1) 15-19.

Dulaney, E.L. (1967). Microbial production of amino acids. In: Peppler, H.J. (Ed) Microbial Technology. Reinhold Publ. Corp. New York pp 308 - 317.

Dutkiewicz, J. (1980). Airborne bacteria as occupational allergens. Umwelt Bundes Amt, Berichte 79 (5) 232 - 242. Cited from Applied Bacteriology Abstracts (1980).

Eka, O.U. (1980). Effects of fermentation on the nutrients status of locust beans. Food Chemistry 5: 303 - 308.

El Dein-Selim, M. S. N., and Khalil, G.A. (1979). Investigations into rhizospere microflora of some plants in Libya. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hgy., 11, 134 (3) 237 - 242. Cited from Applied Bacteriology Abstracts (1980).

Eyeson, K. K and Ankah, E (1975) Composition of Foods commonly used in Ghana. Food Research Institute, Accra.

Fabian, F.W. (1951). Food preservation by use of microorganisms. In: Jacobs M.D. (Ed.) The Chemistry and Technology of Food and Food products 2nd Edition, Interscience Publishers (Division of John Wiley & Sons Inc.) New York.

Fabian, F. W. and Nienhuis, A.L. (1934). Experimental work on cucumber fermentation VII. Bacillus nigrificans n. sp as a cause of pickle blackening. Michigan state Agricultural Experimental station Technical Bulletin 140.

F.A.O. (1970). Amino acid composition of foods and biological data on proteins. FAO Nutrition Studies No. 24 Rome.

Farrar Jr., W.E. (1963). Serious infections due to "non-pathogenic" organisms of the genus Bacillus. American Journal of Medicine 34, 134 - 141.

Fetuga, B.L., Babatunde, G.M. and Oyenuga, V.A. (1974). Protein quantity of some unusual protein feedstuffs. Studies on the African locust bean seed (Parkia filicoidea Welw) British Journal of Nutrition 32 (1) 27 - 36.

Fields, M. L. (1970). The flat sour bacteria. Advances in Food Research 18: 163 - 217.

Fogarty, W.M. (1963). Enzymes Technology 2. Microbial enzymes - production and uses pp. 27 - 30 Dublin. Technical information Division, Institute for Industrial Research and standards.

Fortnagel, P. (1970) Regulation of aconitase and isocitrate dehydrogenase in sporulation mutants of Bacillus subtilis Biochemistry and Biophysics Acta 222 (2) 290-298.

Fortnagel, P. and Lopez J. (1971). Regulation of malate dehydrogenase in sporulation mutants of Bacillus subtilis blocked in the citric acid cycle. Biochemistry and Biophysics Acta 237 (2) 320 - 328.

Freese, E. (1959a). The specific mutagenic effect of base analogues on phage T4. Journal of Molecular Biology 1, 87.

Freese, E. (1959b). On the molecular explanation of spontaneous and induced mutations. Brookhaven Symposia in Biology 12,63.

Fukumura, T. (1976). Hydrolysis of L-a-amino-caprolactam by yeasts. Agricultural and Biological Chemistry 40, 1695-1698.

Garibaldi, J.A., Ijichi, K., Snell, N.S. and Lewis J.C. (1953). Bacillus megaterium for biosynthesis of cobalamin. Industrial Engineering Chemistry 45, 839-946.

Gasson, M.J. (1983). Genetic transfer systems in lactic acid bacteria. Antonie van Leenwenhoek 49, 275 - 282.

Gil, M.C., De La Rosa, M.C., Mosso, M.A. and Garcia Arribas, M.L (1986). Numerical taxonomy of Bacillus isolated from orally administered drugs. Journal of Applied Bacteriology 61, 347-356.

Gilbert, P.J. (1979). Bacillus cereus gastroenteritis. In: Rienman, H. and Bryan, F.L. (Eds). Food borne Infections and Intoxications. Academic Press New York. pp. 495 - 514.

Godfrey A. (1985). Production of Industrial enzymes and some applications in fermented foods. In: Wood, B.J.B. (Ed.) Microbiology of Fermented Foods. Vol.1. Elsevier Pub. Co. pp 345-371.

Goodfellow, M. (1971). Numerical taxonomy. In: Laskin A. I. and Lechevalier, H.A. (Ed.) CRC Handbook of Microbiology Vol. 1 2nd Edition. CRC Press Cleverland pp. 579-596.

Gordon, R.E., Hayes, W.C. and Pang C.H.N. (1973). The Genus Bacillus. Agriculture Handbook No. 427. U.S. Department of Agriculture. Washington D.C.

Halvarson, H. (1973). Formation of lactic acid volatile fatty acids and neutral volatile mono carbonyl compounds in

Swedish fermented sausage. Journal of Food Science 38, 310 - 312.

Hara, T., Aumayr, A., Fujio Y. and Ueda, S. (1982). Elimination of plasmid - linked polyglutamate production by Bacillus subtilis (natto) with acridine orange. Applied and Environmental Microbiology 44, 6 1456 - 1458.

Hayes, W. (1964). The genetics of bacteria and their viruses - studies in basic genetic and molecular biology. Blackwell scientific publications Oxford pp 276 - 361.

Hesseltine, C.W., Smith, M., Bradle, B and Djien, K.S. (1963). Investigations of tempeh, an Indonesian food. In: Developments in Industrial Microbiology. Publd by the American Institute of biological sciences. Washington D.C.

Hesseltine, S.W. (1965). A Millenium of fungi food and fermentations. Mycologia 57, 149-197.

Hill, L.R. (1966). An index to deoxyribonucleic acid base compositions of bacterial species. Journal of General Microbiology 44 419-447.

Holum, J.r. (1969). Introduction to organic and Biological Chemistry. John Wiley and Sons Inc. New York pp 513 - 536.

Huang, H. T. (1961). Production of L-threonine by auxotrophic mutants of Escherichia coli. Applied Microbiology 9,419-424.

Imreh, E.Z., Takacs, J., and Szazados, E. (1979). The value of complementary bacteriological examinations in neoplastic disease of slaughter animals. Acta Veterinary Academy of Science Hungary, 27 (4) 343 - 347.

Irvine, F. R. (1961). Woody plants of Ghana. Oxford University Press, London.

Jay, J. M. (1982). Antimicrobial properties of diacetyl. Applied Environmental Microbiology 44 (3) 525 - 532.

Johnston, D. W. and Bruce, J. (1982). Incidence of thermoduric psychrotrophs in milk produced in the west of scotland. Journal of Applied Bacteriology 52 (3) 333 - 337.

Johnston, J. R. (1985). Strain selection and Improvement. In: Wood, B.J.B. (Ed). Microbiology of Fermented Foods vol. 2. Elsevier pub. Co. London pp. 271 - 291.

Kane, J. F. and Deshpande, K. L. (1979). Properties of glutamate dehydrogenase from Bacillus subtilis. Biochemistry and Biophysics Research Communications 88 (3) 761-767.

Kane, J. F., Wakim, J., and Fischer, R. S. (1981). Regula-

tion of glutamate dehydrogenase in Bacillus subtilis. Journal of Bacteriology 148 (3) 1002-1005.

Kanzaki, T., Kitano, K., Sumino, Y. and Okazaki, Y. (1972). Cited from Nakayama, K. (1982). Amino acids. In: Reed G. (Ed.) Prescott and Dunn's Industrial Microbiology 4th Edition Avi publ. co. Inc. Wesport Connecticut pp. 752. 82.

Kase, H. and Nakayama, K. (1975). L-methionine production by methionine analog - resistant mutants of Corynebacterium glutamicum. Agricultural and Biological Chemistry 39, 153 - 160.

Katznelson, H. (1944). Differentiation of Bacillus polymyxa and Bacillus macerans on the basis of vitamin requirements. Journal of Bacteriology 48, 495.

Kay, W.W. (1981). Purification by affinity chromatography of a membrane dicarbocylate binding protein from Bacillus subtilis. Canadian Journal of Microbiology 27 (8) 795 - 800.

Kelner, A. (1953). Growth, respiration and nucleic acid synthesis in ultra violet - irradiated and in photo reactivated Escherichia coli. Journal of Bacteriology 65, 252.

Kikuchi, M. and Nakao, Y. (1973). Relation between cellular phospholipids and the excretion of L-glutamic acid by a glycerol auxotroph of Corynebacterium alkanolyticum. Agricultural and Biological Chemistry 37, 515 - 519.

Kinoshita, S., Tanaka, K., Udaka, S. and Akita, S. (1957). Glutamic acid fermentation. In: Proceedings of International Symposium on Enzyme Chemistry 2, 464 - 468.

Kisumi, M., Kato, J., Sugiyama, M. and Chibata, T. (1971). Production of L-arginine by arginine hydroxamate - resistant mutants by Bacillus subtilis. Applied Microbiology 22, 987 - 991.

Kisumi, M., Komatsubara, S. and Chibata, I. (1973). Leucine accumulation by isoleucine revertants of Serratia marcescens resistant to a-aminobutyric acid. Lack of both feedback inhibition and repression. Journal of Biochemistry (Tokyo) 73, 107 - 115.

Knight, B.C.J.G and Proom, H. (1950). A comparative survey of the nutrition and physiology of mesophilic species in the genus Bacillus. Journal General Microbiology 4, 508 - 538.

Koburger, J. A., Alvarez, R. J., Appledorf, H. (1981). Nutritional and microbiological changes during production of tostones (fried plantains). Journal of Food Protection 44, (1) 55 - 58.

Kolchinska, I.D., Kvasnikov, E.I., Drindina, L.P. (1970). Biosynthesis of vitamins and amino acids by Bacillus subtilis and Bacillus mesentericus Mikrobiol. Zh. (Kiev) 32 (4) 419 - 423.

Koleshko, O. I., Yu, S., Potayenko, L. and Sharangorich, N. (1982). The incidence and species of heterotrophic bacteria in the lake Naroch. Mikrobiologiya, 51 (1) 143 - 145.

Konda, T. and Tezuka, Y. (1979). Bacterial flora in the water and sediment of lake Motosu-ko an oligotrophic lake in central Japan. Japan Journal of Ecology, 29 (3) 209 - 220.

Konings, W. N. (1975). Localization of membrane proteins in membrane vesicles of Bacillus subtilis. Archives of Biochemistry and Biophysics 167 (2) 570 - 580.

Konuma, H., Takayama, S. and Suzuki, A. (1980). Microbial flora of beef extract imported from Australia, Uruguay, Italy and Brazil. Bulletin of National Institute for Hygiene and Science Tokyo 55 (98) 36 - 40.

Kraft, A.A. (1971). Microbiology of poultry products. Journal of Milk Food and Technology 34, 23 - 27.

Kubota, K., Yokoi, M. and Hirose, Y, (1975). L-serine production from glycine by Corynebacterium glycinophilum and its mechanism. Abstract Meeting Society of fermentation Technology Japan- Osaka 80.

Ladipo, O. O. and Adegboye, R. O. (1976). The economic cost of malnutrition and the price of protein and calories in some selected towns of Oyo, Ogun, and Ondo States of Nigeria. Nigerian Nutrition Newsletter 1: 100 - 117.

Lederberg, J., Lederberg, E. M., Zinder, N. P. and Lively, E. R. (1951). Recombination analysis of bacterial heredity Cold Spring Harber Symposium on quantitative Biology 16, 413.

Lederberg, J. and Tatum, E. L. (1946). Gene recombination in E. Coli. Nature, London 158, 558.

Lerman, L. S. (1961). Structural considerations in the interaction of DNA and acridines. Journal of Molecular Biology 3, 18.

Lerman, L. S. (1963). The structure of the DNA acridine complex. In: Proceedings of national Academy of Science Washington 49, 94.

Leung, W. T. W., Busson, F. and Jardin, C. (1968). Food composition tables for use in Africa. U.S. Department of Health/FAO Bethesda, Maryland.

Lindblow-Kull, A., Shrift, R. and Gherna, L. (1982). Aerobic selenium-utilising Bacillus isolated from seeds of Astragalus crotalariae. Applied and Environmental Microbiology 44 (3) 737 - 743.

Logan, N. A. and Berkeley, R. C. (1981). Classification and identification of members of the genus Bacillus using API test. In: Berkeley, R. C. and Good fellow, M. (Ed). The aerobic endospore - forming bacteria. Academic Press, London. pp. 105 - 130.

Lucke, F. - K. (1985). Fermented sausages. In: Wood, B. J. B. (Ed.) Microbiology of Fermented Foodsvol. 2 Elsevier pub. pp. 41 - 84.

Lysenko, O. and Kucera, M. (1971). Micro-organisms as source of new insecticide chemicals: Toxins. In: Burges, H. D. and Hussey, N. W. (Eds.) Microbial control of insects and mites. Academic press London. pp 205 - 226.

Marr, A. G. and Ingraham, J. L. (1962). Effect of temperature on the composition of fatty acids in E. coli Journal of Bacteriology 8,4 1260.

Matsushima, H., Muratam K. and Mase, Y. (1974). Culture conditions for the microbial production of L-isoleucine from DL-a-bromo-butyric acid. Hakko Kogaku Zasshi52 20 - 27.

Mba, A. U., Njike, M. C. and Oyenuga, V.A. (1974). The proximate chemical composition of the amino content of some Nigerian oil seeds. Journal of the Science of Food and Agriculture 25, 1547 - 1553.

Miles, A. A. and Misra, S. S. (1938). The estimation of the bactericidal power of the blood. Journal of Hygiene Cambridge 38, 732.

Momose, H. and Takagi, T. (1978). Glutamic acid production in biotin - rich media by temperature - sensitive mutants of Brevibacterium lactofermentum a novel fermentation process. Agricultural and Biological Chemistry 42, 1911 - 1917.

Momose, H. (1983). New genetic approaches to amino acid production strains. In: Developments in Industrial Microbiology vol. 24, 109 - 119.

Moon, N. J., Beuchat, L. R., Kinkaid, D. T., Hays E. R. (1982). Evaluation of lactic acid bacteria for extending the shelf life of shrimp. Journal of Food Science 47 (3) 897.

Morcos, S. R., Hegazi, S. M. and El-Damhoughy, S. T. (1973). Fermented foods in common use in Egypt 1. Nutritive value of Kishk. Journal of the Science of Food and Agriculture

24, 1153 - 1156.

Muller, H. J. (1928). The production of mutations by X-rays. In: Proceedings of National Academy of Science Washington 14, 714.

Nakao, Y., Kikuchi, M., Suzuki, M. and Doi, M. (1972). Microbial production of L-glutamic acid by glycerol auxotrophs. 1. Induction of glycerol auxotrophs and production of L-glutamic acid from n-paraffins. Agricultural and Biological Chemistry 36, 490 - 496.

Nakayama, K. (1972). Micro-organisms in amino acid fermentation. In: Terui, G. (Ed). Fermentation Technology Today. Society of fermentation Technology Japan. Osaka.

Nakayama, K. (1982). Amino acids. In: Reed, (Ed) Prescot and Dunn's Industrial Microbiology 4th Edition. Avi Publ. Co. Inc. Westport connecticut. pp. 749 - 801.

Nelson, D. L., Spudich, J. A., Bonsen, P. P. M., Bertsch, L.L, Kornberg, A. (1969). Biochemical studies of bacteria sporulation and germination XVI small molecules in spores. Vol. 4. In: Campell, L.L. (Ed). American Society for Microbiology pp. 59 - 61.

Nestler, E.W., Roberts, C.E., Pearsall, N.N. and McCarthy B.J. (1978). Microbiology. 2nd Edition Holt Saunders pub. Co. New York.

Neumayr, L., Lucke, F. -K. and Lestner, L. (1983). Fate of Bacillus spp from spices in fermented sausage. In: Proceedings of the 29th European Congress of meat research workers, Salsomaggiore pp 418 - 424.

Ngaba, R.R. and Lee, J.S. (1979) fermentation of cassava. Journal Food Science 44, 1570 - 1571.

Nicol, B.M. (1949). Nutrition of Nigerian peasant farmers with special reference to the effect of vitamin A and riboflavin deficiency. Journal of Nutrition 3: 25 - 49.

Nortje, B.K and Vaughn, R.H. (1953). The pectinolytic activity of species of the genus Bacillus. Qualitative studies with Bacillus subtilis and Bacillus pumilus in relation to softening of olives and pickles. Food Resources 18, 57 - 69.

Nwokedi, C.I.C. (1973). Technical report on Pentaclethra macrophylla. Nigerian Stored Products Institute, Lagos.

Oberman, H. (1985). Fermented milks. In: Wood B.J.B. (Ed.) Microbiology of Fermented Foods vol. 1. Elsevier pub. Co. pp 167 - 196.

Obeta, J.A.N. (1983). A note on the micro-organisms associated with the fermentations of seeds of the African oil bean tree (Pentaclethra macrophylla). Journal of Applied Bacteriology 54, 433 - 435.

Odegov, M.I. and Kagan, Y.R. (1980). Increase in phage resistance of starter streptococci by chemical mutagenesis. In: shiler, G.G. (Ed.) Improving efficiency and quality in cheese manufacture. Moscow USSR pp 58 - 59.

Odunfa, S. (1981a). A note on the micro-organisms associated with fermentation of African locust bean (Parkia filicoidea) during iru preparation. Journal of Plant Foods 3, 245 - 250.

Odunfa, S.A. (1981b). Microbiology and amino acid composition of ogiri - a food condiment from fermented melon seeds. Die Nahrung 25, 811 - 816.

Odunfa, S.A. (1982). Biochemical changes during melon fermentation for ogiri production. Qualitas plantarum plant Foods for Human Nutrition 32, 11 - 18.

Odunfa, S.A. (1983). Carbohydrate changes in fermenting locust bean (Parkia filicoidea) during iru preparation. Qualitas plantarum plant Foods for Human Nutrition 32, 3 - 10.

Odunfa, S.A. (1985a). African fermented foods. In: Wood, B.J.B. (Ed.) Microbiology of Fermented Foods Vol. 2. Elsevier Pub. Co. London. pp. 155 - 192.

Odunfa, S. A. (1985b). Biochemical changes in fermenting locust bean (Parkia biglobosa) during iru fermentation Journal of Food Technology 20, 295 - 303.

Odunfa, S.A. and Adewuyi E.Y. (1985c) Optimization of process conditions for the fermentation of African locust bean (Parkia biglobosa) 1. Effect of time, temperature and humidity. Chemistry and Microbiology Technology Lebensm 9, 6 - 10.

Okafor, N. (1975). Microbiology of Nigerian palm wine with particular reference to bacteria. Journal of Applied Bacteriology 38, 81 - 88.

Okafor, N. (1977) Micro-organisms associated with cassava fermentation for garri production. Journal of Applied Bacteriology 42, 279 - 284.

Okafor, N. (1980). A scheme for the improvement of fermented foods of Africa south of the sahara. In: Emejuaiwe, S.O., Ogunbi, O. and Sanni, S.O. (Eds.) Global impacts of Applied Microbiology GIAM VI. Academic Press. London.

Okafor, N., Ijioma, B. and Oyolu, C. (1984). Studies on the microbiology of cassava retting for foo-foo production. Journal of Applied Bacteriology 56, 1 - 13.

Okeoma, J.C. 1984. Comparative analysis of 'Daddawa' with commercial 'synthetic' flavour enhancers. M.Sc. Biochemistry thesis, Ahmadu Bello University Zaria - Nigeria.

Olsen-Sopp, O.J. (1912). Cited from Hesseltine (1965). A millenium of Fungi, food and fermentations. Mycologia 57, 149-197.

Ostover, K and Keeney, P.G. (1973). Isolation and characterization of micro-organisms involved in the fermentation of Trinidad's Cacao beans. Journal of Food Science 38, 611 - 617.

Oyenuga, V.A. (1968). Nigeria's foods and feeding - stuffs. Ibadan University press. Ibadan.

Palumbo, S.A., Rivenburgh, A.I., Smith, J.L and Kissinger, J.C. (1975). Identification of Bacillus subtilis from sausage products and spices. Journal of Applied Bacteriology 38, 99 - 105.

Paunescu, E., Ciolac-Negoescu, A. and Pisica, G. (1964). The effect of Tween 80 and penicillin on the physicochemical properties of the cell wall in mycobacteria. Acad Rep. Populare Romine studii cercetari Biochemistry 7, 83 - 89.

Pavia, D.L., Lampman G.M., and Kriz, G.S. (1976). Introduction to organic laboratory techniques a contemporary approach. W.B. Saunders Co. Philaldephia.

Pederson, C.S. (1971). Microbiology of Food Fermentations. AVI Pub. co. Westport Connecticut.

Platt, B.S. (1962). Tables of representative values of foods commonly used in tropical countries. Special Report Series of Medical Research Council London No. 302.

Platt, B.S. (1964). Biological Ennoblement; Improvement of the nutritive value of foods and dietary regimens by biological agencies. Food Technology 68 (662 - 670) 76.

Popoola, T.O.S. and Akwueshi, C.O. (1985). Micro-organisms associated with the fermentation of soya bean for production of soya bean daddawa (a condiment). Nigerian Food Journal 2 & 3 (3) 194 - 196.

Priest, F.G., Goodfellow, M. and Todd, C. (1981). The genus Bacillus; numerical analysis. In Berkeley, R.C. and Goodfellow (Eds.) The aerobic endospore forming bacteria. Academic press. London pp. 91 - 103.

Promptov, A.N. (1932). The effect of short ultraviolet rays on the appearance of hereditary variations in Drosophila melanogaster. Journal of Genetics 26, 59.

Rangaswamy, B. N. V. and Yathirajan, H.S. (1984). Oxidation of glutamic acid and its rare earth complexes with bromamine - T. Analyst 109, 101 - 102.

Reese, E.T. and Maguire, A. (1969). Surfactants as stimulants of enzyme production by micro-organisms. Applied Microbiology 17 (2) 242 - 245.

Rieman, H. (1963). Safe heat processing of canned cure meats with regards to bacterial spores. Food Technology 17, 39 - 49.

Seenappa, M. and Kempton, A.G. (1981). A note on the occurence of Bacillus cereus and other species of Bacillus in Indian spices of export quality. Journal of Applied Bacteriology 50 (2) 225 - 228.

Sen, S and Chakrabarty, S.L. (1984). Amylase from Lactobacillus cellobiosus isolated from vegetable wastes. Journal of Fermentation Technology 62 (5) 407 - 413.

Shimura, K. (1972). Threonine. In: Yamada, K. Kinoshita, S Tsunoda, T and Aida, K. (Eds.) The microbial production of amino acids. Kodansha Tokyo.

Shugar, D. (1960). Photo chemistry of nucleic acids and their constituents. In: Chargoff, E and Davidson, J. N. (Eds.) The Nucleic acids vol. III Academic press. Inc. New York. p 39.

Siopongco, M.C., and Aspiras, R.B. (1981). Isolation and characterization of the microflora of a commercial pig manure inoculant preparation (1kuton) Phillippine Agriculturist 64 (3) 213 - 223.

Singh, J. and Choppa, A.K. (1982). Accelarated fermentation of milk by nitrosoguanidine induced mutant of Lactobacillus. Journal of Food Science 47, 1027 - 1029.

Smith, N.R., Gordon, R.e. and Clark F.E. (1946). Aerobic mesophilic sporeforming bacteria. U.S. Department of Agriculture Miscellaneous Pub. 559, 112pp.

Sneath, P.H.A. (1962). The construction of taxonomic groups. In: Ainsworth, G.C. and Sneath, P.H.A. (Eds.) Microbiological Classification. Cambridge University press. Cambridge pp 289 - 332.

Spackman. E.H., Stein, W.H., Moore, S. (1958) Automatic recording apparatus for use in the chromatography of amino acids. Analytical Chemistry 30, 1190 - 1206.

Stanton, W.R. (1985). Food fermentation in the tropics. In: Wood B.J.B. (Ed.) Microbiology of Fermented Foods. Vol.2 Elsevier pub. co. London. pp 193-212.

Steinke, P.K.W. and Foster, E.M. (1951). Microbial changes in refrigerated liver sausage. Food Resources 16, 245.

Steinkraus, K.H. (1979). Nutritionally significant indigenous foods involving an alcoholic fermentation. In: Fermented Food beverages in Nutrition. Academic press. London pp 35 - 59.

Sugiyama, Y., Kitano, K. and Kanzaki, T. (1973). The role of copper ions in the regulation of L-glutamate biosythesis. Agricultural and Biological Chemistry 37, 1837 - 1847.

Sulzbacher, W.L. and McLean R.A. (1951). The bacterial flora of fresh pork sausage. Food Technology Champaign 5, 7.

Tanaka, K., Suzuki, T and Okumara, S. (1971). Production of sugars and amino acids from hydrocarbons and petrochemicals by micro-organisms. 5th world petroleum congress Proceedings 5, 165 - 170. Applied Science Publ. London.

Tanaka, Y., Yoshida, H. and Nakayama, K. (1974). Production of L-DOPA by mutants of Pseudomonas metanogenum Agricultural and Biological Chemistry 38, 633 - 639.

Tanner, F.W. (1944). The Microbiology of Foods Garrad Press. Champaign. Illonois.

TertyShny, V.N., Krasnobrizhy, N.Y., Polotnyak, A.D and M.I. Balabushevich. (1981). Effect of molasses composition on glutamic acid biosynthesis by the Corynebacterium glutamicum culture. Microbiol Zh 42 (3) 316 - 320.

Toury, J., Gorgi, R., Favier, J.C. and Savina J.F. (1967). Cited from Campbell-platt, G. 1980. African locust bean (Parkia species) and its West African fermented food product, dawadawa. Ecology of Food and Nutrition 9, 126.

Toury, J., Ware, A., Gorgi, R and Gros, J. (1970). Fish in the diet in Senegal, quantitative and qualitative aspects, methods of preservation. Food Nutrition Africa 8, 6 - 13.

Tribe, D.E., and Choi, Y.J. (1982). Continuous production of phenylalanine using an Escherichia coli regulatory mutant. Biotechnology Letters 4 (4) 223 - 228.

Tsuchida, T., Yoshinaga, F., Kubola, K. Momose, H. and Okumara, S. (1974). Production of L-leucine by a mutant of Brevibacterium lactofermentum 2256. Agricultural and Biological Chemistry38, 1907 - 1911.

Ueda, S., Chiba, A., Kuwabara, 4. (1981). Bacillus spp. from the coating bread crumbs of commercial frozen foods for frying. Journal of Japanese Society of Food Science and Technology 28 (12) 647 - 649.

Ulloa, M. (1980). Indigenous fermented beverages of Mexico. In: Emejuaiwe, S.O., Ogunbi, O. and Sanni, S.O. (Eds.) Global impacts of Applied Microbiology GIAM VI. Academic press London.

Van Veen, A.G. (1957). Fermented protein - rich foods. FAO report No. FAO/57/3/1966.

Van Veen, A.G., Graham, D.C.W. and Steinkraus, K.H. (1968). Fermented rice, a food of Ecuador. Archives Latinoa, Nutrition 18, 363 - 373.

Vaughn, R.H., De Camargo, R., Falanghe, H., Melto - Ayres G. and Serzedello, A. (1958). Observations on the microbiology of the coffee fermentation in Brazil. Food Technology 12 Abstr. No. 173.

Vaughn, R.H. (1985). The microbiology of vegetable fermentations In: Wood, B.J.B. (Ed.) Microbiology of Fermented Foods vol. 1. Elsevier pub. Co. London pp. 49 - 110.

Vielmetter, W. and Schuster, H. (1960). The base specificity of mutation induced by nitrous acid in phage T2. Biochemical and Biophysics Research Communications 2,324.

Voysey, P. (1983). Cited from Wood B.J.B (1985) Microbiology of Fermented Foods. vol. 1 Elsevier Pub. Co. London pp 276.

Wakisaka, Y. and Koizumu, K. (1982). An enrichment isolation procedure for minor Bacillus populations. Journal of Antibiotics 35 (4) 450 - 457.

Watson, J.D. and Crick, F.H.C. (1953). The structure of DNA. Cold spring Harber symposium on quantitative Biololgy 18, 123.

Watson, J.D. (1971). Investigation on the nutrients of some Ghanaian foodstuffs. Ghana Journal of Agricultural Science 4, 95 - 111.

Westhoff, D.C. and Dougherty, S.L. (1981). Characterization of Bacillus species isolated from spoiled ultrahigh temperature processed milk. Journal of Dairy Science 64 (4) 572 - 580.

Wood, B.J.B. (1985). Miscellaneous food-related fermentations. In: Wood B.J.B (Ed.) Microbiology of Fermented Foods vol. 2 Elsevier Pub. Co. London pp. 213 - 236.

Wood, B.J.B and Hodge M.M (1985). Yeast-lactic acid bacteria interactions and their contribution to fermented foodstuffs. In: Wood B.J.B (Ed.) Microbiology of Fermented Foods. Vol. 1 Elsevier Pub. Co. London pp. 263 - 294.

Woolford, M.K. (1985). The silage fermentation. In: Wood B.J.B (Ed.) Microbiology of Fermented Foods Vol. 2. Elsevier Pub. Co. pp. 85 - 112.

Yablonsky, F. (1983). Alteration of membrane permeability in Bacillus subtilis by clofoctol. Journal of General Microbiology 129 (4) 1089 - 1095.

Yokotsuka, T. (1981). Recent advances in Shoyu research. In Charalambous, G and Inglett, G. (Eds) Quality of foods and beverages: Chemistry Technology Vol. 2 Academic Press. New York. pp 171 - 196.

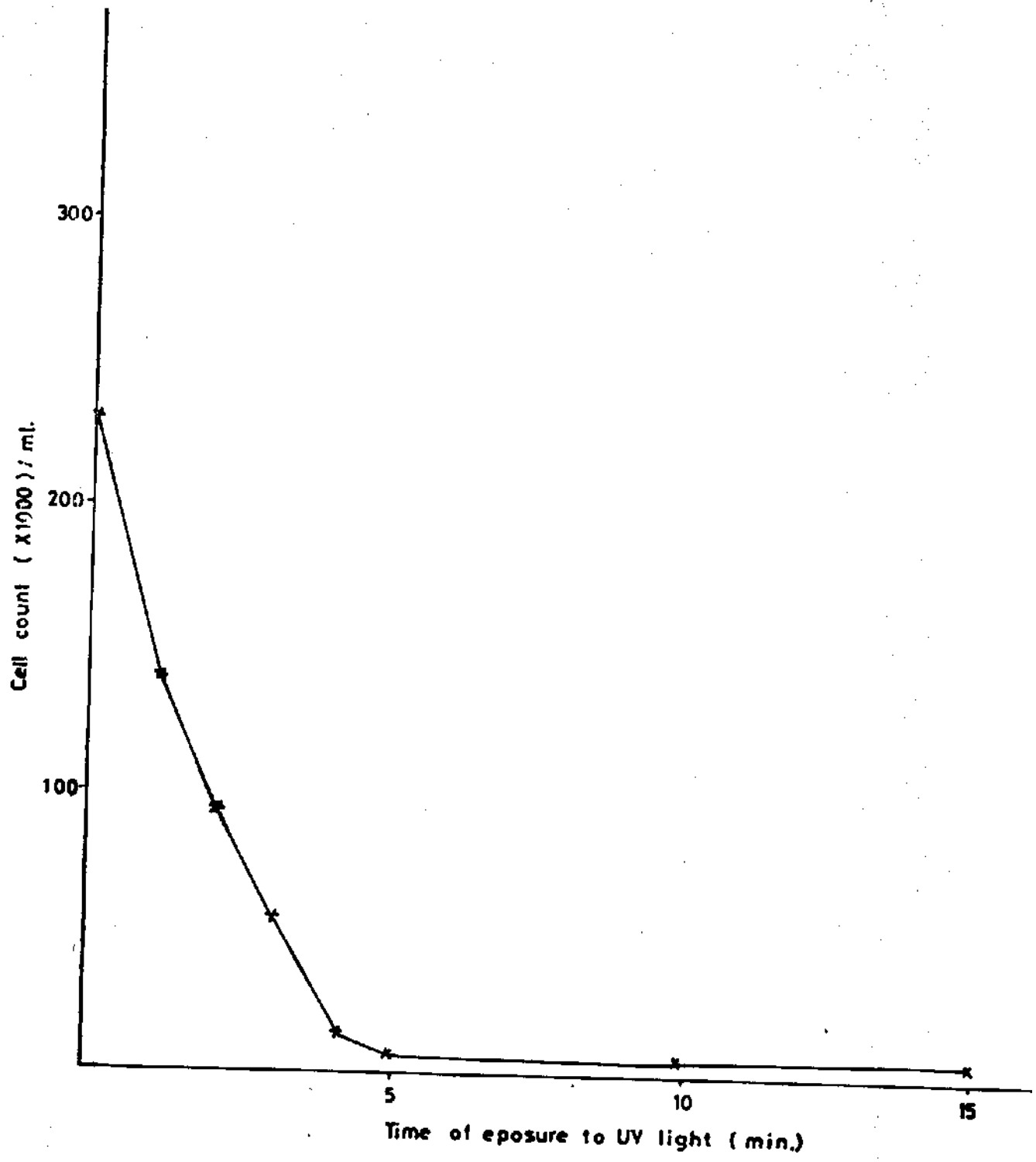
Yokotsuka, T. (1985). Fermented protein foods in the orient with emphasis on shoyu and miso in Japan. In: Wood B.J.B. (Ed.) Microbiology of Fermented Foods Vol. 1. Elsevier Pub. Co. pp. 197 - 248.

Yong, F.M. and Wood, B.J.B. (1976). Microbial succession in experimental soy sauce fermentations. Journal of Food Technology 11, 524 - 536.

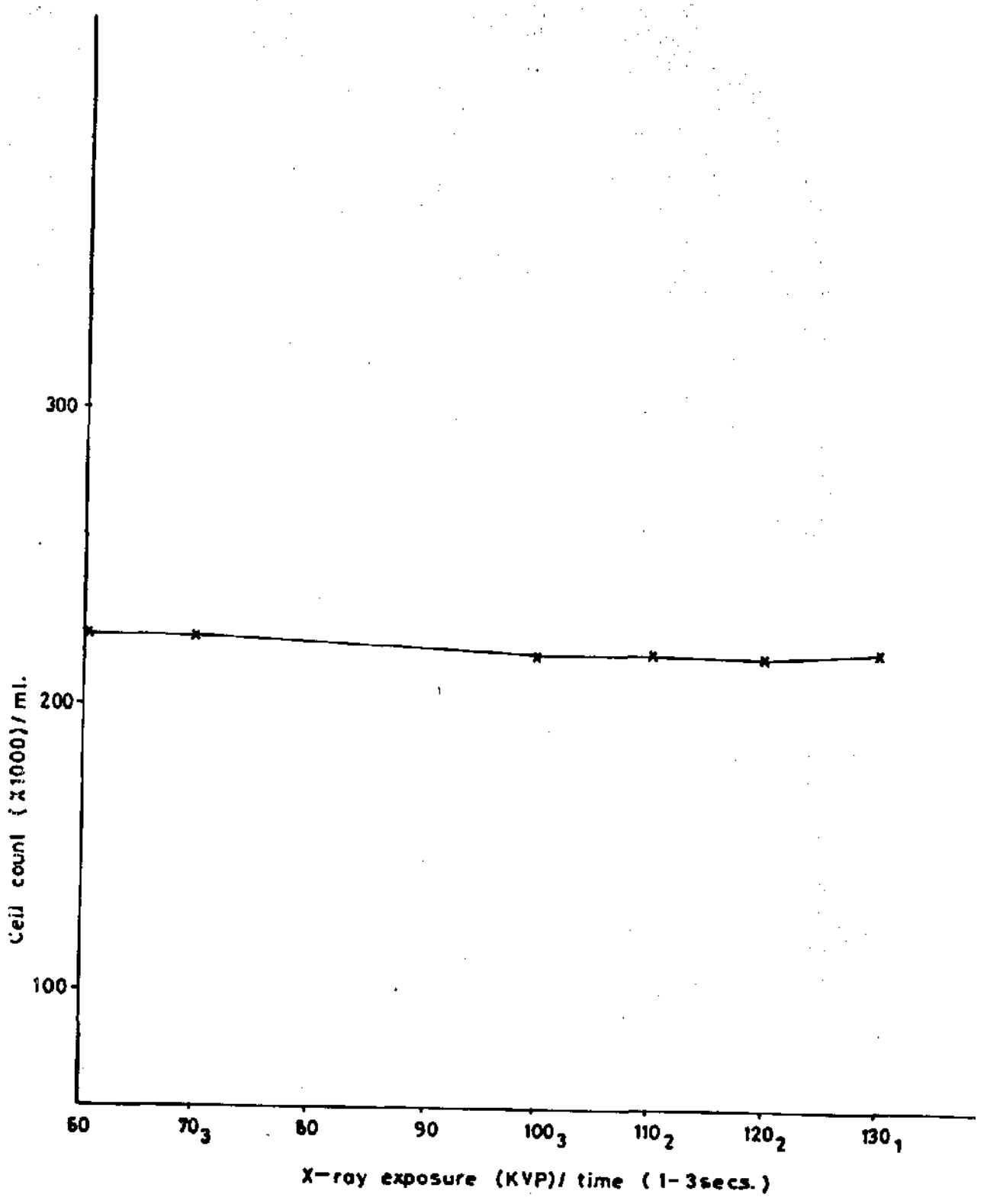
Appendix A: Biochemical characters employed in the identification of the presumptive *Bacillus* isolates from indigenous condiments.

Source of isolation	*Bacillus* isolate code	Biochemical Tests: Catalase	VP	Anaerobic growth	Starch hydrolysis	Gelatin liquefaction	Growth in 7% NaCl	acid + and (gas O glucose)	*Bacillus* species identified
	DS_1	+	+	-	+	+	+	+	*B. subtilis*
	DS_2	+	+	-	-	+	-	+	*B. laterosporus*
	DS_3*	+	+	-	+	+	+	+	*B. subtilis*
Soya bean	DS_4	+	+	-	+	+	+	+	*B. subtilis*
daddawa	DS_5	+	+	-	-	+	+	+	*B. pumilus*
	DS_6	+	+	-	-	+	+	+	*B. pumilus*
	DU_1	+	-	-	-	+	-	+	*B. brevis*
	DU_2*	+	+	-	+	+	+	+	*B. subtilis*
	DU_3	+	+	-	+	+	-	+	*B. macerans*
locust bean	DU_4	+	+	-	+	+	+	+	*B. subtilis*
daddawa	DU_5	+	+	-	-	+	+	+	*B. pumilus*
	DU_9	+	+	+	+	+	-	+	*B. licheniformis*
	DO_1	+	+	-	-	+	+	+	*B. pumilus*
	DO_2	+	+	+	+	+	-	+	*B. licheniformis*
Ofaphehe	DO_3	+	+	-	+	+	+	+	*B. subtilis*
	DO_4	+	+	-	-	+	+	+	*B. pumilus*
	DO_5	+	+	-	+	+	+	+	*B. subtilis*
	DO_6	+	+	-	+	-	-	+	*B. polymyxa*
	DO_7	+	+	-	+	+	+	+	*B. subtilis*
	OG_1	+	-	-	-	+	-	+	*B. brevis*
Ogiri	OG_2	+	+	-	+	+	+	+	*B. subtilis*
	OG_3	+	+	-	+	+	+	+	*B. subtilis*
	DP_1	+	+	-	+	+	+	+	*B. subtilis*
Ugba	DP_2	+	+	+	+	+	+	+	*B. licheniformis*
	DP_3	+	+	+	+	-	-	+	*B. coagulans*

* Isolates from fermenting daddawa - section A 3.1



APPENDIX B: LETHAL EFFECT OF ULTRA VIOLET LIGHT ON THE POPULATION OF B. SUBTILIS- DU_2 IN IRRADIATION BUFFER.



APPENDIX C: EFFECT OF X-RAY EXPOSURE ON THE CELL COUNT OF B. SUBTLLIS-DU$_2$

Appendix D: Biochemical characters of the isolates obtained after exposing *B. subtilis* - DU_2 to radiation and acridine orange.

Mutagen	Isolate	Catalase	VP	Anaerobic growth	Starch hydrolysis	Gelatin liquefaction	Growth in 7% NaCl	Acid + gas (glucose)
	1	+	+	-	+	+	+	+
	2	+	+	-	+	+	+	+
	3	+	+	-	+	+	+	+
	4	+	+	-	+	+	+	+
UV light	5	+	+	-	+	+	+	+
	6	+	+	-	+	+	+	+
	7	+	+	-	+	+	+	+
	8	+	+	-	+	+	+	+
	9	+	+	-	+	+	+	+
	1	+	+	-	+	+	+	+
	2	+	+	-	+	+	+	+
	3	+	+	-	+	+	+	+
	4	+	+	-	+	+	+	+
X-ray	5	+	+	-	+	+	+	+
	6	+	+	-	+	+	+	+
	7	+	+	-	+	+	+	+
	8	+	+	-	+	+	+	+
	9	+	+	-	+	+	+	+
	10	+	+	-	+	+	+	+
	1	+	+	-	+	+	+	+
	2	+	+	-	+	+	+	+
	3	+	+	-	+	+	+	+
	4	+	+	-	+	+	+	+
AO	5	+	+	-	+	+	+	+
	6	+	+	-	+	+	+	+
	7	+	+	-	+	+	+	+
	8	+	+	-	+	+	+	+
	9	+	+	-	+	+	+	+