AN ABSTRACT OF THE THESIS OF

ALCIBIADES HELI MIRANDA-CHAVEZ for the degree of MASTER OF SCIENCE

in MICROBIOLOGY presented on April 28, 1978

Title: CHITINASE PRODUCTION OF ARTHROBACTER SP. BN2

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Abstract approved: ______________________
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A chitinase producing bacterium was isolated from soil and identified as a species of *Arthrobacter*. The organism grew well at temperatures between 27 and 33.5 C, and the chitinase production was highest at 25.5 C. As the temperature increased above the optimum point, the level of enzyme production rapidly decreased, while the rate of decrease was slower at lower temperatures. Optimum pH for both growth and enzyme production was 6.4. Colloidal crab chitin or powdered crab chitin was an excellent substrate for chitinase production and the addition of Fe^{++} and Co^{++} enchanced the enzyme production. Under optimum conditions the organism produced 90 units (μg N-acetylglucosamine/min/ml culture) of extracellular chitinase in 4 to 8 days. Chitinase of the *Arthrobacter* sp. is an inducible enzyme and at least 3% of chitin in the growth medium was necessary for maximum production of the enzyme. Chitinase production by the *Arthrobacter* sp. was greatly increased when the organism was grown with molds (5-fold with *Aureobasidium pullulans*, 12-fold with *Penicillium* sp., and 8-fold with *Aspergillus niger* and *Phanerochaeta chrysosporum*), but cultivation with other bacteria, actinomycetes, and yeasts had no effect.

Chitinase Production of *Arthrobacter* sp. BN2

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Commencement June 1978

APPROVED:

Redacted for Privacy

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Date thesis is presented April 28 , 1978

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A MIS PADRES, ANTENOR Y CUSTODIA

Como pequena compensación a sus denodados sacrificios, desvelos, enseñanzas y ejemplo que supieron brindarme en la forjación de mi caracter.

A MI NOVIA, HERMELINDA JARA CABREJO

Como fiel testimonio de la sacrificada separación que tuvimos que sobrellevar para plasmar en realidad una ambición que encontró comprensión y soporte moral en su amor sincero y epopéyico.

A MI MENTOR CIENTIFICO, PROFESOR JESUS GARCIA ALVARADO

Quien con su excepcional integridad como persona humana, maestro e investigador, me brindó las luces necesarias para hacer de mi escudrinar en las intimidades del frondoso y asombroso mundo de los microbios, la mística de mi vida.

ACKNOWLEDGMENT

I would like to express my deep appreciation to:

Dr. A. W. Anderson, who suggested this area of research to me.

Dr. William Sandine, who substituted for Dr. Anderson while he was on sabbatic leave during the time the final thesis was prepared.

Dr. Youn Han, who provided me with his friendship, advice and ideas along with laboratory facilities.

Jon Beaty, Ms. Sheila Smith and Dr. Francisco Torrella, who graciously proof-read the first manuscript.

The faculty, staff and graduate students of the Microbiology Department, who provided outstanding knowledge and enlightening conversation and, especially Wen Pin Chen and Cleanthes Israelides, with whom I spent many enjoyable hours during my laboratory work.

Latin American Scholarship Program of American Universities, the institution that gave me the oportunity through economic support to fulfill one of my life's dreams: to study and gain experience on a North American campus.

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CHITINASE PRODUCTION OF *ARTHROBACTER* SP. BN2

INTRODUCTION

Although man has learned much about the laws directing natural phenomena, he has not yet learned how to manage his environment properly. Instead, he is constantly damaging or altering his surroundings. As a consequence, humans are now confronted with emerging problems proving difficult both to study and solve. Food and energy shortages, rapid population growth, uncontrolled consumption of non-renewable resources and poisoning of the environment with pollutants are some of the problems being left to future generations. The severity of these problems depends upon how quickly and effectively we develop solutions or, at least, less harmful alternatives. Extreme efforts from scientific, technological, social and political fronts must be undertaken to overcome these problems and their implications. Some of these efforts now are underway on the national, state and local scene in many countries.

In recent decades great strides have been made in gaining knowledge of the metabolism, genetics and ecology of microorganisms which have led to increased understanding of the role microorganisms play in the economy of nature. For example, production of single-cell protein offers promise in the struggle against malnutrition and starvation; production of biomass from waste, when the economics of production and secondary constraints are overcome, may help to alleviate energy and pollution problems. Methane gas production by anaerobic fermentation is now being used in some countries at the farm and village levels. Many chemical products such as

antibiotics, amino acids, organic acids, hormone derivatives, enzymes and insecticides for use in agriculture, medicine and industry are produced from renewable resources by microbial fermentation with little or no pollution.

In the future we will have to depend increasingly on renewable resources derived from the biosphere. A variety of materials has been employed in traditional and modern processes. These raw materials - molasses, corn syrup, corn steep liquor, soybean meal, cottonseed meal, corn meal, meat extract, sulfite waste liquor, whey, n-paraffins, yeast, ethanol and methanol - are relatively few in number and are selected on the basis of their availability and suitability for a specific microbial process. However, other less traditional raw materials or by-products merit attention. Several types from food crops (cereals, cassava, potatoes, yams, sweet potatoes, sago, taro, sesame, etc.) and harvesting and processing wastes (straw, corncobs, husks, stalks, bran, sugar cane bagasse, leaves, cocoa, olive oil, hulls, sisal, papaya, prickly pear, molasses, oilseed cakes, coconut, carob, dates, hops, coffee and grapes), as well as agricultural, forest, urban and industrial waste materials (manure, composts, sewage, municipal garbage) are promising raw materials on the basis of their availability and their ease of assimilation by microorganisms.

The major economic factor related to raw material for microbial processes is the source of carbon. Most of the above mentioned materials are cellulosic in chemical nature; therefore cellulose is the dominant source of carbon for microbial conversion processes.

Since the aforementioned agricultural crops and their processed residues are familiar, available and relatively free from toxic substances, they will maintain their dominance as the carbon source for microbial processes. Nevertheless, we cannot overlook the potential of other suitable and available materials. Chitin is one of these promising raw materials. Since this substance is a polymer of N-acetyl glucosamine, it is a suitable source of carbon as well as nitrogen. Chitin is a potentially valuable marine resource that is obtained as a by-product from seafood operations. There is about half as much chitin recoverable (about 10-20%) from crabs and shrimps as there is edible meat (15-25%) (Brine, 1974; Lubitz et al., 1943; Lovell, 1968). It is estimated that more than 50 million pounds of chitin could be obtained annually from seafood residues now processed, largely for animal feed in the U.S.A. (Brown, 1959; Goodwin, 1949; Lubitz, 1943; Parkhurst, 1943). United States, Department of Commerce (1973)reported that for the Chesapeake Bay-Delmarva area alone, nearly a million pounds of chitin per year is potencially available.

In addition to the vast salt water sources for chitin, fresh water sources also exist. In Louisiana, the Bureau of Commercial Fisheries (1968) reported a commercial catch of 8.6 million pounds of crayfish for the year. At the present time nearly 85% of the live weight of crayfish after processing has no commercial value and its disposal has been an expense for the industry (Lovell et al., 1968).

Although chitin is present in the environment in very large amounts, this polymer has, until recently, not been given the

attention it deserves. By contrast, a tremendous amount of multidisciplinary research has been concerned in the study of cellulose and its derivatives. Among the reasons given to explain this enormously unbalanced effort in the study of two important natural carbohydrates, cellulose and chitin, are the following: (1) chitin has been relatively difficult to collect, isolate and purify due to the fact that most of the chitin-producing organisms are mobile and/or microscopic, (2) chitin is a tough, intractable material difficult to dissolve for purification or further handling except in strong acid or special salt solutions, and (3) little economic incentive has existed so far to incite a systematic study of chitin, its derivatives and its industrial potentials. However, the strongest stimulus for the need for cellulose research is the pollution problem that agriculture waste has created.

Today the annual dumping of thousands of tons of lobster, shrimp and crab carcasses, material that is highly resistant to biodegradation, has also given rise to a new pollution problem. This necessitates the development of alternate methods to dispose of a waste product which, until a few years ago, had an insignificant commercial value and could not be easily destroyed by conventional means. Recognition of this situation has led NOAA's office of Sea Grant, U.S. Department of Commerce, to support a research program at the University of Washington to broaden the efforts to seek and develop economically sound ways of utilizing waste products produced by this type of marine food processing plants. Within this program, chitin is naturally of considerable importance. Moreover,

in the last few years, renewed interest in chitin has been developing because of the numerous industrial applications found for chitin and its derivatives. We, therefore, are tempted to believe in the potential use of chitin because of its availability and suitability in nature as a raw material for microbial fermentations.

The complete degradation of chitin in nature is accomplished by an enzymatic complex borne by numerous organisms. The chitinase system is responsible for the hydrolysis of chitin to N-acetyl-D-glucosamine.

Although the activities of microbial enzymes have been observed and utilized for many centuries, it has been only recently that the use of microbial enzymes has been commercialized and unexpected breakthroughs in enzyme technology are being developed at an ever increasing pace. In 1970 only slightly more than 150 enzymes were available commercially in purified form, and none were immobilized. Today, over 330 enzymes are available, most in a more highly purified state than available six years ago and, in addition, almost 50 enzymes are available in an immobilized (insolubilized) form for direct use (Guilbant, 1976). Enzymes produced by micro-organisms are being used by a large number and variety of industries due to their recognized advantages such as, (1) microbial enzymes impart no toxicity to their substrates under controlled conditions, (2) they can be inactivated readily by heat or a change in pH when their services are no longer needed, and (3) they usually do not need to be removed from the material in which they have directed a chemical reaction.

Chitinases and chitinase-producing microorganisms have a potential to be used whether as a tool in the field of basic research or directly in practical applications that can stimulate their industrial production in the near future. There is, for instance, strong evidence that chitinases may be instrumental in the biological control of phytopathogenic insects and fungi, in the enhancement of antigenicity and/or specificity of fungal preparations used in serological diagnosis of mycosis and in the preparation or improvement of fungal vaccines.

The main constraints on a large-scale production of chitinase seems to depend upon the following limitations: (1) its production apparently requires many steps, extensive time and is difficult to control (Smirnoff, 1975) when produced from the fermentation of microorganisms such as *Streptomyces griseus*, *Bauveria* and *Cordyceps*, (2) the activity of the chitinase preparations sold by chemical suppliers is usually of the order of 1200 nephelometric units (40 Units/mg chitinase, colorimetric method), which is relatively low, and (3) it is difficult to obtain quantities of chitinase exceeding 100 mg and the current price is about 200 dollars per gram. Even though Smirnoff (1975) has patented a process to obtain chitinase from fowl entrails with an activity of 890 nephelometric units, there is a necessity for seeking a more feasible source and process for chitinase production from microorganisms, as this method is more advantageous for enzyme technology practices.

Therefore, this study was carried out with the following goals: First, to search for a microorganism with a high level of chitinase

production. This involved the collection of 62 different soil samples to screen for chitinolytic organisms by means of enrichment techniques and selective differential isolation of potent chitinoclastic organisms. Second, to determine the optimum environmental and nutritional conditions for both growth and chitinase production by the best chitinolytic organism isolated, a culture tentatively placed in the genus *Arthrobacter*. Among the optimum culture factors studied for maximum growth and chitinase production were temperature, pH, effect of several mineral salts, size of inoculum, shaking conditions, incubation time, biological origin, physical state, degree of purity and concentration of chitin in the culture medium. Third, to improve chitinase production by means of mixed culture techniques. To accomplish this, representatives from different microbial groups, bacteria, yeasts and molds were grown together with the chitinolytic culture with the hope that a second organism will metabolize (scavenge) N-acetyl glucosamine which has a repressive effect on chitinase synthesis.

LITERATURE REVIEW

Chitin is a tough, pliable mucopolysaccaride that consists of 2-acetamido-2-deoxy-D-glucopyranose residues held together by beta (1-4) linkages. The molecular weight of chitin is thought to be of the same order of magnitude as that of cellulose. However, as expected for a polymer that is difficult to isolate and purify, a wide range of molecular weights has been reported. X-ray diffraction investigations have shown that chitin contains highly ordered regions giving one of several possible crystallographic patterns: alpha-chitin, beta-chitin and native chitin. Chitin is insoluble in water, organic solvents and cuprammonium reagent. It dissolves with some depolymerization in concentrated mineral acids. It is soluble in anhydrous formic acid and is dispersed in concentrated aqueous solutions containing certain lithium or calcium salts. It is less reactive than cellulose because of its general insolubility. It is slowly N-deacetylated and degraded by strong alkali to give a complex mixture of partially deacetylated products collectively named chitosan. Other derivatives of chitin that have been reported include sulfate, nitrate and xanthate esters (McNeely, 1959; Rudall and Kenchington, 1973).

Tracey (1957) estimates that 10 billion tons of cellulose are annually synthesized on the globe and that chitin is probably the second most important natural polysaccharide on the earth. Without any doubt, chitin is the most abundant polymer of amino sugars. It may well be that chitin is even more abundant than cellulose;

marine copepods alone annually produce several billion tons of chitin, and these arthropods represent only one of many groups of animals that have chitin as an important consitutent of their skeletal supporting systems (Pariser and Bock, 1973; Johnstone, 1908). Jerde and Lasker (1966), have estimated that the population of a single, planktonic crustacean, *Euphasis pacifica*, produces about 1.9×10^{13} g. dry weight molt skeleton per year; 24 to 56% ash remains after the combustion of skeletons. Goodrich and Morita (1977b), assuming that the dry weight skeleton is primarily cuticle and inorganic salts and using Jeauniaux's (1971) report that chitin constitutes 58 to 85% of the dry organic weight in the crustacean cuticle, estimated that the amount of chitin produced annually in molts by *E. pacifica* is from 4.8 to 12×10^{7} metric tons. About 800,000 known insect species rely upon chitin to give them structural support and protection from noxious environmental conditions. Along with arthropods, of which crustacea and insects are important representatives, some members of the annelids, the mollusks and other invertebrates, and still some cephalochordates are also endowed with the capacity of synthesizing chitin. Chitin is the most characteristic component of the cell walls of all fungi with the exception of the Omycetes (Rudall and Kenchington, 1973; Bartnicki-Garcia, 1968).

Chitin has found a lot of industrial applications in the last 20 years. Chitin has medical application as a wound-healing accelerator (Prudden et al, 1970) and its derivative N-formyl chitosan polysulfuric acid has use for patients with primary aldosterism (Conn *et al.*, 1966; Abbot *et al.*, 1966). Chitin is an

essential nutrient in crayfish culture (Kuzenski and Becker, 1974; Lovell et al., 1968). It is also an essential nutrient in selective culture media for enumeration and isolation of Actinomycetes from water and soil (Hsu and Lockwood, 1975; Lingappa and Lockwood, 1961) or for cultivation of chitinophilic fungi (Dogma, 1976). When used as an amender of soils, there results a diminished activity of plant pathogenic fungi (Papavizas, 1976; Huber and Anderson, 1976; Sneh et al., 1971; Guy and Baker, 1977; Buxton and Khalita, 1965; Benzon and Baker, 1970; Maurer and Baker, 1965; Henis et al., 1967; Khalifa, 1965; Singh and Pahde, 1965; Van Vuurde and Schippers, 1971; Vruggink, 1970). Chitin and some of its derivatives have been used as supporters for enzyme or cell immobilization (Stanley et al., 1976; Tsumura et al., 1976), as stabilizers of enzymes (Maeda and Taga, 1976) and in the adsorption of enzymes from organic fluids (Kinoshita and Kozo, 1977). Chitosan-deacetylated chitin, due to its solubility, has found use in technology as a paper-binder for improving strength, dyeing and printing quality (Friedhoff et al., 1975), as an aid in increasing the efficiency of the fiber beating process in paper-making, and as a coagulant in the treatment of water supplies, sewage and industrial waste water (Brine and Austin, 1974; Bough, 1975; Wu et al., 1976). Other chitin derivatives are used as chromatographic supports and adsorbents for collection of metal ions from organic and aqueous solutions and sea water, and for the specific isolation of lectins in a pure form (Muzzarelli and Tubertine, 1969, 1970; Muzzarelli, 1969, 1970, 1971a, 1971b; Muzzarelli and Sipos, 1971; Muzzarelli and Isolati, 1971; Muzzarelli et al., 1970; Muzarelli and Rochetti, 1972; Pruglo et al., 1965, 1968; Banes and Gray, 1977).

Finally, analysis of chitin is performed as an aid in diagnosis of plant and animal diseases produced by fungi (Toppan *et al.*, 1976; Doby *et al.*, 1975; Galun *et al.*, 1976) and as a measure of degree of fungal invasion of stored grains (soybean, corn, etc.)(Donald and Mirocha,1977).

Although enormous amounts of chitin are synthesized each year by arthropods, mollusca and fungi, no large natural areas of chitin settlement or accumulation have been reported in either the marine or terrestrial environments (Goodrich and Morita, 1977a). It can well be stated that chitin is accomplishing an important role in the natural circulation of carbon and nitrogen throughout our globe by an apparently rapid degradation. The hydrolysis of chitin to free N-acetyl glucosamine in nature at a significant rate is mainly performed by microorganisms. These microorganisms possess a complex enzyme system responsible for that hydrolysis, which consists of two main hydrolases whose action is successive. These hydrolases are: (1) chitinase: poly-beta-1-4-(2-acetamido-2-deoxy-D-glycoside) glycan hydrolase: E.C. 3.2.1.14 (Enzyme Nomenclature, 1972) which hydrolyzes the polymers of N-acetyl glucosamine to chitobiose, including tetramers and, to a lesser extent, trimers (Berger and Reynolds, 1958); (2) Beta-N-acetylglucosaminidase (chitobiase): 2-acetamido-2-deoxyglucohydrolase: E.C. 3.2.1.29 (Enzyme Nomenclature, 1972),which hydrolyzes chitobiose (dimer of N-acetyl glucosamine) and chitotriose. Monreal and Reese (1969) and Tiunova *et al.*, (1976b, 1976c) have presented some evidence for the presence of a prehydrolytic factor in the chitinolytic system (CH1) similar to the one in the cellulolytic complex (Ci). They support this opinion on the basis

of their findings that hydrolytic activity against colloidal chitin increases with time of incubation, but activity against "crystalline" native chitin decreases; however, the ratio of the activities of CH1 and chitinase (CHx) in different strains or species would be approximately the same magnitude if only one component was involved in the hydrolysis of both colloidal and native chitin. Berger and Reynolds (1958) have been able to separate and purify the three components from the chitinase system of a Streptomyces griseus culture.

Chitinases, like cellulases, are highly specific enzymes (Jeuniaux, 1966). Removal of the acetyl group from chitin produces a non-susceptible product - chitosan - (Monreal and Reese, 1969); moreover, chitosan is a strong inhibitor of chitinases (Morrisey et al., 1976). Substitution of the N-acetyl group with OH, thus forming cellulose, similarly yields an inappropriate substrate (Monreal and Reese, 1969).

Chitinase production has been observed in representatives from bacteria, fungi, higher plants and animals. Among bacteria, chitinase production is a characteristic with relatively wide distribution as shown in Table 1. Indeed, the initial report of the biological degradation of chitin was presented by Benecke (1905). He reported a bacterium, Bacillus chitinovorus, capable of using chitin as a food source. As a general rule, bacterial chitinases are inducible extracellular enzymes (Monreal and Reese, 1969; Chigaleichik and Pirieva, 1976a; Sundarray and Bhat. 1972: Reynolds, 1954: Clarke and Tracey, 1956; Jeauniaux, 1966). Chitinase production

TABLE 1. Distribution of Chitinase Production Among Bacteria.

Organism	Reference
Serratia marcescens BKM	Chigaleichik *et al.* (1976a)
S. marcescens B-851	Podwaite and Cosenza (1976)
S. marcescens QMB1455, QMB1466	Monreal and Reese (1969)
Serratia spp., 36 strains	Molise and Drake (1973)
Streptomyces sp. QMB1359	Monreal and Reese (1969)
Streptomyces spp., C-10,C-14	Reynolds (1954)
Streptomyces sp. FL 44	Laborda *et al.* (1974)
Streptomyces spp. 42 strains	Tiunova *et al.* (1976a)
S. californicus 45	" "
S. kurssanovi 75	" "
Streptomyces orientalis	Tominaga and Tsujisaka (1976a)
S. griseus	Berger and Reynolds (1958)
Streptomyces sp. 2B, 3C	Skujins *et al.*, (1970)
S. violaceus	Wrigert (1962)
S. antibioticus	Jeauniaux (1959)
S. albus 26,1,25,28,29	Tiunova *et al.* (1976b)
S. aburaviensis 86	" "
S. albidoflavus 12,13	" "
S. albireticuli 52	" "
S. annulatus 2	" "
S. argenteolus 100	" "
S. badicus 55	" "
S. californicus 44,46	" "
S. griseoflavus 20	" "
S. griseolus 47,48,49,70	" "
S. griseoincarnatus 106	" "
S. griseorubens 71	" "
S. griseostramineus 72	" "
S. griseus 6,7	" "
S. griseoviridis 101	" "
S. Halstedii 32,33,34,35	" "
S. hachijoensis 73	" "
S. candidus 35	" "
S. canescens 58,3	" "
S. cellulosae 42,44,43	" "
S. chinensis 103	" "
S. cinereoruber 60	" "
S. cinnamomeus 85	" "
S. coelicolor 37	" "
S. cremeus 62	" "
S. cyaneofuscatus 63	" "
S. diastaticus 23	" "

Table I. (Continued)

S. endus 99	Tiunova et al. (1976b)
S. flavovireus 16,17,18,19	"
S. flavus 5	"
S. leoridus 77	"
S. levoris 8,9	"
S. longisporus-flavus 96	"
S. odorifer 10	"
S. olivoreticuli 89	"
S. olivoverticillatus 50	"
S. platensis 88	"
S. rimosus 31	"
S. rochei 94	"
S. roseolus 80	"
S. rubiginosohelvolus 98	"
S. rutgersensis 14,15	"
S. scabies 11	"
S. spiroverticillatus 82	"
S. toxytricini 83	"
S. variabilis 84	"
S. satsumaensis	Sietsma (1971)
S. cinerosus 3a	Shklyar and Apanasenko (1976)
S. venezuelae RA	Benitez (1976)
Pseudomonas hydrophila 7810	Clarke and Tracey (1956)
P. ichthyosmia 8049	"
P. pyocyaneae RES 2650	"
P. chitinovorans	Veldkamp (1955)
P. aurantiaca 518c	Tiunova et al. (1973b)
Bacillus circulans WL-12	Tanaka et al. (1970)
Bacillus sp. R-4	Tominaga (1976b)
B. thuringiensis var, thurigiensis	Chigaleichik (1976)
B. thuringiensis var. dendrolimus	Smirnoff et al. (1976)
B. " " tuviensis	Chigaleichik (1976)
B. " " kenyae	"
B. " " alesti	"
B. " " sotto	"
B. " " subtoxicus	"
B. " " morrisoni, 2 strains	"
B. thuringiensis var. tolvorth, 2 strains	"
B. " " caucasicus	"
B. " " thompsoni	"
B. " , 273 strains	"
Clostridium sp.	Timmis et al. (1974)

Table I. (Continued)

C. septicum 284, 285	Clarke and Tracey (1956)
C. welchii 8246	"
C. chitinophilum	Billy (1969)
Achromobacter liquefaciens 301	Chigaleichik *et al.* (1976b)
Pectobacterium phytophthorum 113	Tiunova *et al.* (1973b)
Enterobacter liquefaciens 11 strains	Monreal and Reese (1969) Molise and Drake (1973)
Chromobacterium essayanumm NCTC 4618	Clarke and Tracey (1956)
C. indicum 2847	"
C. prodigiosum 3804, 4612	"
C. violaceum 8683	"
Klebsiella aerogenes RES 1912,8197, 418,M20,243	"
K. cloacae, 5920,6027,8155,8168	"
K. ozaenae 5051,5053	"
K. pneumoniae 5054,5055	"
K. rhinoscleromatis 5046,7799	"
Vibrio sp.	" Dastidar (1968)
Vibrio cholerae subgroup I 6560,7252	Clarke and Tracey (1956)
V. el tor 30	"
Aeromonas liquefaciens	Chigaleichik *et al.* (1976a)
Alteromonas marinopraesens, 14 strains	Baumann *et al.* (1972)
Arthrobacter sp.	Morrisey *et al.* (1976)
Cytophaga sp.	Peterson *et al.* (1965), Hocking and Cook (1972), Bacon *et al.* (1965), Veldkamp (1955)
Cytophaga johnsonii, 25 strains	Sundarraj and Bhat (1972), Stanier (1947)
Erwinia sp. M23	Clarke and Tracey (1956)
Sorangium sp. 3C	Peterson *et al.* (1966), Gillispie and Cook (1965)
Beneckea sp.	Cook and Lofton (1973)
Cellvibrio fulvus QM B18	Monreal and Reese (1969)
Bacterium sp. QMB1589	"
Irregular coliformes, 6 strains	Clarke and Tracey (1956)

is also a property widely distributed among fungi as can be seen in Table 2. Fungi seem to secrete chitinase as a true extracellular enzyme and/or release chitinase during their autolysis, both in presence or absence of chitin in the medium (Tiunova et al., 1973b; Monreal and Reese, 1968; Cohen, 1974; Tracey, 1955b; Lahoz et al., 1976; Reyes et al., 1977; Tayama, 1967; Claus, 1961; Musilkova and Tencl, 1970). It is thought that fungi may synthesize chitinases as components of the physiological systems linked with their growth cycle (Iten and Matile, 1970; Vessey and Pegg, 1973; Ko and Lockwood, 1970; Cohen, 1974).

Since the first report of chitinase synthesis in plants by Grassman et al., (1934), who identified chitinase in sweet almond emulsion, the chitinase production characteristic has been sought to a small extent in higher plants. However, chitinases have been identified in seeds, leaves, stems and roots of some woody and herbaceous plants. The plant chitinases seem to be located in the vascular tissue of the plants that can produce them (Wargo, 1975; Pegg, 1976; Abeles et al., 1971; Amagase et al., 1972; Powning and Yrzykievicz, 1965; Zechmeister and Toth, 1939). Table 3 shows some examples of higher plants reported in the literature as chitinase producers.

Chitinase is synthesized by a wide range of animals, including both vertebrates and invertebrates. Chitinases are secreted by the pancreas and the gastric mucosa, and identified in blood, lymph and lymphomyeloid tissues of insectivorous fishes, amphibians and reptiles, as well as insectivorous birds and mammals (Jeauniaux, 1961; Lundblad et al., 1974). Regarding marine vertebrates, Barmington (1957), and

TABLE 2. Distribution of Chitinase Production Among Fungi.

Beauveria bassiana	Claus (1961), Leopold and Samsinakova (1970), Samsinakova and Misikova (1973)
Paecilomyces favinosus	"
P. variottii	"
P. heliothis	"
Cephalosporium coecorum	"
Metarrhizium anisophiae	"
Aspergillus parasiticus	"
Aspergillus niger	Lahoz *et al.* (1976)
Aspergillus fumigatus QM45	Monreal and Reese (1968), Kojima and Koase (1965)
A. oryzae	Otakara (1962), Tiunova (1973b)
A. melleus	Tiunova (1973b)
A. ochraceus	"
A. terreus 30,48,Thorm	"
A. ustus	"
Aspergillus sp. 2 strains	"
Penicillium oxalicum	Lahoz *et al.* (1976)
P. javanicum	Jackson and Gay (1976)
P. lilacinum	Monreal and Reese (1968)
P. chrysogenum	Tiunova (1973b)
P. purpurogenum	Musilkova and Tenci (1970)
Neurospora crassa	Reyes *et al.* (1977), Lahoz *et al.* (1976),
Chytriomyces hyalinus	Reisert and Fuller (1962), Reisert (1972)
Myriococcum albomyces	Okasaki and Irzuka (1971)
Polystictus versicolor	Lahoz *et al.* (1976)
Nectia galligena	"
Trichoderma viride	De Viries (1973), Monreal and Reese (1968), Toyama (1967)
Myrothecium verrucaria QMB1259	Monreal and Reese (1968), Tiunova (1973b)
Mucor subtilissimum QM 460	Monreal and Reese (1968)
Conidiobolus villosus QM929	"
Penicillium decumbens	Tiunova (1973b)
Trichoderma sp. 1,14	"
Trichotecium roseum 41	"
Verticillium alboatrum	Vessey and Pegg (1973)
Aphanomyces astaci	Unestam (1966)
Helminthosporium victoriae	Ko and Lockwood (1970)

Table 2. (Continued)

Glomerella cingulata	Ko and Lockwood (1970)
Fusarium solani	"
Cordyceps sp.	Smirnoff (1977a)
Coprinus lagopus	Iten and Matile (1970)
Coprinus comatus	Tracey (1955)
Phallus impudicus	"
Fistulina hepatica	"
Bovista plumbea	"
Lycoperdon depressum	"
L. giganteum	"
L. pyriforme	"
Boletus edulis	Chmeilnicka *et al.* (1970)
Agaricus bisporus	"
Suillus bovinus	"
Leccinum scabrum	"
Xerocomus badius	"
Cantharellus cibarius	"
Paxillus involutus	"
Hydnum imbricatum	"
Gyromitra esculenta	"

TABLE 3. Distribution of Chitinase Production Among Higher Plants.

Plant	Reference
Acer saccharum (maple)	Wargo (1975)
Quercus rubra (red oak)	"
Q. velutina (black oak)	"
Q. alba (white oak)	"
Lycopersicum esculentum (tomato)	Pegg (1976), Pegg and Vessey (1973)
Phaseolus vulgaris	Abeles *et al.* (1971), Bahl and Agrawal (1969)
Humulus lupulus (hop)	Abeles *et al.* (1971)
Turnip	Bernier *et al.* (1974)
Papaya	Howard and Glazer (1967)
Almond	Zechmeister *et al.* (1938)
Wheat	Powning and Trzykiewicz (1964)
Bean	" "
Cabbage	" "
Warath, *Telepea speciosissima*	" "
Carrot	" "
Snapdragon	" "
Beetroot	" "
Pea	" "
Onion	" "
Pumpkin	" "
Lettuce	" "
Grass, *Agrostis tenuis*	" "
Nepenthes spp.	Chandler (1976)
	Amagase *et al.* (1972)
Drosera whittakeri	"
Drosera binata	"

TABLE 4. Distribution of Chitinase Production Among Animals.

Animal	Reference
Mammals:	
Perodicticus potto	Dandrifosse (1975)
Bos taunus (adult and calf)	Lundblad *et al.* (1974)
Ovis sp. (sheep)	"
Capra sp. (goat)	"
Suis sp. (pig)	"
Rattus norvegicus	Frankignoul and Jeauniaux (1965)
Mus musculus	"
Cricetus frumentarius	"
Cavia porcellus	"
Erinaceus europaeus	"
Rhinolophus ferrum-equinum	Jeauniaux (1961)
Fish:	
Carassius auratus	Jeauniaux (1961)
Scylliorhinus canicula	Sedallian (1968), Fange *et al.* (1976), Alliot and Bocquet (1967)
Raja radiata	Fange *et al.* (1976)
Lateolabrax japonicum	Okutami (1964)
Seriola quinqueradiata	"
Hippoglossoides dubius	"
Stichaeus grigorjewi	"
Gadus macrocephalus	"
Mustelus manazo	"
Chimaera monstrosa	Fange *et al.* (1976)
Etmopterus spinax	"
Melanogramus aeglefinus	"
Gadus morhua	"
Pollachius pollachius	"
Lophins piscatorius	"
Anarhichas lupus	"
Clupea harengus	"
Glyptocephalus cynoglossus	"
Gasterosteus aculeatus	Jeauniaux (1961)
Birds:	
Gallus gallus	Berger and Weiser (1957)
Passer domesticus	Jeauniaux (1961)
Turdus merula	"

Table 4. (Continued)

Amphibia:	
Rana temporaria temporaria	Dandrifosse (1975)
Reptiles:	
Lacerta viridis	Jeauniaux (1961), Micha (1974), Dandrifosse and Schoffeniels (1966)
Clemmys cappica	Jeauniaux (1961)
Emys orbicularia	Micha *et al.* (1974)
Coelenterata:	
Metridium semile fimbriatum	Elyakova (1972)
Vermes:	
Polichaeta:	
Chaetopterus variopedatus	Elyakova (1972)
Neoamphitrite figulus	"
Serpula vermicularis	"
Nerlis cyclurus	"
Sipunculidea:	
Physcosoma japonica	Elyakova (1972)
Brachiopoda:	
Coptothiris grayi	Elyakova (1972)
Echinodermata:	
Patiria pectinifera	Elyakova (1972)
Distolasterias nipon	"
Lysastrosoma anthosticta	"
Cucumaria fraudatrix	"
Strongylocentrotus interdius	"
Echinarachnius parma	"
Aschelminthes:	
Ascaris serum	Lestan (1968), Justin (1969), Ward (1972)
A. diaperinus	Saxena (1972)
Moniliformis dubius	Edmonds (1966)

Table 4. (Continued)

Octolasium cyaneum	Tracey (1951)
O. lacteum	Devigne and Jeauniaux (1961)
Lumbricus terrestris	"
Allolobophora caliginosa	"
A. chorotica	"
A. icterica	"
A. conga	"
Dendroboena subrubicunda	"
Eisenia fetida	"
E. rosea	"
Lumbricus rubellus	"
Mollusca:	
Gastropoda:	
Philaleopoldvillensis sp.	Van Coillie *et al.* (1973)
Aemaea pallida	Elyakova (1972)
Littorina manschurica	"
L. brevicula	"
L. squalida	"
Tectonatica janthostoma	"
Umbonium suturale	"
Helix poliemphala	Kimura *et al.* (1965)
H. pomatia	Lundblad *et al.* (1976)
Achatina fulica	Okutani (1966)
Bivalvia:	
Glycymeris albolineatus	Elyakova (1972)
Mactra sulcataria	"
Spisula sachalinensis	"
Crenomytilus grayanus	"
Modiolus difficilis	"
Patinopecten yessoensis	"
Chlamys seviftii	"
Tellina lutea	"
Pitaria pacifica	"
Cephalopoda:	
Ommastrephes sloanipacificus	Okutani and Kimata (1964)
Polypus dofleine	"
Loricata:	
Ischnochiton sp.	Elyakova (1972)

Table 4. (Continued)

Arthropoda:	
Insects:	
Cyphomyrmex lattine	Martin (1973)
Apterostigma sp.	"
Sericomyrmex sp.	"
Atta sp.	"
Ectatomma ruidum	Martin (1976)
Eciton burchelli	"
Aphaenogaster treatae	"
Dolichoderus taschenbergi	"
Formica ulkei	"
F. dakotensis	"
F. pallidefulva	"
Periplaneta americana	Waterhouse *et al.* (1961), Bernier (1974)
Bombix mori	Beaulaton (1969)
Mauduca sexta	Bade (1974)
Anthereae permyi	Beaulaton (1969)
Crustacea:	
Hapalogaster dentata	Elyakova (1972)
Pachycheles stevensi	"
Pagurus ochotensis	"
Cancer pigmaeus	"
	Jeauniaux (1965)
Hemigrapsus sanguineus	Elyakova (1972)
Maja sp.	Jeauniaux (1965)
Diplopoda:	
Orthoporus ornatus	Nunez (1976)
Ascaroid:	
Aleuroglyphus oratus	Akimov (1976)
Caloglyphus berlesei	"
C. absoloni	"
C. molitor	"
Tunicata:	
Halocynthia aurantium	Elyakova (1972)
H. roretzi	"
H. styela clava	"

Table 4. (Continued)

Protozoa:

Hartmanella glebae	Tracey (1955)
Hartmanella sp.	"
Schizopyrenus erythaenusa	"

Goodrich and Morita (1977b) have pointed out that there is no substantial evidence for these groups of animals producing chitinase. Goodrich and Morita (1977b) have found a direct correlation between the chitinoclastic bacteria population and chitinase activity in fish stomach contents. Moreover, they were able to show that a fish (*Europhrys bison*) deprived of its microbial intestinal flora possesses no chitinase activity. On the other hand, Okutani (1966) has indicated the presence of both "bacterial" and "non-bacterial" (i.e., fish) chitinases in the digestive tract of the Japanese seabass *Lateolabrax japonicus*. Lundblad *et al.* (1974), Jeauniaux (1966) and Fange *et al.*, (1976) support the existence of non-bacterial chitinases produced by animals, for they were able to demonstrate these hydrolytic enzymes in gland tissues, serum, and carefully washed gastric and intestinal mucosa of several animals where bacterial presence was eliminated and, therefore, bacterial origin of chitinases. Chitinases have not been found in omnivorous mammals such as cat and man (Jeauniaux, 1961; Lundblad *et al.*, 1974). Chitinase activity of a significant level has been detected in different tissues of numerous invertebrate groups as shown in Table 4. Chitinase activity has been detected in molting fluid and in extracts of molting or molted cuticle of arthropods (Devigne and Jeauniaux, 1961; Bade, 1974; Kimura, 1973; Waterhouse and McKeller, 1961). Chitinase activity has been observed in the epidermis of nematodes during the hatching process (Justin, 1969; Rogers, 1958; Edmonds, 1966), and in different glandular tissues of the digestive systems of many coelenterates, nematodes, polychaetes and oligochaetes, mollusca, echinodermata, tunicata, arthropods and brachiopoda

(Jeauniaux, 1966; Elyakova, 1972).

The bacterial chitinases, like many hydrolytic enzymes, are performing a catabolic function and, therefore, chitinases furnish bacteria the capacity for using chitin as a carbon and nitrogen source (Okafor, 1966). Consequently, chitinolytic bacteria will have not only the ease of colonizing and thriving in environments with chitin as an exclusive or dominant carbon source, but might also be equipped with an accessorial weapon to limit or eliminate chitin-containing organisms such as fungi and arthropods. Chitinoclastic bacteria have been shown to be able to hydrolyze fungal cell walls, insect cuticles and peritrophic membranes (Lockwood and Lingappa, 1963; Tsujisaka _et al._, 1973; Laborda _et al._, 1974; Morrisey _et al._, 1976; Jackson and Gay, 1976; Moore _et al._, 1975; Carter, 1957; Smirnoff, 1974a, 1974b; Smirnoff and Valero, 1972; Kalucy and Daniel, 1972). It must be emphasized here that the lytic activity of bacterial chitinases, at least in natural conditions, becomes valuable when it is concerned with the activities of other hydrolytic enzymes, toxins and antibiotics (heterolysis) often produced as well by chitinoclastic bacteria (Lloyd _et al._, 1965; Laborda _et al._, 1974; Tominaga and Tsujisaka, 1976a, 1976b). Chitinoclastic bacteria have been found in enormous quantities in the digestive tracts of all detritivorous, insectivorous and fungivorous animals, and in the tentacles of insectivorous plants (Okutani, 1966; Goodrich and Morita, 1977b; Chandler and Anderson, 1976). These microorganisms, owing to their ability to synthesize chitinase and other hydrolytic enzymes, may

be supplying their hosts with metabolizable intermediates. Insectivorous plants, for instance, can grow in a barren region due to the fact that their chitinolytic flora, possessing cooperative chitinolytic and proteolytic enzyme systems, can digest intact insects in a strongly acid medium. This prevents putrefaction and supplies organic carbon, nitrogen and phosphates directly to the plants (Amagase et al., 1972). It appears that the main role of the chitinoclastic bacteria is their association with the turnover of the universally distributed chitin, particularly in the marine environment considering its enormous invertebrate population.

Several attempts have been made to determine rates of chitin degradation in nature, especially in the sea. Seki (1965) reported that 10^{10} chitinoclastic bacteria could decompose 30 mg of chitin per day at 25°C. Chau (1970) reported 80 to 130 ug of chitin per hour were decomposed by chitinoclasts at 22°C. Liston et al. (1965) showed the rate of chitin decomposition in Puget Sound sediments as 18.8 mg per day, while Hood and Meyers (1973) demonstrated a rate of 87 mg chitin per day per total chitin in "in situ" studies of a salt-marsh environment. Seki (1965) has calculated on the basis of his findings that the rate of total decomposition of chitin in the ocean is 140 days at 15°c, 370 days at 5°c, and 500 days below 5°c. These data indicate that in marine waters at 5°c and below there should be a net accumulation of chitin, since the amount produced within a year would not be degraded within the following year, assuming a constant amount was produced annually (Goodrich and Morita, 1977a). However, Goodrich and Morita (1977a) were unable to

demonstrate chitinase activity in a water column and sediments, indicating that a significant portion of chitin decomposition may be carried out in the stomachs of fish and digestive tracts of invertebrates living in sediments. Goodrich and Morita (1977a), assuming favorable conditions for growth and metabolism of the chitinoclastic bacteria and an abundant supply of chitin, have estimated that with a population of 1 x 10^5 fish, a chitinase activity rate of 300 g N-acetyl glucosamine produced/g dry weight/h , and an average dry weight stomach content of 50 g., the species Enophrys bison would be responsible for the production of about 14 metric tons of NAG/year or the decomposition of about 16 metric tons of chitin. As a consequence, the recycling of organic matter, in this case chitin, by bacterial flora with degradative capabilities (chitinolytic action) is another example of the ecological role microorganisms are playing in our environment.

It seems that fungi, able to secrete exocellular chitinases, are not contributing significantly to the chitin turnover in nature. Intracellular fungal chitinases may be involved in the regulation of cell wall growth. Cohen (1974), Middlebrok and Preston (1952) and Ortega et al. (1974), have reported some evidences of a role for chitinase in growth regulation of sporangiophores of the fungus Phycomyces elongates. Although the cause of lysis of fungal mycelia in soil is a controversial question, there is sufficient support for both heterolytic and autolytic mechanisms. The autolysis hypothesis is strongly supported by Ko and Lockwood (1970), and Lloyd et al. (1965). Iten and Matile (1970), and Matile and Wiemken (1967) consider

chitinase as a member of a special group of lysosomal enzymes with no conspicuous function since they are synthesized shortly before autolysis begins in fungal gills. It is not clear yet whether the fungal cells actively secrete chitinase into the walls or whether passive release occurs from cells whose metabolic activity has ceased. However, in some fungi, chitinases seem to mediate in the efficient propagation of spores of these fungi since they are released from fruiting bodies by autolysis (Vessey and Pegg, 1973; Iten and Matile, 1970), and in the dissolution of the septal wall and nuclear migration during mating of two compatible monokaryons (Wessels and Marchant, 1974).

The presence and role of chitinase in higher plants has remained unexplained or conjectural since chitin has not been reported as a constituent of higher plants and chitinase is a hydrolytic enzyme. Hydrolytic enzymes such as RNase, proteases or glucanase in plants represent essentially degrading or catabolic enzymes concerned with solubilizing cellular components prior to translocation, for example, from the leaf into other parts of the plant. However, absence of chitin in plants does not rule out the existence of some polymer of NAG in plant tissue difficult to localize. Recently aminopolysaccharides have been found in higher plants (Gladyshev, 1962; Pusztai, 1964). If the presence of chitinase is common in plants and occurs simultaneously with aminopolysaccharides, for instance in seeds, this raises the possibility of some metabolic connection between the two, although the relationship between the enzyme and its possible substrates is as yet unknown (Powning and Yrzykiewicz, 1965). A

second possibility for presence of chitinase in higher plants is that this enzyme has an antibiotic and protective role in plants. That is, it does not attack endogenous carbohydrates but rather protects plants from fungal pathogens by digesting, along with other hydrolytic enzymes, the invading fungal cell walls. Many accounts of the in vivo intracellular digestion of fungi occur in the literature (Hartley, 1950; Burges, 1939, Blackhurst and Wood, 1963, Dixon and Pegg, 1969; Scheffer and Walker, 1954; Sinha and Wood, 1967; Taylor and Frentje, 1968 and Wilhelm and Taylor, 1965). Moreover, Pegg and Vessey (1973) have identified increased chitinase activity in xylem sap and tissue extracts of infected tomato cultivars. Abeles et al. (1971) have shown that ethylene, which is produced by the plant as a defense against infection by bacteria, viruses and fungi, increases chitinase production in Phaseolus vulgaris in vivo and in vitro.

Among animals, chitinases, those from arthropods have been clearly demonstrated to be associated with ecdysis processes, and those from nematodes with hatching events. One of the characteristics of insects and other arthropods is that they are enclosed within a relatively inextensible exoskeleton which must be cast at intervals to permit growth. To assist in shedding, and presumably to conserve material, the old cuticle prior to discard is thinned by a molting fluid which has long been assumed to possess chitinolytic and proteolytic activities (Bade, 1974; Kimura, 1973; Waterhouse and Mckellar, 1961). Chitinase may also have a more general function in the body of insects and arthropods than digestion of the cuticle

at molting, since its activity has been demonstrated during the intermoult. For instance, it is possible that mechanisms which result in the production of large amounts of chitin at molting are diverted with the participation of chitinase to other purposes during the intermoult, a period when regular chitin synthesis is known to be involved only in the production of the perithrophic membrane (Waterhouse et al, 1961). The permanent presence of chitinase in adult arthropods suggests that chitinase may be playing a definite function (Waterhouse and Mckellar, 1961). The chitinase present in alimentary tracts of arthropods may be performing a true digestive function since remains of arthropods, especially cuticles, have been found there. Specimens eating their own exuvia have also been reported, and lichens and fungi are a food base for many other arthropods (Wooten and Crowford, 1975; Friedman and Galun, 1974; Martin <u>et al.</u>, 1973; Waterhouse and McKellar, 1961). Even in the absence of exogenous chitin in the food, their peritrophic membrane is always present. Layers of this membrane are produced at intervals and surround the food in the midgut. Possibly these layers are being continuously attacked by chitinase from the moment they are formed so that they are excreted (Waterhouse and Mckellar, 1961).

Because chitinase is synthesized and secreted by the pancreas and gastric mucose of insectivorus or detrivorus fish, amphibians and reptiles as well as by the gastric mucose of some insectivorus birds and mammals chitinase must be accomplishing an essentially digestive function in these animals. Jeauniaux (1963) suggests that organisms adapted to a more specialized, noninsectivorus diet

have lost the chitinase biosynthetic systems which their evolutionary forebearers had. Thus, insectivorous habits and chitinase production are considered to be primitive conditions in vertebrates, especially in mammals. It is noteworthy that the site of chitinase secretion seems to show an evolution towards a greater specialization of tissues; for instance, the secretion of chitinase is a property of the whole epithelium and of pancreatic islets in the goldfish, while it is restricted to the gastric mucosa and the pancreas in Lacerta and to the gastric mucose alone in bats (Jeauniaux, 1961). The presence of chitinase activity in blood of some vertebrates may be an indication that not only lyzozyme but also chitinase has a defense function in these animals. Chitinase in the blood probably offers protection against invading chitin-containing organisms such as parasitic crustaceans or fungi (Fange et al, 1976; Glynn, 1969).

Chitinases and chitinolytic microorganisms present a potential for practical applications. In basic research, chitinases have been instrumental in the study of various physiological phenomena. They have been essential in the realization that chitin is the material responsible for the rigidity and resistance of fungal cell wall, arthropodal cuticle, insectal peritrophic membrane, tenia and tracheal epicuticle (Beaulaton, 1969). Chitinases have helped to establish the correlation between cellular morphogenesis and fungal cell wall structure (Wang and Bartnicki-Garcia, 1968 and Benitez et al., 1976). Chitinases have attained importance in the elucidation of the biochemical basis of fungal growth, dimorphism and sexual

differentiation (Christias and Baker, 1967; Wang and Bartnicki-Garcia, 1968) as well as the understanding of host-parasite relationship in plants susceptible to pathogenic fungi (Tanaka _et al._, 1970). Chitinases have been used to study the localization, distribution and arrangement of the chitin-polysaccaride and chitin-protein complexes within the fungal cell wall and arthropodal cuticle, thus providing a tool for evolutionary and comparative studies (Carbonell _et al._, 1970; Jansons and Mickerson, 1970; Hunsley and Burnet, 1970 and Bartnicki-Garcia and Reyes, 1964). Chitinases have been used to prepare fungal protoplasts. Protoplasts are a novel system to study many problems associated with cell structure, growth, nutrition, biosynthesis and antibiotic action (Villanueva and Garcia-Acha, 1971). Studies in fungal protoplasts have provided a great deal of information useful to the control of plant pathogenic fungi (Moore and Peberdy, 1976; Laborda _et al._, 1974; Moore, 1975; Iten and Matile, 1970; De Vries and Wessels, 1973; Sietsma, 1971; Peberdy and Issac, 1976; Chattaway _et al._, 1976; Binding and Weber, 1974). Chitinases have been fundamental in understanding the occurrence and frequency as well as molecular events of autolysis and heterolysis phenomena in fungi (Vessey and Pegg, 1973). They have been essential in the elucidation of biochemical events operating in arthropodal molting and hatching in nematodes (Edmonds, 1966), and in the establishment of their distribution, origin, biological role and relationship with other hydrolytic enzymes.

From a practical point of view, potential applications of chitinases and chitinolytic microorganisms as well are extensive: (1) in taxonomic and evolutionary purposes, (2) enzyme release,

(3) analytic quantitation of chitin, (4) enhancement of antigenicity or specificity of fungal antigens, (5) preparation of fungal vaccines, (6) biological control of plant pathogenic insects and fungi (7) nutritional enhancement or production of single-cell protein, (8) favorable modifications of native chitin for its industrial applications, (9) production of industrially important substances in mixed cultures.

The chitinase biosynthesis characteristic promises to be an important trait useful in taxonomic and evolutionary studies. Among bacteria, chitinase seems to be synthesized by special groups of species. For instance, the genera Serratia and Streptomyces have been shown to have the highest numbers of species with chitinase activity (Baumann et al., 1972 and Molies and Drake, 1973).

Chitinases have been suggested for the efficient release of industrially important wall-bound enzymes such as trehalase, invertase, alpha amylase, glucanases, proteases, etc. Chang and Trevithick (1972) have demonstrated that chitinase released about 80% of the invertase and 60% of the trehalase from wall preparations of Neurospora crassa. They had failed to dissociate these enzymes from the walls with chemical reagents believed to be acceptable for these purposes.

Chitinases, like other enzymes used in enzymatic methods of analysis, can serve as a sensitive tool for specific analytic determination of chitin or at least as a useful check on chemical methods. There is the advantage that the end product is only NAG and any doubt about the acetylated form of the original material

is removed (Tracey, 1955). Chitin is normally determined by total hydrolysis followed by estimation of glucosamine and sometimes of acetic acid. This method, however, is not specific as the enzymatic method even if it is preceded by extensive purification of the material, a process that often includes alkali treatment with risk of deacetylation. And yet, glucosamine is broken down to a variable extent during acid hydrolysis and in crude preparations it may arise from substances other than chitin (Skujins et al., 1965; Tracey, 1955; Kanetsuna et al., 1969).

Chitinases may be an excellent means to enhance the antigenicity or at least the specificity of the fungal antigens that have application in diagnosis of mycoses. Odds et al. (1974) have demonstrated that treatment of Histoplasma capsulatum and Blastomyces dermatitidis cell wall preparations with chitinase appeared to increase the specificity against pooled crossreacting antisera. Moreover, chitinases appear to be important in the improvement or preparation of fungal vaccines. It is widely recognized that attempts at developing fungal vaccines have been so far practically unsuccessful. However, Levine et al. (1960) and Collins and Pappagianis (1973) have been able to prepare a killed Coccidiodes inmitis spherules vaccine since the treatment of the spherules with chitinases seems to increase the antigenic mass involved in stimulation of protective antibody synthesis.

Chitinases, at the present stage, have proved tremendously successful in biological control of some insect and fungal pests. Smirnoff (1971) studied the effect of various concentrations of chitinase added to preparations of Bacillus thuringiensis on larvae

of Choristoneura fumiferana which were inoculated orally by placing a drop of the various suspensions to be tested on the artificial diet. Bacillus thuringiensis is known to provoke an enterotoxinosis-septicemia in different species of Lepidoptera. Smirnoff found that larval mortality by commercial B. thuringiensis was advanced 4 to 6 days for the various temperatures tested when chitinase was added. While a pure culture preparation of the bacillus provoke about 50 to 60% mortality, in the treatments using combined bacilli and chitinase, all the larvae succumbed to infection. Smirnoff and Valero (1972), studying the metabolic disturbances in C. fumiferana infected by B. thuringiensis alone or with chitinase added, observed that the differences between healthy and infected larvae or pupae were generally more obvious when the insects were infected by the combination B. thuringiensis and chitinase. Smirnoff et al. (1973a) showed that larva mortality was higher on a plot treated by the chitinase-supplemented sample; foliage protection was better as well. He sprayed two 100-acre plots of balsam fir heavily infested with spruce budworm with a water-based formulation of a liquid concentrate containing 25% commercial B. thuringiensis and 25% non-volatile additive. The spray formulation used in 1 plot contained 5.5 g chitinase added to 200 gal of material. Smirnoff et al. (1973b) extended the field trial to a 10,000 acre stand of mature balsam fir which had a budworm population of about 21 larvae/18 in branch-tip. The B. thuringiensis plus chitinase formulation produced a larva mortality between 84% and 93% compared with 39% to 53% in the control plot, and the foliage protection was 47%.

In a new trial, using 1,000 acres of balsam fir forest severely infested by C. fumiferana, the B. thuringiensis-chitinase formulation sprayed by means of an Avenger aircraft, caused a larva mortality of 88% and a foliage protection level of 70% (Smirnoff et al., 1973c). Smirnoff (1974a, 1974b, 1976, 1977) developed a new, more compact formulation making B. thuringiensis plus chitinase treatments more economical and competitive with chemical insecticides. This new formulation, sprayed at 0.5 gal/acre, permitted dispersion of 7 BIU and 5.5 mg/acre chitinase allowing high mortality (about 90%) and good foliage protection. Morris (1976), after two years of experiments in biological control of spruce budworm, reported the following correlation of potentiality of different formulations: Thuricide plus chitinase $>$ Dipel plus chitinase $>$ Dipel $>$ chitinase alone $>$ Thuricide alone. Thuricide and Dipel are commercial preparations of B. thuringiensis: Thuricide contains serotype 5, var. galleriae while Dipel consists of serotype 3, var. alesti. Smirnoff (1974c) has also observed that chitinase increases the efficiency of B. thuringiensis in controlling the insect, Lambidina fiscellaria (Lepidoptera, Geometridae).

In addition, chitinolytic organisms have been found to be potent pathogens for insects and fungi. Podwaite and Cosenza (1976) reported a strain of Serratia marcescens 922A to be highly pathogenic for early instar larvae from the gypsy moth, Lymantria dispar, which is a potent defoliator presenting a definite threat to hardwood forest stands and forested recreational areas. Kalucy and Daniel (1972) have shown that Aeromonas punctata can affect larvae of

Anopheles annulipes. Chitinase, as well as a phospholipase which is also detected, allows the bacterium to penetrate the homocoel through the cuticle or intestinal epithelium of weakened larvae. The ability to produce chitinase and phospholipase appears, then, to be advantageous to bacteria if circumstances enable them to multiply in the gut and produce sufficient quantities of these enzymes to penetrate the peritrophic membrane and epithelial cells. Koths and Gunner (1967) utilized a predaceous Arthrobacter sp. isolated from the carnation rhizosphere for the protection of carnations against Fusarium stem rot. This bacterium actively lyses living Fusarium mycelium in liquid medium due to its capacity to secrete chitinase and other hydrolytic enzymes. Hocking and Cook (1972) demonstrated that mixobacteria of the group Cytophaga characterized by producing chitinase when introduced into peats before or after sowing seeds of 4 species of conifer colonized the rhizosphere of the developing seedling without affecting seedling development. But, in subsequent inoculation with pathogenic fungi, there was significantly less mortality, chlorosis and stunting than in a control without myxobacteria. Many seedlings for reforestation are now being produced in small discrete containers and, on these seedlings, loses and stunting due to root pathogens occur widely and unpredictably (Hocking and Cook, 1972).

Chitin amendment of soils has been shown to decrease saprophytic growth and disease severity of Fusarium wilt of peas (Guy and Baker, 1977; Khalifa, 1965), Fusarium wilt of radish (Mitchell and Alexander, 1961), bean root rot (Maurer and Baker, 1964, 1965; Snyder, 1963, Sneh et al., 1971), common scab of potato tubers produced by

Streptomyces scabies (Vruggink, 1970), wheat root foot rot (Buttler, 1961) as well as inhibiting infection by plant parasitic nematodes Pratylenchus penetrans, Tylenchorhynchus dubies, and Haplolaimus sp. (Miller, 1976). Chitin amendment results seem to be dependent upon environmental modification caused by enhanced growth and activities of chitinolytic organisms whose chitinases and other hydrolytic enzymes will eliminate any fungi, insects or nematodes present in the amended soil (Ko and Lockwood, 1970; Miller and Sands, 1977). Moore et al. (1975) have shown that several chitinolytic organisms lyse both autoclaved and actively growing intact Trichophyton rubrum. They speculate that chitinolytic organisms may be essential in lysing pathogenic dermatophytes in soil and, thereapeutically, chitinases may be useful for topical treatment of dermatophytosis. These findings raise the possibility of using chitinase or chitinase-producing organisms to destroy or control dermatophytes and systemic mycosis agents in endemic regions, or may even be useful for protecting stored grains from fungal spoilage.

Chitinases and chitinolytic organisms may be fundamental in the direct production of single-cell protein using chitin as raw material, taking advantage of the fact that enormous amounts of chitin are being accumulated as by-products from seafood operations (Lovell et al., 1968). Moreover, chitinases or chitinolytic organisms could be used to break down fungal cell walls of fungi grown as a source of single-cell protein. It is recognized that one of the constraints in the production of fungal single-cell protein is the difficulty of breaking down cell walls to increase their

digestability and nutritional quality. Micropolyspora 434, a chitinase producer, and Myriococcum albomyces have shown a synergistic effect in the lysis of cell walls of Candida utilis, a widely-used organism in single-cell protein production.

Chitinases or chitinolytic organisms may be beneficial in altering native chitin in such a way as to render this polymer into a form better suited for direct industrial applications (Brine and Austin, 1974). Finally, there is the possibility that chitinases, or the organisms producing them, can hydrolyze chitin into suitable carbon, nitrogen and energy sources for the growth of other industrial organisms in either mixed or sequential systems.

Most of the efforts in chitinase research have been devoted to studies of optimum conditions for chitinase activity, and to chitinases properties and purification (Tominaga and Tsujisaka, 1976a, 1976b; Tiunova et al., 1976a; Berger and Reynolds, 1958; Wadstrom, 1971; Skujins et al., 1970; Sundarraj and Bhat, 1972; Otakara, 1962, 1963; Gillispie and Cook, 1964; Reisert, 1972; Tracey, 1955; Reynolds, 1954; Chigaleichik and Pirieva, 1976; Chigaleichik et al., 1976a; and Morrisey et al., 1976). In most of these studies chitinase production was conducted at the known optimum conditions for growth of the organisms employed.

Relatively few reports exist in the literature regarding the best culture conditions for maximum biosynthesis of chitinases by chitinolytic microorganisms. Reynolds (1954) studied the speed of chitin breakdown of the three most active organisms he isolated from soil - two streptomyces and a bacterium - using both submerged

and static incubation. Clarke and Tracey (1956) compared the chitinase production on liquid and on solid media of Klebsiella pneumoniae and K. rhinoscleromatis. They also determined the degree of chitinase production by several bacterial strains in relation to incubation time. Monreal and Reese (1969) reported the adaptative character of chitinases produced by a strain of Serratia marcescens. Morrisey et al. (1976) studied the effect of various carbon sources on the appearance of chitinase in cell-free culture supernatants of Arthrobacter p-35 strain. They also determined the effect incubation time, concentration of chitin in the culture medium, size of chitin powder particles, Tween 80 and the presence of fungal mycelium had on chitinase production by the Arthrobacter p-35 strain. Chigaleichik et al. (1976a) have studied the incubation time, pH, and temperature effects on the production of chitinase by Serratia marcescens BKM 851 using three forms of chitin as substrate: crab shells, demineralized crab shells and highly purified chitin. Chigaleichik and Pirieva (1976) reported results of chitinase production by a culture of Aeromonas liquefaciens using demineralized crab shells as substrate. Chigaleichik(1976) correlated the chitinolytic activity of a strain of Bacillus thuringiensis with the appearance of spores and toxic crystals in the culture fluid. Chigaleichik et al. (1976b) have studied in detail the influence of temperature, aeration, pH and rate of culture medium addition on the biosynthesis of chitinase by a culture of Achromobacter liquefaciens 301 under conditions of batch and continuous cultivation. Tiunova et al. (1976a), testing several culture media, determined the optimum incubation

time for maximum chitinase yield from *Streptomyces kurssanovii* both in a flask and a fermentor, and compared the dynamics of chitinase biosynthesis on native and colloidal chitin.

The data in the few reports mentioned above, concerning the influence of diverse environmental factors on growth and chitinase production of microorganisms capable of synthesizing it, will be compared with those obtained in this study with the isolate *Anthrobacter* sp. BN2.

MATERIALS AND METHODS

Culture Media

Reynolds' Enrichment Medium. (REM)

This culture medium developed by Reynolds (1954) has the following composition: K_2HPO_4, 0.7 g; KH_2PO_4, 0.3 g; $MgSO_4 \cdot 5H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.1 g; $ZnSO_4$, 0.001 g; distilled H_2O, 1 liter; powdered chitin, 2.5 g.

The pH was adjusted to 7.0. The phosphates and the powdered chitin were autoclaved separately in order to avoid precipitate formation. They were brought to a temperature of 45 C and mixed together in the indicated proportions with the mineral salts solution which makes up the rest of the medium.

This medium was used for the enrichment and isolation of chitinolytic organisms from 32 soil samples.

Reynolds' Chitin Agar. (RCHA)

This culture medium has the same composition as REM medium except it contains agar and colloidal chitin rather than powdered chitin.

In addition to being used for the isolation of chitinolytic organisms from 32 out of 64 different soil samples, this medium was also employed for the maintenance of organisms with recognized chitinolytic capacity as well as for the determination of the proportion and degree

of chitinolysis for the highest chitinase-producing cultures isolated in this study.

The stock cultures were transferred monthly, incubated for 72 h. at 25 C and then stored at 4 C.

Morrisey's Chitin-Mineral Salts Medium. (MCHMSM)

This is the medium employed by Morrisey *et al.* (1976) on their studies of chitinase production by *Arthrobacter* sp. It has the following composition:

Solution A:	glucose, 0.1% (w/v)	100 ml
Solution B:	Yeast Extract, 0.02% (w/v)	100 ml
Solution C:	Mineral salts solution	800 ml
Solution D:	Powdered (or colloidal) chitin	10 g

The mineral salts solution was prepared from stock solutions to give the following final concentration per liter of medium:

KH_2PO_4	4 g	K_2HPO_4	3 g
$MgSO_4 \cdot 7H_2O$	0.2 g	H_3BO_3	5.6 mg
$MnCl_2 \cdot 4H_2O$	3.7 mg	$CoCl_2 \cdot 6H_2O$	0.7 mg
$CuSO_4 \cdot 5H_2O$	0.4 mg	$ZnSO_4 \cdot 7H_2O$	0.5 mg
$NaMoO_4 \cdot 2H_2O$	1.5 mg	$FeSO_4$	1.0 mg
$CaCl_2$	10.0 mg	Distilled H_2O	800.0 ml

The final pH of the medium was 6.8.

The phosphates, chitin and glucose solutions were autoclaved separately, cooled to 45-50 C and added to the other solutions (solutions B and C) which were autoclaved together. This avoided precipitate formation.

This medium was used in all the studies carried out for the determination of optimum cultural conditions for chitinase production by the most active chitinase producer isolated.

N-Acetyl-D-Glucosamine Medium (NAGM)

Two versions of this medium were used which differed from each other only in the amount of N-acetyl glucosamine added. The basic composition of the medium is as follows:

N-acetyl glucosamine	5 (or 1) g
Mineral salts solution, (Solution C of MCHMSM medium)	1 l

This medium was used to survey utilization of NAG as unique carbon, nitrogen and energy source as well as the uptake rate of NAG by the different organisms used in the mixed culture experiments.

Semisolid Chitin Medium (SSCHM)

This medium is of the same composition as the MCHMS medium except that it contains chitin powder at the concentration of 20%. It was prepared as follows:

Ten g amounts of chemically purified chitin powder (24 mesh), were dispensed in a series of 250 ml. Erlenmeyer flasks. After being autoclaved at 120 C for 20 min, each flask received 40 ml of a solution having the following composition:

Sol A, glucose 1%	4 ml
Sol B, yeast extract 0.2%	4 ml
Sol C, Mineral salts sol	32 ml

This medium was employed to investigate chitinase production on semisolid conditions by the chitinolytic strain Arthrobacter sp. BN2

grown both in pure and in mixed culture conditions.

Shell-Protein Chitin Medium (SHPCHM)

This medium has the following composition:

Mother shell protein solution, diluted 10 times	1 l
Chitin (colloidal or powder)	5 g

The mother shell-protein solution was prepared as follows: The alkaline solutions which were changed each 24 h (See Deproteinized chitin preparation) period just after cooling the heated KOH soaked crab shells, during deproteinization, were pooled, then neutralized to pH 7.0 and filtered through whatman #1 filter paper to eliminate any non-proteinic insoluble material. About 4 l of shell protein solution were obtained out of 100 g crab shells.

This medium was used to determine if the alkaline protein solution produced in the purification process of chitin from arthropodal shells is useful as an ingredient culture medium for the growth and chitinase production of chitinolytic organisms.

Preparation and Sources of Chitin Substrate

In this study chitin was supplied from the following sources:

(i) Chemically pure chitin powder
(ii) Tanner's chitin powder
(iii) Crab shell chitin
(iv) Crayfish chitin
(v) Colloidal chitin

Chemically pure chitin powder was purchased from Calbiochem Lab. This chitin was used in the preparation of colloidal chitin needed for the enzyme assay. It was also employed as sole

carbon and nitrogen source (as a powder or converted to colloidal chitin) in the medium used for studying the optimum cultural conditions for maximum chitinase production and maximum growth of Arthrobacter sp. BN2.

Tanner's chitin powder was a gift from Dr. A. Smith. It came in two grades, 90% and 45% purity. The 90% pure chitin was employed as the substrate during the screening program for chitinolytic organisms from soil samples. The 45% pure chitin was used preferentially to prepare Reynolds' chitin medium (RCHA), employed essentially as a storage medium. The 45% chitin was also deproteinized to obtain shell-protein solution for preparing chitin medium.

Crab and crayfish chitin were both prepared as demineralized and deproteinized chitin. These chitin substrates were employed to determine their potential as a substrate for chitinase production during bacterial fermentation.

The demineralized powdered chitin was prepared as follows (Benton, 1935): Shells were scrubbed as free as possible of flesh and dirt and then were subjected to cold 1% HCl, which was changed each 24 h for a period of about 7 weeks. The limp leathery shells were washed, cut into pieces of suitable size and shape and finally ground through a 0.5 mm mesh screen Wiley mill.

The deproteinized powdered chitin was prepared as follows: Demineralized shells immediately previous to being cut and ground were washed with distilled water and soaked in 2% KOH for 96 h. During this period they were stirred several times, and at each 24 h interval, brought to a temperature just below boiling, cooled and replaced with fresh 2% KOH. Proteins, other organic materials (except chitin) and

most of the pigment were dissolved out in the alkaline liquid. The material was washed with distilled water free of alkali, extracted 3 times in boiling ethyl alcohol (this step may be omitted), dried, and ground through a 0.5 mm mesh screen Willey mill.

Colloidal chitin was prepared from chemically pure chitin powder (Calbiochem), or 90% Tanner chitin powder as follows (Goodrich, 1976): Five hundred ml concentrated sulfuric acid (specific gravity 1.84) and five hundred ml distilled water were previously cooled to 2 C and then mixed in a 3 l flask. This mixture was cooled to 2 C and stirred continually using a magnetic stirrer (Bellco). The temperature was maintained by placing the flask in ice in a 5 C controlled environment incubator. This 50% H_2SO_4 solution was then added to 50 g of pure chitin powder, and placed in a 3 l flask. This chitin was allowed to dissolve for 18 to 24 h. It was then reprecipitated in 15 liters of distilled water previously cooled to 5 C in a 50 l carboy. The H_2SO_4-chitin mixture was poured into the distilled water through a Buchner funnel, using a glass wool filter. After the colloidal chitin had settled out of solution, most of the liquid was decanted. The remaining solution tion was centrifuged in 250 ml Nalgene centrifuge bottles at 10,400 x g for 15 min at 0 C. The supernatant was poured off and the chitin was pooled. Potassium hydroxide was used to adjust the pH to 7.0 ± 0.5. This solution was poured into 4.8 cm diameter viscose dialysis tubing. The tubes were placed in running tap water for 3 days and then in in running distilled water for 24 h to remove excess ions. The tubes were hung to air dry. When the chitin in the tube had reached the consistency of very thick paste, it was removed and resuspended in

distilled water or in citrate-phosphate buffer, pH 6.0 to a concentration of about 0.1 g/ml. The colloidal chitin was stored in 200 ml dilution bottles after autoclaving at 120 C for 20 min. The colloidal chitin diluted in citrate-phosphate buffer was used as substrate in the chitinase assay. For this test, the colloidal chitin was diluted 20 times with citrate-phosphate buffer to give a final concentration of 5 mg/ml. The colloidal chitin diluted in distilled water was used in the preparation of colloidal chitin media.

Chitinolytic Culture Source and Isolation

Sixty-four garden and forest soil samples collected in and around Corvallis were employed in the screening for chitinolytic organisms. Dilutions of 10^{-4}, 10^{-5}, and 10^{-6} were made with each of the soil samples. Aliquots of 1 ml from each dilution of 32 of the soil samples were used to inoculate 125 ml Erlenmeyer flasks containing 30 ml Reynolds' Enrichment Medium. The flasks were incubated at room temperature on a shaker until turbidity was observed. The flasks were monitored for 2 weeks. From flasks showing turbidity, 1 ml aliquots were taken and transferred to flasks containing fresh Reynolds' enrichment medium and incubated under the same conditions as mentioned previously until turbidity was again observed (usually between 48 h and 5 days). From the second series of flasks showing growth, streaks were made on chitin agar plates. Colonies showing clear chitinolytic zones were preserved on chitin agar slants. Having chitin as a unique carbon, nitrogen and energy source the enrichment medium employed here was expected to allow only the growth of chitin-hydroly ing organisms.

One ml aliquots from each dilution of the remaining 32 soil samples were streaked directly on chitin agar plates. The plates were incubated at room temperature and examined for the appearance of chitinolytic zones around the colonies The plates were checked every 24 h for 2 weeks. The colonies showing chitinolytic zones were streaked on chitin slants.

Chitinase Assay

The enzyme assay was performed in 20 x 100 mm screw-capped test tubes. The following procedure was used (Jeauniaux, 1966). In these tubes were placed 2 ml of diluted colloidal chitin, 1 ml of 0.6 M-citrate- 1.2 M phosphate buffer, pH 6.0, 1 ml of crude enzyme preparation, and 0.1 ml of 0.5% (w/v) merthiolate, added as a preservative. The tubes were incubated on a shaking water bath at 37 C for 0, 1, 2 and 6 hours. After each incubation time, 2 ml of the enzyme preparation were withdrawn and centrifuged at 18,000 x g at 2 C for 10 minutes to remove chitin residues. 0.5 ml aliquot of the supernatant was used in the N- acetyl glucosamine assay. The activity is expressed as μg acetyl glucosamine liberated per hour per mililiter of culture.

N - Acetyl - D - Glucosamine Assay

The Morgan-Elson (1934) method as modified by Reissig et al. (1955) was the assay procedure used here. This method serves for the colorimetric determination of N-acetyl hexosamines. Therefore, the method is not a direct measure of the quantity of chitinase but rather it is a measure of the end-product of the chitinase system.

Reagents used were a Borate buffer (1.12 M boric acid plus 0.56 M

potassium hydroxide, pH 9.1) and a solution of the color reagent, p-dimethylamino benzaldeyde (DMAB). This solution was prepared by adding 10 g. DMAB to 100 ml glacial acetic acid with 12.5% (w/v) concentrated hydrochloric acid and diluted 9 times with glacial acetic acid before the assay.

The assay was performed as follows: 0.1 ml borate buffer was added to tubes containing 0.5 ml sample. This mixture was heated in a boiling water bath for exactly three minutes and cooled immediately in cold running water or an ice bath. Three ml of the diluted DMAB were added at this time. The mixture was mixed and incubated in a water bath at 37 C for exactly 20 minutes, and finally cooled again in running water or an ice bath. The tubes were read at 585 nm in a Bausch and Lomb Spectronic 20 within 20-30 minutes. A standard curve was constructed with solutions of N-acetyl glucosamine (5, 10, 20, 30, 40, and 50 μg NAG/ml of medium.

Temperature Effect Experiment

Temperature optima for growth and chitinase production were determined for the chitinolytic *Arthrobacter sp.* BN2 using a temperature gradient incubator (Scientific Industries Inc., Mineola, New Jersey). The gradient was set to include temperatures from 6 to 45 C. Fifteen ml of sterile colloidal chitin broth (MCCHB) were added to each previously sterilized L-shaped tube. The tubes were placed in the gradient incubator and allowed to equilibrate for approximately 2 hours. Each tube received a 1.5 ml inoculum prepared from a 24 h culture of *Arthrobacter* sp. BN2 which had been grown on enriched nutrient agar

plates (yeast extract, 0.5%, peptone, 1.0%, NaCl, 0.5%, and beef extract, 0.3%) at room temperature. The culture for the inoculum was harvested and resuspended with saline solution to a concentration which would yield about 1×10^6 cells/ml when 1.5 ml of this suspension was added to the 15 ml volume in the L-shaped tube. Growth and chitinase production both were monitored once every 24 h for 5 days. Growth was determined by plate counts using the spread plate technique on chitin agar.

pH Effect Experiment

pH optima for growth and chitinase production were determined for the chitinolytic Arthrobacter sp. BN2 using Morrisey's medium modified by adding phosphates or other buffer stock solutions to obtain a pH value ranging from 3.4 to 8.6.

Sorenson's phosphate buffer stock solutions were employed for a pH range from 6.0 - 8.6. Appropriate amounts of solution A (0.2 M NaH_2PO_4) and solution B (0.2 M NaH_2PO_4) were mixed in 250 ml Erlenmeyer flasks, autoclaved at 120 C for 15 min and diluted 1:1 with Morrisey's medium to a final volume of 30 ml.

Phthalate buffer stock solutions were used to prepare the pH range 3.4 - 5.6. Varying amounts of solutions A (0.2 M potassium acid phthalate) and B (0.2 M sodium hydroxide) were mixed, autoclaved at 120 C for 15 min, and diluted 1:1 with sterile Morrisey's medium to a final volume of 30 ml.

Each flask received a 5 ml inoculum of the Arthrobacter sp. BN2 culture. The inoculum was prepared in the same fashion as for the temperature experiment. All flasks were incubated at room temperature in

a shaker at 180 rpm. Growth, chitinase activity and pH were determined at 24 h intervals for 5 days. Growth was monitored using plate counts by the spread plate technique on chitin agar.

Chitin Concentration Experiment

For this experiment the mother colloidal chitin suspension, diluted in distilled water to a concentration of 0.5 g/ml, was employed. The proper amounts of mother colloidal chitin, sterile distilled water and double concentrated Morrisey's medium to obtain the desired final chitin concentrations in the culture medium are described below.

Mother colloidal chitin (0.1 g/ml)	Sterile distilled water	Doubled conc. Morrisey's Medium	Final conc. in medium (%,w/v)	Final medium volume
0.30 ml	14.70 ml	15.00 ml	0.10	30.00 ml
1.50 ml	12.50 ml	15.00 ml	0.50	30.00 ml
3.00 ml	12.00 ml	15.00 ml	1.00	30.00 ml
6.00 ml	9.00 ml	15.00 ml	2.00	30.00 ml
9.40 ml	5.60 ml	15.00 ml	3.00	30.00 ml

The 250 ml Erlenmeyer flasks containing 30 ml of medium at different chitin concentrations were inoculated with 3.0 ml of the *Arthrobacter* sp. BN2 culture. The inoculum was prepared as described in the temperature experiment. The flasks were incubated at room temperature in a shaker at 180 rpm.

For the experiments involving mixed cultures, the inocula of the symbiotic organisms, *Escherichia coli*, *Micrococcus* sp., *Candida albicans*, and *Aureobasidium pullulans* were prepared from 24-h cultures grown on dextrose yeast extract plates. The cells were harvested with sterile saline solution and diluted to give a final concentration in the medium

of 1×10^5 organisms/ml. The inocula of the symbiotic molds (*Penicillium* sp., *Aspergillus niger*, and *Phanerochaetae chrysosporium*) were prepared from 3 day cultures grown on potato dextrose plates. The growth was harvested with sterile saline solution and brought to a concentration of 1×10^5 spores/ml of medium. The inoculum of *Volvariella volvaceae* was prepared from a 5 day submerged culture on a dextrose-mineral salts medium.

Growth and chitinase activity were monitored at 24 h intervals for 6 days.

Mineral Salts Effect

This experiment was performed to determine the effect different mineral salts in the Morrisey's medium might exert on growth and chitinase production of the chitinolytic organisms being studied.

The three different concentrations of mineral salts in the base medium which were used are these: (i) at the concentrations which they are present in the original Morrisey's medium, (ii) 15 mg/100 ml, and (iii) 0.3 mg/100 ml medium. The base medium had the following composition: chitin, 2.0%; yeast extract, 0.02%; glucose, 0.1%; KH_2PO_4, 0.4%; and K_2HPO_4, 0.3%. Thirty ml of medium was dispensed in 250 ml Erlenmeyer flasks and inoculated as was specified for the above experiments.

The following controls were run in parallel: Base medium without any mineral salt (except phosphates), base medium without yeast extract, base medium without glucose and base medium without glucose and yeast extract.

N-Acetyl Glucosamine Uptake

The NAG medium, which has N-acetyl glucosamine as a sole carbon, nitrogen and energy source, was used to monitor N-acetyl glucosamine uptake by the different organisms employed in the mixed culture experiments. The culture medium was prepared with one of two different NAG concentrations, 0.5% and 0.1% (w/v).

Amounts of 30 ml of sterile NAG medium were dispensed into 250 ml Erlenmeyer flasks and inoculated with the organisms being examined. The inoculum of *Arthrobacter* sp. BN2 was prepared as for the temperature experiment except the bacterial suspension was washed three times with sterile saline solution. The inoculum consisted of 3 ml of culture for a final concentration of 10^6 bacteria/ml.

Inocula of *Streptomyces* cultures were prepared from 48-h cultures grown on enriched nutrient agar plates. The cell material was harvested and washed three times with saline solution and added to the NAG medium for a final concentration of 0.1 mg cell material/ml. This concentration corresponds approximately to 10^7 bacteria/ml medium.

Fungi inocula were obtained from 4-day cultures grown on enriched nutrient agar plates. The spores were harvested and washed 3 times with saline solution. The fungal suspension was added to NAG medium to give a final concentration of 0.1 mg spores/ml.

Protein Determination

Protein determinations were carried out according to Lowry's Method (Lowry *et al.*, 1951). The following reagents were employed:

Reagent A:

2% Na_2CO_3 in 0.10 N NaOH

Reagent B1:

1% $CuSO_4 \cdot 5H_2O$

Reagent B2:

2% Na-K tartrate

Reagent B:

Mixture of equal volumes of reagent B1 and B2

Reagent C:

49.0 ml reagent A plus 1.0 ml reagent B mixed immediately before use.

The assay was performed as follows: A two ml sample from the culture flasks was placed in centrifuge tubes and centrifuged for 15 min at 18,000 x g. Then 0.5 ml aliquots from the supernatants were withdrawn, mixed in test tubes with 5 ml of reagent C, and allowed to stand for 10 min. One half ml of 1.0 N Folin-phenol reagent was added with rapid stirring and the tubes stood 30 min. at room temperature. The tubes were read at 750 nm on a spectronic 20. A standard curve was constructed using bovine serum albumin.

RESULTS

I. Screening Program

To isolate chitinolytic microorganisms from the 64 soil samples collected in the Corvallis area two techniques were employed: the enrichment method and direct plating on chitin agar.

Forty-two soil samples were positive for chitinolytic cultures and, in total, 56 chitinolytic isolates were obtained. As can be seen in Table 5, the direct plating technique yielded a larger number of soil samples from which chitinolytic cultures were isolated as well as a larger number of chitinolytic cultures than the enrichment technique. Twenty-six out of 32 soil samples were positive for chitinolytic organisms by the direct plating technique, while sixteen out of 32 samples were positive by the enrichment technique. Thirty-five and 21 chitinolytic cultures were obtained by the direct plating and enrichment techniques respectively. Hence, the direct plating technique accounted for 61.9% of the positive soil samples and 62.5% of the chitinolytic cultures whereas the enrichment technique accounted for 38.1% of the positive soil samples and 37.5% of the chitinolytic isolates (Table 6).

In addition, as illustrated in Figure 1, it took from 2 to 7 days of incubation to visualize chitinolytic activity of the cultures by the direct plating technique and from 2 to 15 days by the enrichment technique. In the direct plating technique, chitinolytic activity was recognized by the appearance of a visible clear area around the colonies. In the enrichment technique, the first clue of the presence of chitinolytic activity was the appearance of turbidity (growth) in the flasks; this was

TABLE 5. Distribution of soil samples and chitinolytic cultures regarding the number of chitinolytic cultures obtained per soil sample and the screening technique employed.

No. chitinolytic cultures per sample	Enrichment technique (32 samples)		Direct plating technique (32 samples)		Total (64 samples)	
	soil samples (#)	chitinolytic cultures (#)	soil samples (#)	chitinolytic cultures (#)	soil samples (#)	chitinolytic cultures (#)
0	16	-	6	-	22	-
1	11	11	19	19	30	30
2	5	10	5	10	10	20
3	-	-	2	6	2	6
Total	32(16)[a]	21	32(26)	35	64(42)	56

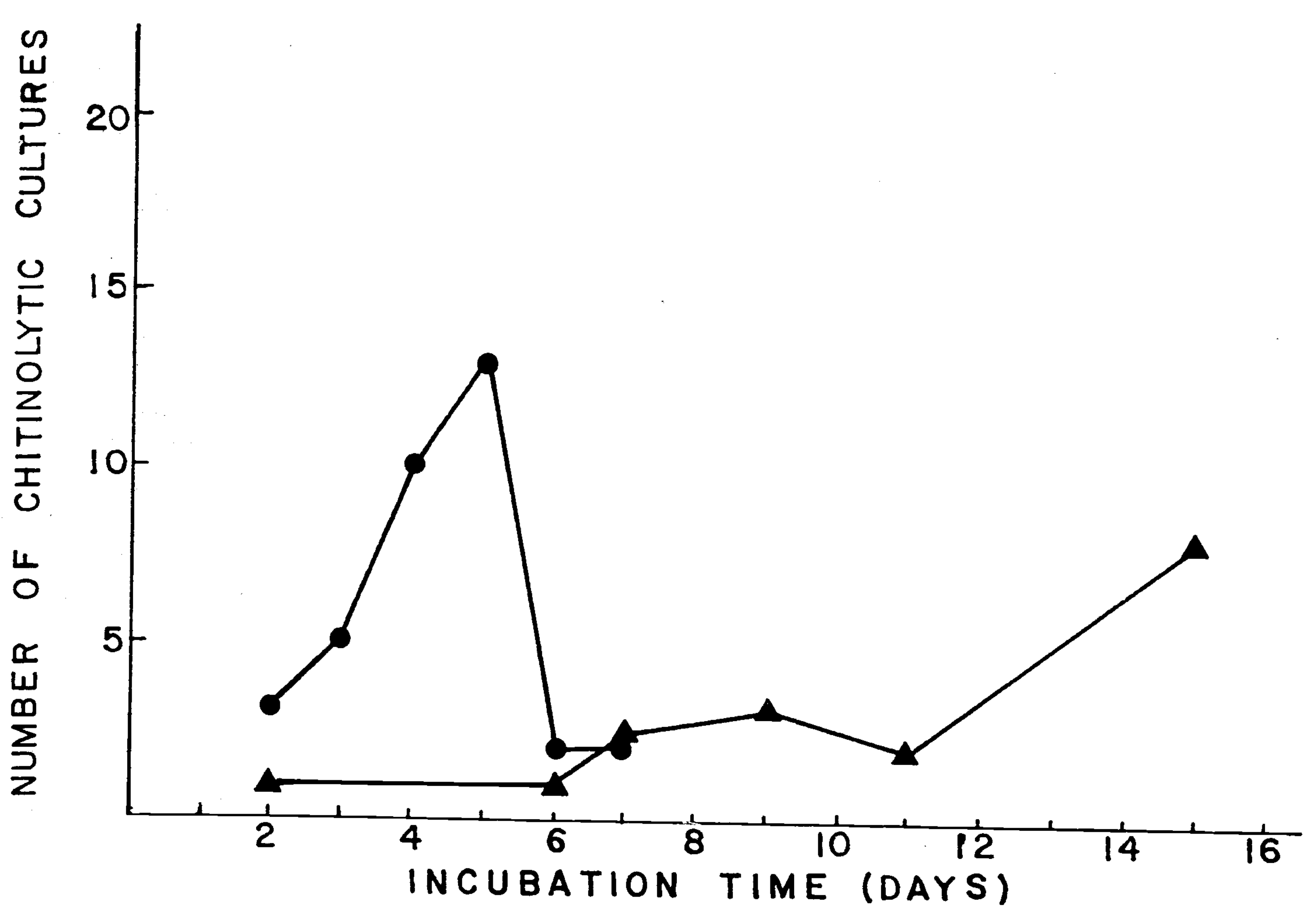
[a]The numbers in parentheses correspond to the number of soil samples from which chitinolytic cultures were obtained for each screening technique or in relation to the total of soil samples used.

TABLE 6. Distribution of soil samples positive for chitinolytic organisms and total chitinolytic cultures obtained with respect to screening method.

Method	Positive Soil Samples		Chitinolytic Cultures	
	Number	%	Number	%
Enrichment Technique	16	38.10	21	37.50
Direct Plating technique	26	61.90	35	62.50
Total	42	100.00	56	100.00

Figure 1. Numbers of chitinolytic cultures versus incubation time. Direct plating technique, ●——● ; enrichment technique, ▲——▲ . Chitinase production was recognized once a visible clear area was seen around colonies (direct plating) or when growth (turbidity) was demonstrated in flasks (enrichment technique).

Figure 1.



subsequently confirmed on chitin plates. It is assumed that in the culture medium used in screening for these organisms only those with the ability to metabolize chitin will be able to thrive, since chitin is present as the sole carbon, nitrogen and energy source.

It must be noted, on the other hand, that chitinolytic activity was checked for only up to 18 days of incubation. The main goal at this phase of the study was to isolate the most active chitinolytic organisms and not all possible chitin hydrolysing species present in the soil samples.

The 56 different chitinolytic cultures isolated belonged to three of the major microbial groups: 12 cultures were true bacteria, 39 actinomycetes and 5 fungi. Thus, actinomycetes constituted 69.64% of the chitinolytic cultures followed by true bacteria, 21.43% of the total, and finally, fungi, representing 8.93%.

When examining the incubation time at which chitinolytic cultures first showed their chitinolytic activity on chitin plates (Table 7) most bacterial cultures needed 2 or at the most 5 days, most actinomycetes at least 3 to 5 days, and the fungi usually more than 6 days.

When considering the number of organisms per gram of soil[1] the true bacteria appeared to be the quantitatively dominant group of chitinoclastic organisms ($\log_{10}$ of number of colonies/g soil, 5.23) followed by the actinomycetes ($\log_{10}$ number of colonies/g soil, 4.84) and the fungi (log of number of colonies/g soil, 4.3) (Table 7 and Figure 2).

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[1]The number of organisms per gram soil is expressed as the average of number of colonies of each group per gram soil from the 32 soil samples processed by the direct plating technique in the screening program.

TABLE 7. Distribution of the chitinolytic cultures isolated during the screening program in relation to the major microbial groups in which they belong, and the incubation time at which the chitinolytic activity was first shown.

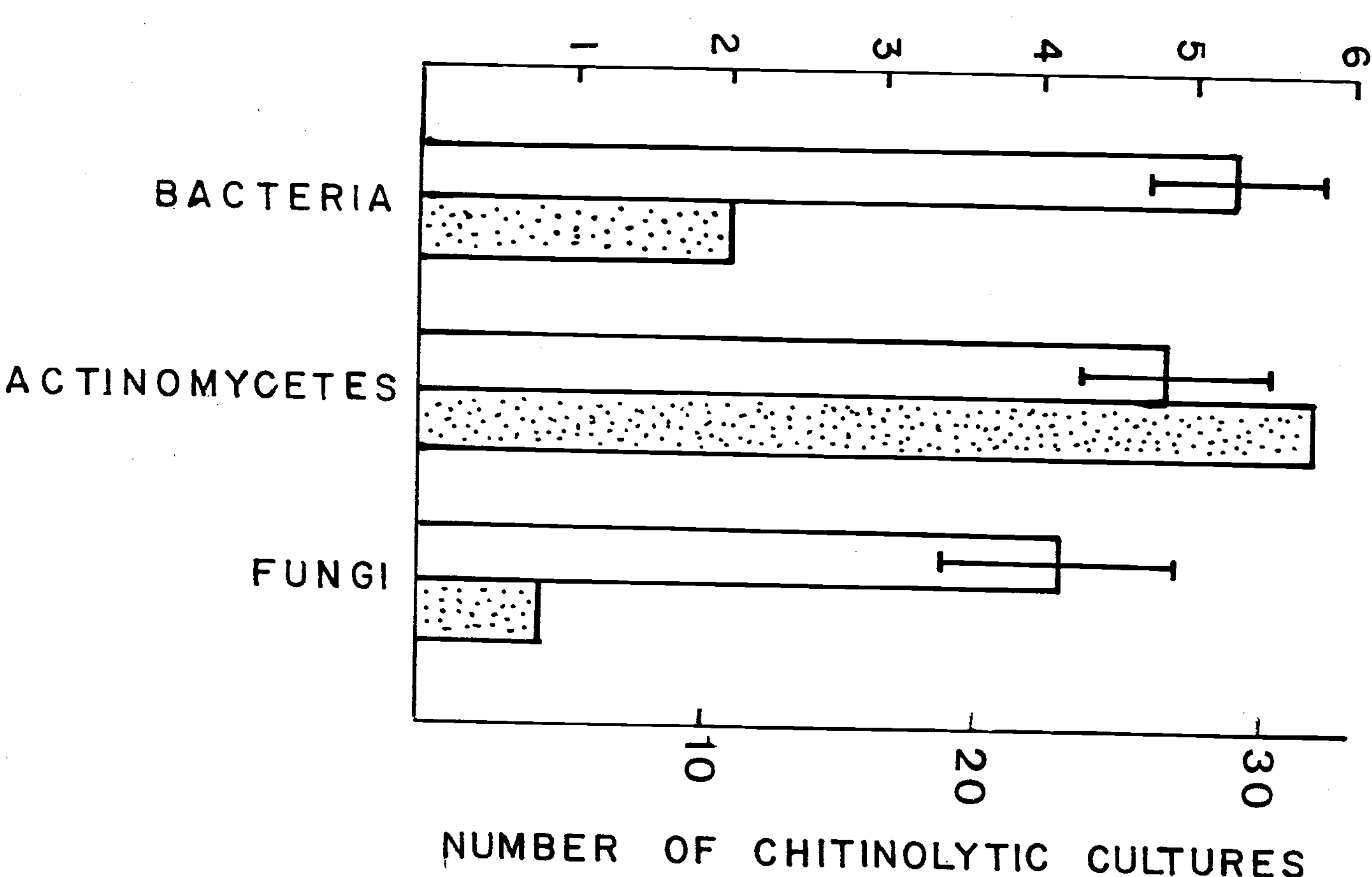
Incubation Time of the First Chitinolytic Activity Appearance (days)	Number of Chitinolytic Cultures			
	True Bacteria	Actinomycetales	Fungi	Total
≤ 2	5	-	-	5
3-5	5	33	1	39
6 or more	2	6		12
TOTAL:	(21.43%)	(69.64)	5 (8.23)	56
Log. of the Average Number of Colonies Per Gram Soil Sample[a]	5.23	4.84	4.30	

[a]These numbers correspond to the logarithms of the mean of the numbers of bacterial, actinomycetal and fungal colonies per gram soil totaled from the 32 soil samples processed by the direct plating technique during the screening for chitinolytic organisms.

Figure 2. Relationship of the number of chitinolytic cultures isolated in the screening program and the number of chitinolytic microorganisms per gram of soil sample to the major microbial groups with which the chitinolytic cultures belong. Log_{10} of number of organisms per g soil sample, ▭ . Number of chitinolytic cultures, ▭.

Figure 2.

LOG_{10} Nº ORGANISMS PER GRAM SOIL SAMPLE



In the second phase of the screening program the 56 chitinolytic isolates were cultured in baffled 250 ml Erlenmeyer flasks containing enriched nutrient broth at room temperature. The bacterial isolates were incubated for 24 h, while the fungi were grown for 72 h in the same medium supplemented with 0.025 g/100 ml sterile Tween 80. After the appropriate incubation time, a drop of each culture was innoculated by puncture on a premarked point on chitin plates (10 cultures per plate) and incubated for one week at room temperature. Each day, growth and progress of the width of the chitinolytic clarification zones were noted. It was assumed that the width of the clarified zone was a function of the concentration of chitinase produced by the organisms. This was conducted to determine: (1) the time at which visible chitinolytic activity (clearing of chitin surrounding colonies) first appeared, and (2) the maximum chitinase production, expressed as the radius of the zone of clearing (in mm) around the colonies once zone enlargement ceased.

On the basis of this experiment, 7 strains were selected as the most active chitinolytic organisms, and further tested, quantitatively, for chitinase production. This was ascertained by determining the amount of N-acetyl glucosamine, main by-product of the hydrolysis of chitin, which was produced by the organisms in shaking culture. Table 8 shows the maximum chitinase activity for these 7 strains, both on chitin agar and in submerged cultivation, as well as the time at which the maximum chitinase production was attained in the shaking cultures. From these data, the bacterial isolate BN2 (Arthrobacter sp.) was chosen for further studies as it had the highest chitinase activity (12.70 units) and

TABLE 8. Chitinase production by the 7 most active chitinolytic organisms selected among the 56 organisms isolated from the 62 soil samples used in the screening program.

Strain	Chitin Agar[a]	Submerged Culture[b]	
	Maximum chitinase act.[c]	Maximum chitinase activity (U)[d]	Incubation time at which the maximum chitinase production was observed (days)
Bacteria:			
BN1	5.5	8.63	4
BN2 (*Arthrobacter* sp.)	5.5	12.70	4
Actinomycetes			
St1 (*Streptomyces albus*)	4.5	11.16	5
St2 (*S. griseus*)	3.5	5.75	4
St3	2.5	5.41	6
Fungi:			
F-1	2.50	3.21	6
F-2	2.50	0.83	7

[a]Chitin agar is the same medium used during the selection of chitinolytic organisms by direct plating technique.

[b]The chitin broth used was that of Morrisey's (see material and methods) with 2% chitin).

[c]Maximum chitinase activity on chitin agar is expressed as the radius of the zone of clearing (in mm) around the colonies. It was controlled for 7 days.

[d]A unit of chitinase is defined here as the amount of enzyme required to release 1 microgram of NAG per hour per ml medium.

required only 4 days to achieve the level reached.
It also produced the largest chitinolytic area (5.5 mm) on chitin plates among the 56 organisms tested. In addition, *Arthrobacter* sp. BN2 grew well in chitin media. It reached a growth rate of 0.86 divisions per hour in colloidal chitin broth.

The isolate BN2 has been placed tentatively in the genus *Arthrobacter* on the basis of cultural and biochemical characteristics examined (Table 9).

II. Cultural Conditions for Maximum Chitinase Production and Growth by *Arthrobacter* sp. BN2.

Temperature Effect

When the isolate *Arthrobacter* sp. BN2 was cultured in chitin broth at a wide range of temperatures, the resulting growth curves were unique for each temperature of the gradient. The gradient covered the following temperatures: 2 C, 6 C, 8.5 C, 11 C, 13.5 C, 16.0 C., 18.0 C., 20.0 C, 22.0 C, 23.5 C, 25.5 C, 27.5 C, 30.5 C, 32.5 C, 35.5 C, 37.5 C, 40.0 C, and 42.5 C.

In Figure 3, only the growth curves that were thought essential for determining the optimum, maximum, and minimum growth temperatures were plotted. As can be seen in Table 10 , the aforementioned temperature profiles varied depending upon the incubation times. After 24 h of incubation, the optimum temperature was 33.5 C, after 48 h, 22.7 C; after 72 h, 96 h, and 118 h, 24.5 C. The minimum growth temperature, after 24, 72, 96, and 118 h was 6 C; and after 48 h, 9.0 C. The

TABLE 9. Characterisitics of the chitin-utilizing organism *Arthrobacter* sp. BN2.

Morphological:

FORM: pleomorphic; old cells, coccoid (almost 100%); young cells, rod-shaped with wide variation in length.

SIZE: 0.5 - 2 u length, 0.3 - 0.7 u wide, 24 h old culture.

GRAM STAIN: variable; young cells, usually gram positives; old cells, usually gram negatives.

MOTILITY: motile

OTHERS: usually as isolated cells, non-spore forming, capsulated.

Cultural:

AGAR COLONIES: 1-2 mm (48 h old), opaque, cream, smooth, sticky, circular, flat; cells die off at 3 days.

BROTH: uniformly turbid

GELATIN STAB: slow hydrolysis

BHI: no growth

Biochemical:

LITMUS MILK: unchanged (7 days)

STARCH: hydrolyzed

NITRATE REDUCTASE: positive

VP: negative

CATALASE: positive

GLUCOSE: acid (by oxidation)

NH_3 PRODUCTION: positive

Figure 3. Growth curves described by *Arthrobacter* sp. BN2 when cultured at different temperatures during five days. L-shaped tubes with 2% chitin were inoculated with a 4 x 10^5 cells/ml aliquot of *Arthrobacter* sp. BN2, 24-h old. The temperature gradient covered the following temperatures: 2, 6, 8.5, 11.0, 13.5, 16.0, 18.0, 20.0, 22.0, 23.5, 25.5, 27.5, 30.5, 32.5, 37.5, 40.0, and 42.5 C. Growth curves plotted correspond to those temperatures required to determine optimum, maximum and minimum growth temperatures in relation to incubation time. 6.0 C, ○; 9.0 C, ●; 22.7 C, △; 24.5 C, □; 31.0 C, ■; 33.5 C, ▲; 36.5 C, ◇; 42.5 C, ◆.

Figure 3.

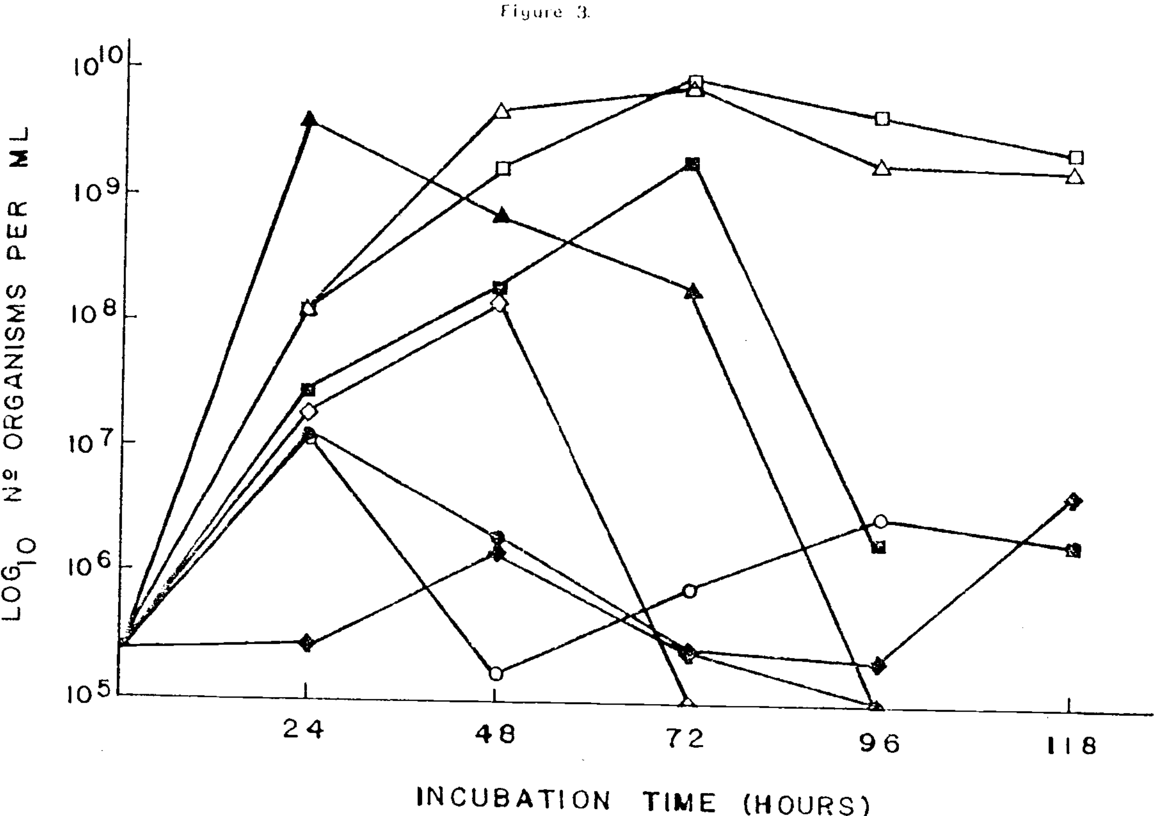


TABLE 10. Growth and growth rate temperatures of *Arthrobacter* sp. BN2 versus incubation time.

Temperature (° C)	Incubation Time (hours)				
I. Growth Temperature	24	48	72	96	118
Optimum	33.5	22.7	24.5	24.5	24.5
Maximum	36.5	42.5	33.5	31.0	31.0
Minimum	6.0	9.0	6.0	6.0	6.0
II. Growth Rate Temperature					
Optimum	33.5	24.5	18.5	16.0	8.5

maximum growth temperature after 24 h was 36.5; after 48 h, 42.5 C; after 72 h, 33.5 C; and after 96 h and 118 h, 31.0 C.

As expected, when maximum growth rates of _Arthrobacter_ sp. BN2 were plotted against each temperature of incubation, the growth rate increased slowly as the temperature of incubation increased from temperature 2 C to 24.5 C, reached a plateau between 24.5 to 33.5 C and, abruptly decreased from 33.5 C to 42.5 C (Figure 4).

Table 11 and Figure 4 illustrated the influence of temperature on chitinase biosynthesis by _Arthrobacter_ sp. BN2. The minimum temperature at which chitinase activity could be detected was 8.5 C, while the maximum temperature was 35.5 C. The optimum temperature for the highest chitinase production was in the range of 18.0 C - 25.5 C. The maximum chitinase activity was observed at 25.5 C throughout the incubation time. In Figure 4 can be seen that chitinase production slowly decreases below 25.5 C yet rapidly decreases above 25.5 C.

pH Effect

As depicted in Figure 5, _Arthrobacter_ sp. BN2 was able to grow at pH values from 5.0 to 8.0 yet failed to grow at pH values outside of this range. The maximum cell yield was reached at pH 6.0 after 96 h of incubation. The maximum growth rate was observed at pH 6.5 (Figure 6).

Chitinase production was observed when the isolate grew at pH values from 5.0 to 7.5 (Figure 6). The maximum chitinase production was achieved at pH 6.5 after 96 h of incubation. The biosynthesis of chitinase was significant at pH's 6.0, 7.0, and 7.5. It is interesting to note that significant levels of chitinase synthesis were observed

Figure 4. Total growth, growth rate and chitinase production curves described by *Arthrobacter* sp. BN2 at different temperatures. Total growth, ○—○, expressed as number of organisms per ml was determined by microscopic count. The isolate was cultured in chitin broth for 72 h. Growth rate ●—● ; chitinase production △—△ . Each point in the curves correspond to the average of at least two observations.

Figure 4.

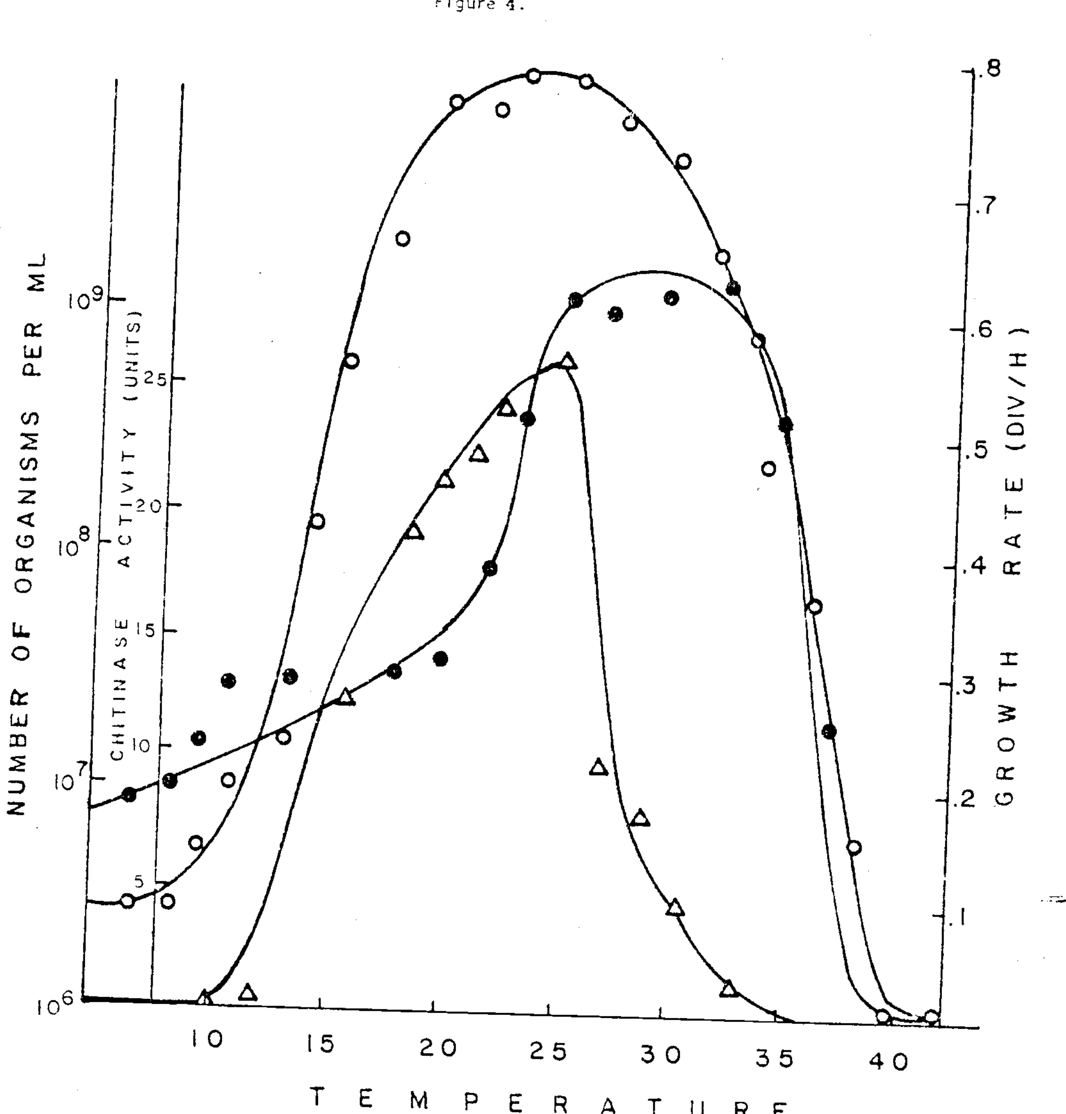


Figure 5. Growth curves described by *Arthrobacter* sp. BN2 when cultured at different pH values during five days. At the pH values of 3.4, 3.8, 4.6 and 8.6, growth could not be detected. pH 5.4, △ ; 6.0, ● ; 6.4, ○ ; 7.0, ▲ ; 7.4, □ ; 8.0, ■ .

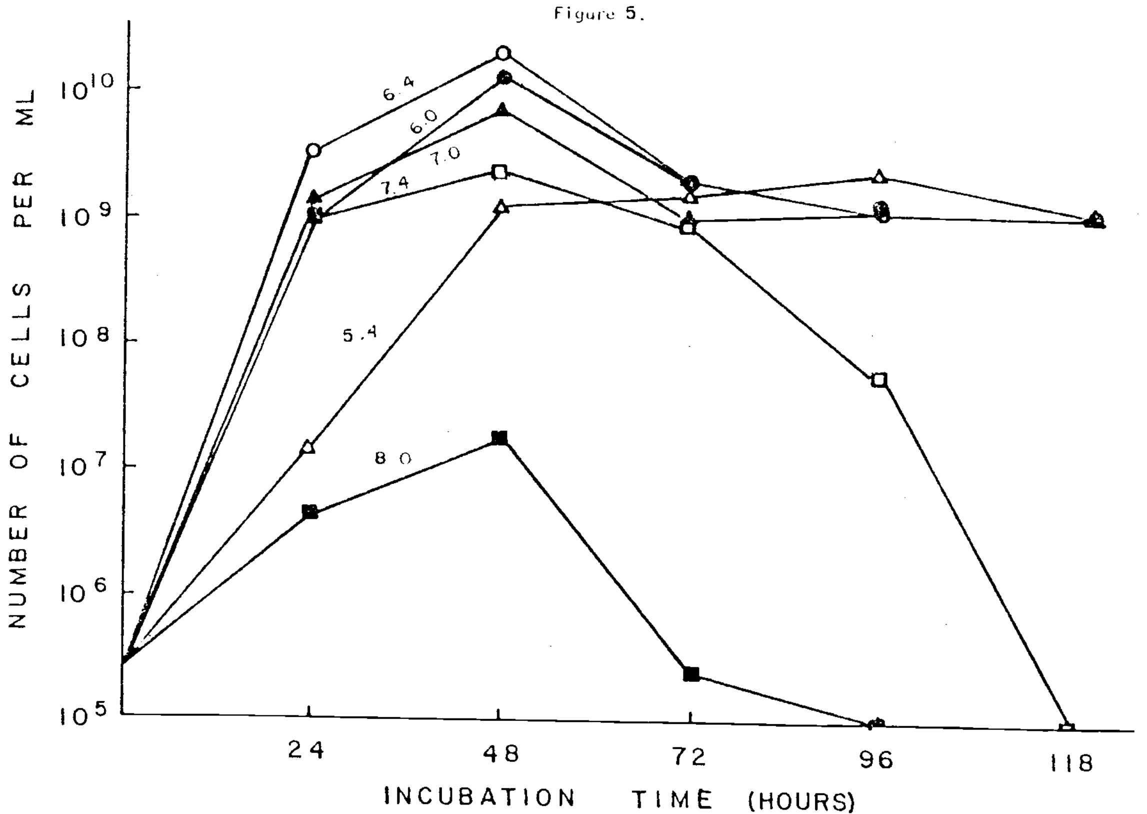
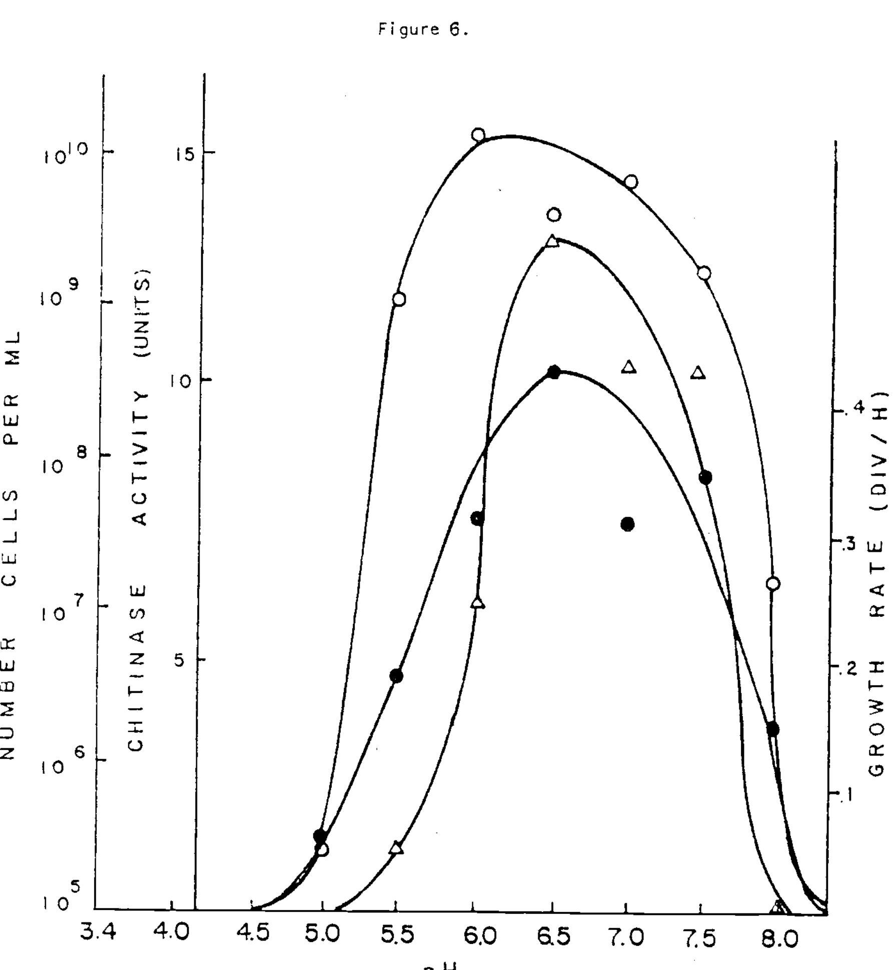


Figure 5.
NUMBER OF CELLS PER ML
10^10
10^9
10^8
10^7
10^6
10^5
6.4
6.0
7.0
7.4
5.4
8.0
24
48
72
96
118
INCUBATION TIME (HOURS)

Figure 6. Chitinase production, total growth and growth rate curves of *Arthrobacter* sp. BN2 at various pH values. *Arthrobacter* sp. BN2 was cultured in chitin broth at 24.5 C for five days. The chitin broth contained 0.5% colloidal chitin. Total growth O—O was determined at 48 h of incubation. Growth rate ●—● during the first 48 h of incubation. Chitinase activity Δ—Δ was measured at 96 h of incubation, except for pH 5.5, when chitinase activity was measured at 48 h of incubation.

79

Figure 6.



at pH values of the buffer medium one unit below neutrality and only 0.5 unit above it.

Incubation Time Effect

Figures 3 and 5 illustrate how the incubation time can influence the effects which factors such as temperature and pH have on growth and chitinase biosynthesis of Arthrobacter sp. BN2. Under the culture conditions used here (25.5 C, pH 6.4, 2% colloidal chitin medium), the optimum incubation time for the maximum growth rate of Arthrobacter sp. BN2 was 24 h, the maximum cell yield was obtained after 72 h, and maximum chitinase biosynthesis was noted after 96 h. It should be emphasized that these conclusions may be true only under the culture conditions used here. Indeed, as shown later, the optimum incubation time for growth and/or chitinase biosynthesis varies depending upon factors such as chitin concentration, chitin purity, etc. Although chitinase activity was observed at 24 h of incubation it reaches significant values after the culture entered into stationary phase. It is interesting to note that growth rate declines to near zero after 72 h of incubation, and, thereafter it begins to increase slowly in parallel with chitinase biosynthesis. After the fifth day of incubation the chitinase concentration in the medium begins to decrease abruptly (Figure 7, Table 11 and Table 12).

Chitin Concentration Effect

To study the influence that different concentrations of chitin in the culture medium may have on the biosynthesis of chitinase by the

Figure 7. Influence of incubation time on biomass yield, growth rate and chitinase biosynthesis by *Arthrobacter* sp. BN2 cultured in 2% colloidal chitin medium at 24.5 C, at pH 6.5. Biomass yield, O—O . Growth rate, ●—● . Chitinase production, □—□ .

Figure 7.

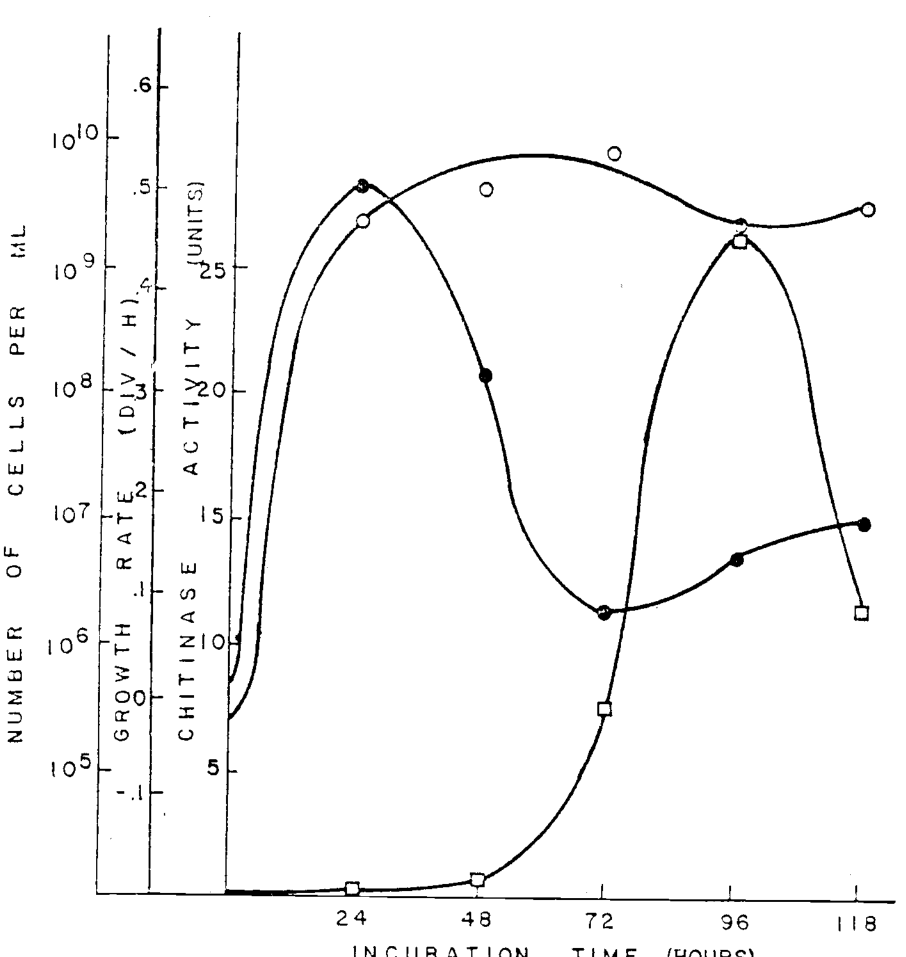


TABLE 11. Chitinase production by Arthrobacter sp. BN2 cultured at different temperatures versus incubation time.

Temperature (^{o}C)	Incubation Time (hrs.)				
	24	48	72	96	118
6.0	-	-	-	-	-
8.5	.18	.30	.06	.09	.19
11.0	.17	.30	.11	.70	.20
13.5	.20	.40	4.21	13.68	9.20
16.0	.20	.40	3.42	12.21	10.00
18.0	.20	.40	5.27	19.12	10.14
20.0	.20	.40	6.58	21.33	12.93
22.0	.19	.38	6.71	22.06	12.80
23.5	.20	.39	6.89	23.53	11.35
25.5	.20	.38	7.74	26.47	11.62
27.5	.19	.39	2.37	9.61	10.27
30.5	.18	.31	2.24	9.09	9.19
32.5	.18	.28	2.37	4.04	2.97
35.5	.17	.15	1.10	2.09	1.62
37.5	-	-	-	-	-
42.5	-	-	-	-	-

Chitinase production is expressed as chitinase units present in culture fluid.

The culture conditions are the same used for temperature effects on growth.

TABLE 12. Effect of pH on growth and chitinase production. Numbers of organisms/ml, log. of numbers of organisms/ml, growth rate, chitinase activity (units) and final pH of medium at different initial pH of medium vs. incubation time.

Initial pH		24 h.	48 h.	72 h.	96 h.	120 h.
3.4 3.8 4.6				NO GROWTH		
5.0		4×10^5	5.57×10^5	-	-	-
5.4	# Bact/ml	1.47×10^7	$.6 \times 10^9$	1.84×10^9	3.6×10^9	$.6 \times 10^9$
	Log # Bact/ml	7.17	8.78	9.26	9.56	8.78
	Growth Rate	.1910	.2505	.0467	.0280	.0747
	Chitinase Act.	.10	1.42	.02	.02	.11
	Final pH	6.0	6.65	6.3	6.3	6.8
6.0	# Bact/ml	$.27 \times 10^9$	16.8×10^9	1.9×10^9	1.12×10^9	$.67 \times 10^9$
	Log # Bact/ml	8.43	10.23	9.28	9.05	8.83
	Growth Rate	.3123	.1721	-.0908	-.0220	-.0214
	Chitinase Act.	.2	.72	2.0	6.26	1.29
	Final pH	7.5	8.3	8.55	7.8	8.25
6.4	# Bact/ml	4.73×10^9	3.25×10^{10}	2.44×10^9	$.88 \times 10^9$	$.49 \times 10^9$
	Log # Bact/ml	9.67	10.51	9.39	8.94	8.69
	Growth Rate	.4316	.0802	-.1079	-.0425	-.0244
	Chitinase Act.	.28	1.12	1.48	13.14	3.79
	Final pH	7.4	7.3	7.25	7.25	7.4

Continued next page.

TABLE 12. (Continued)

Initial pH		24 h.	48 h.	72 h.	96 h.	120 h.
7.0	# Bact/ml	2.1×10^9	8.4×10^9	$.7 \times 10^9$	$.3 \times 10^9$	$.1 \times 10^9$
	Log # Bact/ml	9.32	9.92	8.85	8.48	8.00
	Growth Rate	.3018	.0578	-.1035	-.0383	-.0458
	Chitinase Act.	.13	.65	.75	10.29	2.11
	Final pH	7.14	7.45	7.6	7.5	7.5
7.4	# Bact/ml	$.58 \times 10^9$	3.6×10^9	$.13 \times 10^9$	7.8×10^7	
	Log # Bact/ml	8.76	9.56	8.11	7.89	
	Growth Rate	.3442	.0761	-.1384	-.0213	NO GROWTH
	Chitinase Act.	.04	.23	.6	10.09	2.14
	Final pH	7.4	7.4	7.8	7.7	7.8
8.0	# Bact/ml	6.1×10^6	2.4×10^7	4×10^5		
	Log # Bact/ml	6.79	7.38	5.60	NO GROWTH	NO GROWTH
	Growth Rate	.1544	.2461	-.1706		
	Chitinase Act.	.04	.02	.03	.02	.02
	Final pH	7.85	8.0	8.0	8.0	8.0
8.6				NO GROWTH		

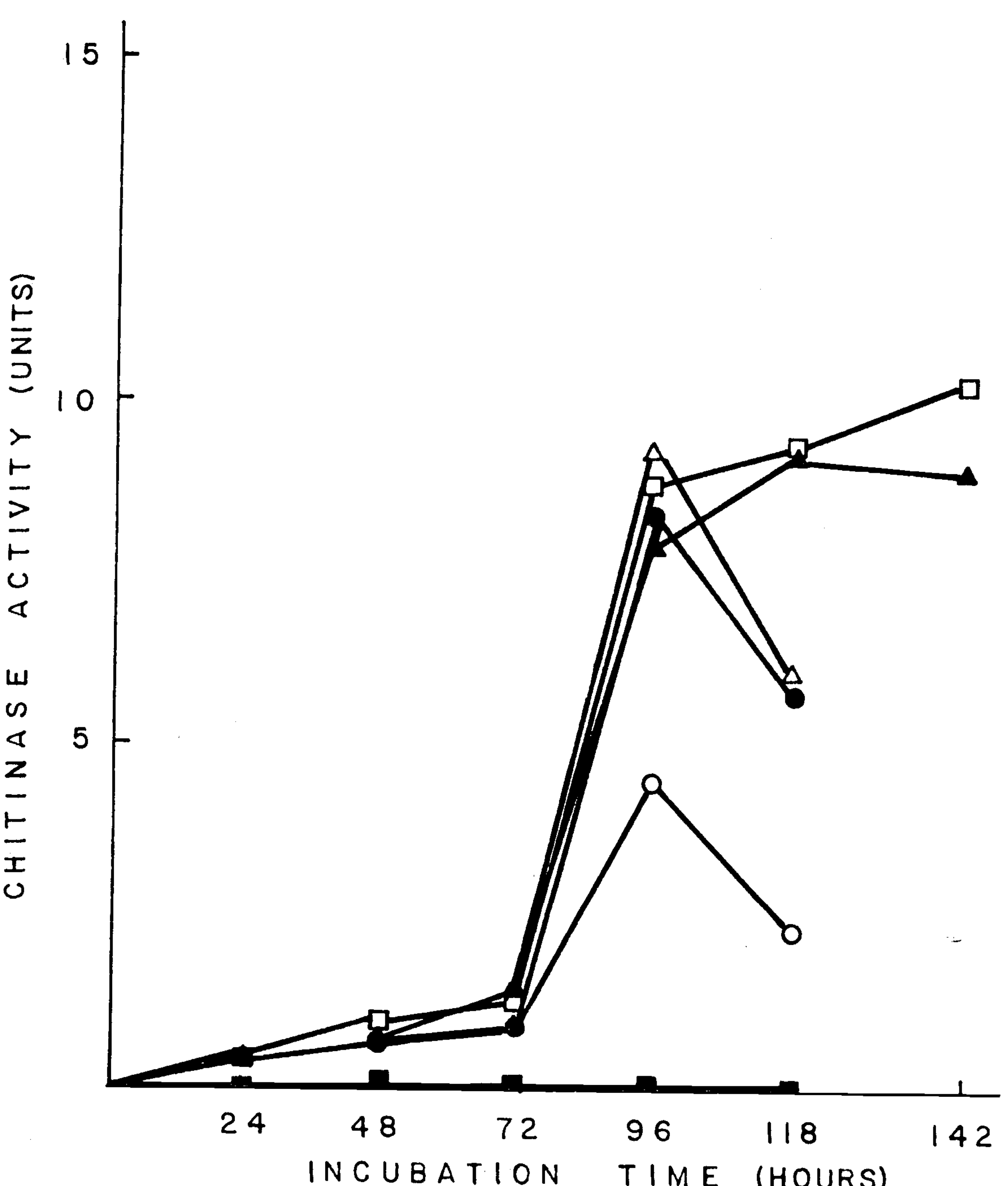
isolate Arthrobacter sp. BN2, the following powdered chitin concentrations were tested: 0.1%, 0.5%, 1.0%, 2.0% and 3.0% (w/v). As depicted in Figure 8 no significant difference in the extracellular chitinase levels was observed between the different chitin concentrations during the first three days of incubation; only very low levels of chitinase activity were detected during this time. The maximum chitinase production was reached after the fourth day of incubation for 0.1, 0.5, and 1.0% concentrations of chitin; after the fifth day for 2.0% and after the sixth day for 3.0% chitin. Therefore, the lower the concentration of chitin in the medium the earlier the peak chitinase production is reached, and conversely, the higher the concentration of chitin in the medium, the higher the chitinase biosynthesis and the later this maximum is reached. Although chitinase production increased as the time of incubation time proceeded for all the concentrations of chitin, it increased at a more rapid rate at the higher concentrations than at the lower ones.

Mineral Salts Effect

An adequate culture medium for the study of chitin degradation by microorganisms was thought to be one that could supply sufficient types and amounts of mineral salts and other essential nutrients and result in both optimum growth and enzyme biosynthesis. Among a series of media used in microbial chitinase production, the medium developed by Morrisey et al. (1976) using an Arthrobacter sp. was considered one of the most complete for the Arthrobacter sp. BN2. Hence, it was employed in most of the experiments reported here. In order to verify the actual role of

Figure 8. Chitinase biosynthesis by Arthrobacter sp. BN2 at different chitin concentration versus incubation time. Culture was grown at 24.5 C and pH 6.4. Powdered chitin was used as a substrate. No chitin in medium, ■—■ ; 0.1% chitin, ○—○; 0.5% chitin, ●—● ; 1.0% chitin, △—△ ; 2.0% chitin, ▲—▲; 3% chitin, □—□ . Each point is the average of two observations.

Figure 8.



each mineral salt as well as the glucose and yeast extract in Morrisey's medium, these substances were added to or removed from the complete base medium[2], one by one.

As is illustrated in Table 13 using the chitinase production levels (chitinase activity or Monod rate) obtained with the complete base medium as a standard, three types of influences on chitinase biosynthesis could be established: (1) reduction of chitinase production, (2) enhancement of chitinase production, or (3) the absence of any effect. The addition of H_3BO_3, $MnCl_2$, $NaMoO_4$ and $CaCl_2$, or the removal of glucose does not result in any change in the chitinase biosynthesis level when compared to the complete base medium. The addition of $MgSO_4$, $CuSO_4$ and $ZnSO_4$ exerted a slight inhibition of the synthesis rate of the enzyme. Finally, the addition of $FeSO_4$ and $CoCl_2$ and the elimination of yeast extract brought about a relatively significant augment in the chitinase production; the following factors can be ordered by their relative effects on the enzyme synthesis level, from most to least enhancement: removal of yeast extract > addition of $FeSO_4$ > removal of yeast extract and glucose > addition of $CoCl_2$.

Effect of the Size of Inoculum of Arthrobacter Sp. BN2

The size of the inoculum of Arthrobacter sp. BN2 did not vary the chitinase production significantly, at least after 96 h of incubation when when the chitinase assay was performed (Table 14). It is important to note that chitinase synthesis did vary in relation to the size of the

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[2] Complete base medium is the Morrisey's Medium lacking all the mineral salts except phosphates.

TABLE 13. Chitinase production of *Arthrobacter* sp. BN2 as a function of different mineral salts in the culture medium.

Mineral Salt	Cell Yield (log. of viable cells/ml)	Chitinase Synthesis: Activity (Units)	Chitinase Synthesis: Monod's Rate (Activity/$\log_{10}$ # cell/ml)
H_3BO_3	9.52	7.0	.74
$MgSO_4$	9.84	6.0	.61
$MnCl_2$	9.78	6.8	.70
$CoCl_2$	9.78	8.2	.84
$CuSO_4$	10.00	6.3	.63
$ZnSO_4$	10.02	6.3	.63
$NaMoO_4$	9.87	6.8	.69
$FeSO_4$	9.76	12.0	1.23
$CaCl_2$	9.73	7.3	.75
Controls:			
All Salts	10.46	7.5	.72
CBM[1]	9.30	7.0	.75
CBM-yeast ext.	9.47	14.0	1.48
CBM-glucose	9.85	7.0	.71
CBM-yeast ext. & glucose	9.30	10.0	1.08

1. CMB: complete base medium was KH_2PO_4, 0.4%, K_2HPO_4, 0.3%; glucose, 0.1%; yeast extract, 0.02%; and powdered chitin, 0.5%. Mineral salts added at the concentration present in Morrisey's medium. Flasks were incubated for 4 days in a shaker at room temperature.

TABLE 14. Chitinase biosynthesis by *Arthrobacter* sp. BN2 in pure and mixed culture with *Aureobasidium pullulans* as a function of the size of inoculum of *Arthrobacter* sp. BN2.

Inoculum size (ml)	CHITINASE PRODUCTION		
	Pure Culture (P)	Mixed Culture (M)	Ratio M/P
0.1	13.76 (9.84)[a]	27.86	2.02
0.5	13.84 (9.72)	32.73	2.36
1.0	14.08 (9.90)	29.17	2.07
2.0	14.17 (10.02)	33.52	2.36
3.0	14.12 (9.79)	31.93	2.26

Inoculum of *Arthrobacter* sp. BN2 was prepared from a 24-h old culture with about 10^7 cells/ml added to 30 ml of medium contained in 250 ml Erlenmeyer flasks. In mixed culture, the inoculum size of *A. pullulans* (10^5 cells/ml) was 0.1 ml. Chitinase activity was determined after 96 h of incubation.

[a]logarithm of number of cells/ml after 96 h of incubation.

culture inoculum early in the incubation period. However, the chitinase production at these times were too low to be considered significant.

Effect of the Source and Degree of Purity of Chitin

Chitinase production by *Arthrobacter* sp. BN2 was compared using chitin from the following sources: crab and crayfish chitin, powdered and colloidal chitin, pure, partially purified (demineralized or deproteinized) and impure (washed) chitin. As depicted in Table 15, the maximum chitinase synthesis was reached using colloidal chitin (12 units) followed by pure powdered crab chitin (4.5 units). Crab chitin seems to be a better chitin source for chitinase production than crayfish chitin. The chitinase activities obtained with demineralized crab and crayfish were 2.0 and 0.5 units, respectively.

When using washed powdered crab shells, the chitinase production was of the same magnitude as that obtained using demineralized powdered crab shells and that obtained with partially purified powdered crayfish chitin. On the other hand, no chitinase activity could be detected in either N acetyl glucosamine medium or enriched nutrient agar. The chitinase assay was performed with maximum of 3 hours of incubation at 37 C, so that very low chitinase activities may not have been detected.

There appears to exist a relationship between the degree of purity of the chitin source and the changes observed in the pH of the medium. The more impure the source of chitin, the higher the pH at the end of the experiment.

TABLE 15. Chitinase biosynthesis by Arthrobacter sp. BN2 as a function of the nature of chitin used in the culture medium.

Chitin[a] Source	Final pH	$\log_{10}$ of number of organisms per ml	chitinase Activity (units)	Differential rate: chitinase units / $\log_{10}$ # org per ml
Pure powdered crab chitin, 24 mesh	6.85	8.71	4.5	0.52
Pure colloidal crab chitin	7.00	9.50	12.0	1.26
Demineralized powdered crab shells, 24 mesh	7.60	8.17	2.0	0.24
Washed powdered crab shells, 24 mesh	8.10	8.04	2.2	0.27
Partially purified (demineralized & deproteinized) powdered crayfish chitin, 24 mesh	7.80	7.98	1.7	0.21
Demineralized powdered crayfish chitin, 24 mesh	8.10	7.67	0.5	0.06
N-acetyl glucosamine medium	6.80	9.13	-	-
Enrichment nutrient medium	7.00	8.93	-	-

[a]Chitin added at 0.2%, cultures incubated for 96 h in a shaker at RT.

TABLE 16. Chitinase biosynthesis in the mixed culture of *Arthrobacter* sp. BN2 with *Aureobasidium pullulans* as a function of the nature of chitin source used.

Chitin Source	Final pH	logarithm$_{10}$ number organisms		chitinase activity (units)	Differential Rate: chitinase units / log$_{10}$ # org. per ml	Ratio Pure/ Mixed
		BN2	*A. pullulans*			
Pure powdered crab chitin, 24 mesh	7.0	8.70	7.60	36.5	4.20	8.08
Pure colloidal crab chitin	7.0	8.95	7.74	37.0	4.12	3.27
Demineralized powdered crab shells, 24 mesh	7.3	8.49	7.16	19.0	2.24	9.33
Washed powdered crab shells, 24 mesh	7.9	7.82	7.13	10.9	1.39	5.15
Partially purified powdered crayfish chitin (24 mesh)	7.5	8.24	7.71	12.0	1.46	6.95
Demineralized powdered crayfish chitin (24 mesh)	8.0	7.62	7.51	8.05	1.05	2.10
N-acetyl glucosamine Medium	6.8	8.65	7.12	-	-	-
Enrichment Nutrient Medium	7.0	8.83	7.34	5.5	0.62	-

Static and Shaking Cultivation Effect

As is illustrated in Table 17, Arthrobacter sp. BN2 was able to show higher chitinase production in shaking conditions than in static cultivation. In addition, it was found that the chitinase synthesis was slightly greater in a shaker at 200 rpm than at 100 rpm. Finally, higher chitinase production was observed in tubes than in flasks (13.78 and 8.30 units, respectively).

III. Classes of Chitin-Degrading Cells in the Culture Arthrobacter sp. BN2 as a Function of the Magnitude of Their Chitinolytic Activity

To determine if there was any variation among individual organisms with respect to chitinolytic capacity, Arthrobacter sp. BN2 was cultured for 24 h in chitin broth, diluted and plated on chitin agar. After 96 h of incubation at 25.5 C, about 2790 colonies were analyzed for chitinolysis on the basis of the presence and size (in mm) of the radius of the clarification zone around the colonies. Three categories were established arbitrarily: super-producers, medium-producers and low-producers. As shown in Table 18, the super-producers comprised about 13% of the total colonies screened while the medium and low producers about 45% and 42% respectively. Subsequently, 8 colonies of each category were chosen for further study. Like the wild type culture above, they were cultured for 24 h in chitin broth, dilutions were plated on chitin agar and after 96 h of incubation, the widths of the clarification zones around the resulting colonies were measured. In each category of colonies examined, the low and medium producers always predominated the super-producers

TABLE 17. Chitinase production by *Arthrobacter* sp. BN2 on static and shaking culture conditions.[a,b]

	Chitinase production (Units)	
	Pure Culture	Mixed Culture (BN2 + *A. pullulans*)
Static:	4.69	17.23
Shaking:		
100 rpm (flask)	7.80	23.69
200 rpm (flask)	8.30	24.82
200 rpm (tube)	13.78	37.08

[a] Flasks were incubated at room temperature for 96 h.

[b] Medium contained 1.0 % colloidal chitin.

TABLE 18. Classes of chitinase producing cells in Arthrobacter sp. BN2 on the basis of the size of clarification zone around colonies.

Type of cells	Radius of clarification zone (mm)	Relative frequency (%)			
		A	B	C	D
Super producers	≥ 5	12.86	18.54	16.23	13.95
Medium producers	2 - 4	44.64	39.03	54.05	34.89
Slow producers	< 2	42.50	32.43	29.72	51.16

Chitin plates inoculated with young (24 h old) culture of Arhtrobacter sp. BN2 in colloidal broth were incubated at room temperature for 96 h.

A. Frequencies using the wild type strain, 2,790 colonies were analyzed.
B. Frequencies using eight super producers clones, 3,215 colonies were analized.
C. Frequencies using eight medium producers clones, 2,984 colonies were analized.
D. Frequencies using eight slow producers clones, 3,990 colonies were analized.

(Table 18). Moreover, the relative frequencies of each category were quite similar to each other in the populations (colonies) examined from the original culture and from the three subcultures of *Arthrobacter* sp. BN2. The relative frequency of a given category in the progeny of the subculture of that category was slightly higher than that obtained from the progeny of the original culture. The super-producers usually had larger colonies than the two other categories.

IV. Chitinase Production of the Mixed Culture of *Arthrobacter* sp. BN2 with Different Organisms

To find a cultural partner that would give *Arthrobacter* sp. BN2 a higher capacity for chitinase synthesis, *Arthrobacter* sp. BN2 was symbiotically grown with representatives of the following microbial groups: bacteria, actinomycetes, yeasts and molds. As illustrated in Table 19, the bacteria *Escherichia coli*, *Bacillus subtilis*, and *Micro-ccocus* sp. neither enhanced nor inhibited chitinase production in *Arthrobacter* sp. BN2. The actinomycetes *Streptomyces albus* and *S. gri-seus*, which by themselves are chitinase-producers, gave rise to a higher chitinase synthesis in the mixed culture. The yeast *Candida utilis*, and the mushroom *Volvariella volvaceae* did not alter the chitinase synthesis rates of *Arthrobacter* after 4 days of incubation, although slight in-crease in chitinase synthesis was noted in these mixed cultures after eight days of incubation. The molds *Phanerochaetae chrysosporium*, *As-pergillus niger*, and *Penicillium* sp. and the yeast-like mold *Aureobasi-dium pullulans* triggered enormous chitinase production under the mixed culture conditions tested here. After 4 days of incubation, *A. pullulans*

TABLE 19. Chitinase production of Arthrobacter sp. BN2 in mixed culture with different microorganisms.

SYMBIOTIC ORGANISM	CHITINASE PRODUCTION: 4 day incubation			8-day incubation
	Chitinase Act. (units)	Specific Activity	NAG Accumulation (ug/ml)	Chitinase Activity
Arthrobacter sp. (alone)	5.0	0.046	≤ 5	6.45
E. coli [a]	5.0	0.046	≤ 5	7.99
Bacillus subtilis [a]	5.73	0.050	≤ 5	5.05
Micrococcus sp. [a]	5.0	0.040	≤ 5	6.99
Candida utilis [a]	4.67	0.036	≤ 5	10.66
Volvariella volvacoae [c]	4.33	0.040	≤ 5	8.49
Streptomyces albus [b]	19.17	0.173	40	14.65
Streptomyces griseus [b]	11.83	0.120	70	8.33
Aureobasidium pullulans [a]	25.67	0.263	≤ 5	30.00
Phanerochaetae chrysosporium [b]	41.17	0.310	≤ 5	39.96
Aspergillus niger [b]	42.00	0.320	≤ 5	83.25
Penicillium sp. [b]	60.00	0.700	≤ 5	31.30

Chitin concentration in medium: 2% (w/v).
Inocula: Arthrobacter sp.: 5 ml of 10^7 cell/ml suspension.
Symbiotic organisms: [a]0.5 ml of 10^5 cell/ml suspension.
[b]0.5 ml of 10^5 spores/ml suspension.
[c]A heavy loop of mycelium.

increased chitinase production about 4 times, P. chrysosporium and A. niger about 8 times and Penicillium sp. about 12 times. After 8 days of incubation by these molds, the degree of enhancement did suffer some variations but still remained relatively high, especially with A. niger.

V. Effect of Environmental and Cultural Factors on Chitinase Production of the Mixed Culture Arthrobacter Sp. BN2 with Aureobasidium Pullulans

A. pullulans was selected as the symbiotic organism in further studies of the mixed culture system because of the ease in determining its population size, its relatively high growth rate and its metabolic versatility.

Chitin Concentration Effect

As was the case of the pure culture, the influence of the chitin concentration in the culture medium varied in relation to incubation time. As is shown in Figure 9 , the higher the chitin concentration in the medium, the higher the level of chitinase biosynthesis that was reached. Furthermore, in considering the maximum chitinase biosynthesis for each chitin concentration, the lower the chitin concentration, the earlier the maximum biosynthesis was achieved (Table 20). Examining the chitinase production of both pure and mixed cultures after 4 days of incubation, the ratio of chitinase production in the mixed culture to that in the pure culture did not fluctuate significantly as a function of the chitin concentration in the medium (Table 14). On the other hand, when the ratio of maximum chitinase production in the mixed culture

to the maximum in the pure culture was calculated, a direct relationship was seen between the enchancement of chitinase biosynthesis and the concentration of chitin in the medium (Table 20). It is interesting to note that the mixed culture of *Arthrobacter* sp. BN2 with *A. pullulans* in a non-chitin medium (Morrisey's medium lacking chitin) was able to show chitinase production at levels comparable to those obtained when a low-chitin concentration was used. However, the chitinase activity was not detected when *A. pullulans* grown alone in chitin medium, at least under the conditions used here for the enzyme assay (Figure 9).

Incubation Time Effect

Considering the maximum cell yield and the maximum chitinase production as a function of the incubation time, Table 21 shows that, after 24 h of incubation, the maximum cell yield obtained was with 1.0% chitin concentration and the maximum chitinase production with 0.1% chitin concentration. After 48 h of incubation, the maximum cell yield reached was with 2.0% chitin and the maximum chitinase synthesis with 1.0%. After 72 and 96 h, the maximum cell yields were reached with 3.0% and the maximum chitinase synthesis with 1.0%. After 120, 144, and 168 h the maximum cell yields and chitinase production were achieved with 3.0% chitin concentration. Therefore, the maximum cell yield and chitinase production at the early incubation times were obtained with the lower chitin concentrations, but at the later incubation times, the maxima were obtained with the highest chitin concentrations (usually 3%).

Figure 9. Chitinase biosynthesis by the mixed culture of *Arthrobacter* sp. BN2 and *Aureobasidium pullulans* at different chitin concentrations versus incubation time. No chitin in medium, ○—○ ; *A. pullulans* grown alone, ◇—◇ ; 0.1% chitin, ●—● ; 0.5% chitin, □—□ ; 1.0% chitin, ■—■ ; 2.0% chitin, △—△ ; 3% chitin, ▲—▲ .

Figure 9.

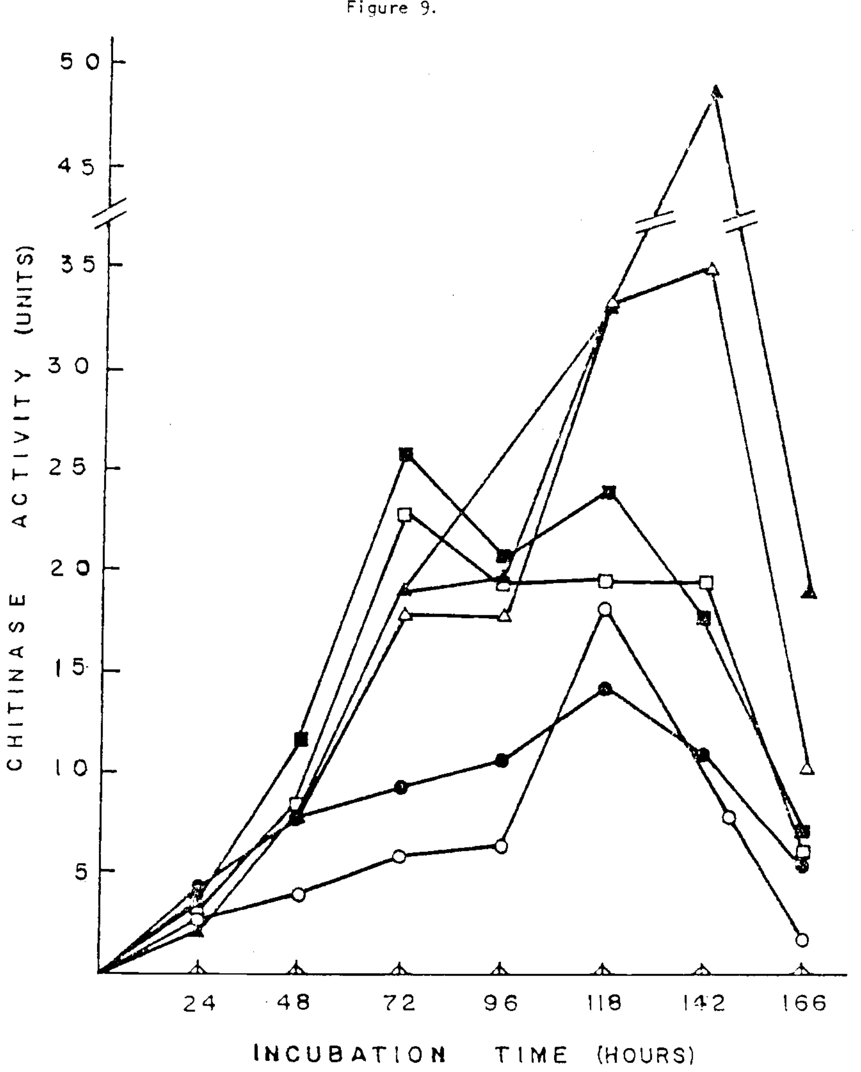


TABLE 20. Maximum levels of chitinase biosynthesis by the pure culture of *Arthrobacter* sp. BN2 and in symbiosis with *Aureobasidium pullulans* as a function of the chitin concentration in the medium.

chitin concentration % (w/v)	CHITINASE SYNTHESIS (UNITS)				
	PURE CULTURE		MIXED CULTURE	Ratio	Ratio
	P_1[a]	P_2[b]	M_1[c]	$\frac{M_1}{P_1}$	$\frac{M_1}{P_2}$
0.1	4.52 (96 h)[d]	2.56 (120)	14.50 (120)	3.21	5.66
0.5	8.40 (96 h)	1.10 (72)	22.80 (72)	2.71	20.72
1.0	9.32 (96 h)	1.42 (72)	26.16 (72)	2.81	18.42
2.0	9.34 (120 h)	9.15 (144)	40.00 (144)	4.28	4.37
3.0	10.20 (144 h)	10.20 (144)	49.14 (144)	4.82	4.82

[a] P_1 = maximum chitinase production by the pure culture at the respective chitin concentration.

[b] P_2 = chitinase activity (of the pure culture) at the incubation time of maximum chitinase activity in the corresponding mixed culture.

[c] M_i = maximum chitinase production by the mixed culture at the respective chitin concentration.

[d] The number in parentheses refer to the incubation time at which the chitinase activity values were obtained.

TABLE 21. Maximum cell yields and chitinase activity of the mixed culture of *Arthrobacter* sp. BN2 and *Aureobasidium pullulans* as a function of incubation time.

Incubation Time (h)	MAXIMUM CELL YIELD			MAXIMUM CHITINASE PRODUCTION	
	BN2	*A. pullulans*	Chitin conc.[a]	Chitinase Units	Chitin Conc.
24	3.09×10^{10}	7.9×10^{6}	1.0	4.17	0.1
48	1.12×10^{10}	1.51×10^{7}	2.0	12.00	1.0
72	1.35×10^{10}	3.47×10^{7}	3.0	26.16	1.0
96	2.24×10^{10}	3.98×10^{7}	3.0	21.16	1.0
120	2.63×10^{9}	1.35×10^{7}	3.0	38.13	3.0
144	2.34×10^{9}	1.15×10^{7}	3.0	49.14	3.0
168	2.24×10^{9}	1.70×10^{6}	3.0	19.00	3.0

[a]Chitin concentration values at which the maximum cell yields and chitinase biosynthesis were observed.

Mineral Salts Effect

To study the possible effects of the different mineral salts in Morrisey's medium at different concentrations on the chitinase production by the mixed culture of *Arthrobacter sp.* with *A. pullulans*, each of the mineral salts was added separately to the complete basal medium (CBM) at three different concentrations. These concentrations were as follows: at the level they are present in the original formula by Morrisey, 3 mg per 100 ml medium, and 15 mg per 100 ml medium. The mineral salts concentration in the original Morrisey's medium is much less than 3 mg/100 ml medium. The level of chitinase production observed in the complete basal medium was used as a standard.

As can be seen in Table 22, three effects on chitinase production can be drawn: (1) no alteration of the chitinase production, (2) enchancement of the chitinase production, and (3) inhibition of the chitinase production.

When the effects of Morrisey's concentration were studied, it was seen that the addition of $CuSO_4$ and removal of glucose did not affect the chitinase synthesis. The additions of $NaMoO_4$, $CaCl_2$, and to a slight extent, $FeSO_4$, as well as the addition of all salts together resulted in an enhancement of chitinase activity. The removal of yeast extract or the removal of yeast extract and glucose together, both enhanced chitinase production. The additions of H_3BO_3. $MgSO_4$, $MnCl_2$, $CoCl_2$, or $ZnSO_4$ each inhibited chitinase synthesis.

When the effects of mineral salts added at the concentration of 3 mg per 100 ml medium were examined, the additions of $MnCl_2$ or $CaCl_2$ did

not affect chitinase production. The additions of $MgSO_4$ or $FeSO_4$ enhanced it, and the additions of $CoCl_2$, $CuSO_4$, $ZnSO_4$ or $NaMnO_4$ inhibited chitinase production.

When the effects of mineral salts added at the concentration of 15 mg per 100 ml medium were observed, the additions of $CaCl_2$ or H_3BO_3 enhanced the chitinase production, but the additions of all the other mineral salts individually inhibited it.

Hence, increasing the concentrations of the salts $MnCl_2$, $CoCl_2$, $CuSO_4$, $ZnSO_4$, $NaMoSO_4$ inhibited chitinase synthesis in the mixed culture of *Arthrobacter* sp. with *A. pullulans*, while increasing the concentration, at least up to 15 mg/100 ml medium, of H_3BO_3 and $CaCl_2$ and, to some extent, $MgSO_4$ and $FeSO_4$ enhanced chitinase production (Table 22).

Effect of the Inoculum Size of A. Pullulans

As shown in Table 23, successively larger inocula of *A. pullulans* at the levels used here, resulted in increased levels of the chitinase production.

Effect of Adding A. Pullulans After Various Periods of Incubation of Cultures of Arthrobacter Sp. BN2

As shown in Table 24, the highest chitinase activity was observed when *A. pullulans* was added to the culture of *Arthrobacter* sp. after 24 h of incubation. Thereafter, the maximum chitinase production decreased as the time before inoculation of *A. pullulans* increased.

TABLE 22. Effect of different concentrations of several mineral salts on chitinase production by the mixed culture of *Arthrobacter* sp. BN2 and *Aureobasidium pullulans*.

MINERAL SALTS	cell yield (log_{10} # org/ml) Morrisey's Concent.		CHITINASE PRODUCTION: Chitinase Activity (Units)			Differential Rate (Chitinase Act/ log # mg/ml)	Specific Activity	
	BN2	*A. pullulans*	Morrisey's Conc.	3 mg/ 100 ml	15 mg/ 100 ml	Morrisey's conc.	3 mg/ 100 ml	15 mg/ 100 ml
H_3BO_3	8.30	7.75	14.0	10.0	54.0	1.69	0.36	1.95
$MgSO_4$	10.11	7.30	8.5	58.0	14.0	0.84	2.86	3.79
$MnCl_2$	9.89	8.04	20.0	24.0	3.0	2.02	0.87	0.54
$CoCl_2$	9.77	7.88	11.0	5.0	3.0	1.13	0.15	0.32
$CuSO_4$	9.67	8.10	26.0	15.0	15.0	2.69	0.81	0.81
$ZnSO_4$	9.64	8.12	20.0	18.0	3.0	2.07	0.39	0.54
$NaMoO_4$	9.59	8.11	32.0	7.0	5.0	3.34	0.25	0.14
$FeSO_4$	9.54	7.97	29.0	52.0	10.0	3.04	2.35	1.81
$CaCl_2$	9.54	8.11	38.0	24.0	30.0	3.98	1.44	1.62
All salts:								
(1)	10.18	7.0	40.0			0.98		
[a]			3.5					
CBM: (1)	9.45	8.08	26.0	26.0	26.0	2.75		
(2)			3.5				1.49	1.49
CBM-Yeast ext.	9.72	8.15	40.0			4.12		
CBM-glucose	9.15	8.21	26.0			2.84		
CBM-Yeast ext. and gluc.	9.48	8.15	32.0			3.38		

[a]Pure culture of *Arthrobacter* sp. BN2.

TABLE 23. Effect of the size of inoculum of *A. pullulans* on chitinase production by the mixed culture of *Arthrobacter* sp. with *A. pullulans*.

Inoculum size[a] (ml)	Chitinase production[b] (Units)
0	8.03
0.1	20.12
0.5	23.45
1.0	26.37
3.0	30.80
5.0	34.70

[a] *Arthrobacter* sp. BN2 inoculum: 5 ml of 10^7 cells/ ml suspension.

[b] Chitinase activity was read after 96 h of incubation.

TABLE 24. Chitinase production by the mixed culture of *Arthrobacter* sp. BN2 with *A. pullulans*[d] as a function of the time of incubation at which inoculum of *A. pullulans* was added to the growth of *Arthrobacter* sp. BN2.

Incubation Time (Hours)	Chitinase Production (Units)[a]
0	28.08
24	36.19
48	23.76
72	17.89
Control [b]	9.50

[a] Chitinase activity was measured after 96 h of incubation

[b] No addition of *A. pullulans* inoculum.

[c] Medium with 2.0 % chitin concentration.

[d] Inocula:

A. pullulans, 0.5 ml of 10^5 cells/ml suspension.

Arthrobacter sp. BN2, 5 ml of 10^7 cells/ml suspension.

Static and Shaking Cultivation Effect

As was demonstrated for pure culture, shaking cultivation allowed a higher chitinase production by the mixed culture than in static conditions. The chitinase production was greater after agitation of the culture at 200 rpm than at 100 rpm; chitinase levels for the mixed culture were higher when tubes, rather than flasks, were used as culture vessels (Table 17).

Effect of the Source and Degree of Purity of Chitin

The same type of influence observed in the pure culture was observed again in the mixed culture (Table 16). Consequently, crab chitin was a better source for chitinase synthesis than crayfish chitin; purified chitin was better than partially purified (demineralized), deproteinased and washed chitin. Examining the ratio of levels of chitinase synthesis by the pure culture to that by the mixed culture, the ratios corresponding to purified chitin powder (8.08), demineralized powdered crab chitin (9.33), washed powdered crab chitin (5.15), and partially purified powdered crayfish chitin (6.19) were higher than that for colloidal chitin (3.27). The highest chitinase production in both culture types was achieved from using colloidal chitin in the medium.

VI. Chitinase Production of *Arthrobacter* sp. BN2 as a Function of the Addition of Autoclaved or Live Culture of *A. pullulans* in Chitin and Non-Chitin Culture Media

In order to determine if viable cells of *A. pullulans* are needed for the enhanced chitinase synthesis in the mixed culture, *Arthrobacter*

BN2 was grown with viable or autoclaved cells of A. pullulans in presence or in absence of chitin as a carbon source in Morrisey's medium. As is illustrated in Table 25, the enhancement is observed only with viable cells of A. pullulans. However, chitin was also necessary for the high chitinase synthesis in the mixed culture, since Arthrobacter in mixed culture with A. pullulans in a non-chitin medium did not show the characteristic high chitinase biosynthesis observed in presence of chitin.

VII. N-Acetyl Glucosamine Uptake

If the mold partner must be alive to contribute to the enhancement of chitinase production in the mixed culture, how does the mold trigger the synthesis of chitinase of Arthrobacter sp. BN2? Is there a derepression of the synthesis of chitinase as a result of a rapid consumption of the by-product, N-acetyl glucosamine, by the mold? N-acetyl glucosamine has been shown to repress the synthesis of chitinase (Monreal and Reese, 1968; Morrisey et al., 1976). A large number of fungi use N-acetyl glucosamine to synthesize chitin, the main component of their cell walls. The accumulation of N-acetyl glucosamine in the culture fluid during the growth of Arthrobacter sp. BN2 was measured both alone and in mixed culture. During the first 5 days of incubation the concentration of N-acetyl glucosamine in the culture fluid was never higher than 5 μg/ml. On the other hand, cultures of the chitinolytic actinomycetes Streptomyces albus and S. griseus did show an ever increasing accumulation of N-acetyl glucosamine in the culture fluid.

To understand how molds trigger chitinase synthesis in Arthrobacter

TABLE 25. Chitinase production by *Arthrobacter* sp. BN2 under different associations with *A. pullulans*.

Organism	Chitin in medium	Chitinase Production (Units)
Arthrobacter sp BN2	Yes	12.0
Arthrobacter sp BN2	No	-
BN2 + *A. pullulans*	Yes	44.0
BN2 + *A. pullulans*	No	9.4
BN2 + autoclaved *A. pullulans*	Yes	11.7
BN2 + autoclaved *A. pullulans*	No	7.4

and other species, an extensive study of the kinetics of the synthesis of chitinase in both pure and mixed system is needed. One can begin by measuring the uptake rate of N-acetyl glucosamine by Arthrobacter sp. BN2, the chitinolytic bacterium BN1, Streptomyces albus, S. griseus as well as the molds A. pullulans, Aspergillus niger and Penicillium sp. by using a medium which contained N-acetyl glucosamine as unique carbon and nitrogen source. If the molds do enhance chitinase synthesis by a rapid uptake of N-acetyl glucosamine, thus limiting or eliminating its repressive effect, they should also show a relatively high uptake rate in a medium with N-acetyl glucosamine as the sole C and N source. As can be seen in Table 26, the data presented did not supply any important insight to either prove or disprove the hypothesis presented above. Rather, the results are ambiguous. The chitinolytic organisms seem to have a higher NAG intake than the molds in both pure and mixed culture. Of course, in a NAG medium where no chitinase synthesis can be detected, the results cannot be directly extrapolated to what would happen in a chitin medium where chitinase synthesis occurs.

VIII. Chitinase Production by Semisolid Fermentation

Semisolid fermentation has been demonstrated as having excellent advantages for the production of microbial enzyme. To test the ability to produce chitinase using this technique, the chitinolytic organisms Arthrobacter sp. BN2, Streptomyces albus, S. griseus and the bacterium BN1, as well as BN2 in mixed culture with some molds, were grown in Morrisey's medium with 20% chitin powder (w/v). Arthrobacter sp. in mixed culture with A. pullulans also was grown in Morrisey's medium

TABLE 26. N-Acetyl glucosamine uptake by some organisms in pure or mixed culture.

Organisms	PURE CULTURE: Maximum Intake Final conc./Initial Conc.	PURE CULTURE: NH_3 % of control	MIXED CULTURE: Organisms combinations	MIXED CULTURE: Maximum Uptake Final conc./Initial Conc.	MIXED CULTURE: NH_3 conc. (% of control)
Arthrobacter sp. BN2[a]	.357	32	BN2 + *Penicillium* sp.	.543	34
BN3[a]	.519	26	BN2 + *A. pullulans*	.413	37
			BN2 + *A. niger*	.398	24
Streptomyces albus[a]	.445	17	*S. albus* + *Penicillium* sp.	.632	30
			S. albus + *A. pullulans*	.406	38
Streptomyces griseus[a]	.750	25	*S. albus* + *A. niger*	.537	42
Penicillium sp.	.552	26	*S. griseus* + *Penicillium* sp.	.366	29
			S. griseus + *A. pullulans*	.469	24
Aspergillus niger	.727	40	*S. griseus* + *A. niger*	1.015	84
Aureobasidium pullulans	.979	90	Control: (uncultured medium)	1.000	100

[a]Chitinolytic organisms.

NOTE: Chitinase activity was also measured. No chitinase production was detected under the conditions used. The maximum uptake of NAG was reached after 4 days of incubation except for the molds tested in pure culture. These reached their maximum NAG uptake after 3 days of incubation. After 6 days of incubation *S. griseus* had eliminated their partners except *Penicillium* sp. which showed some but poor growth. *S. albus* did not kill off their partners but strongly limited their growth.

with 5% colloidal chitin. As shown in Table 27, a relatively high chitinase synthesis was observed when colloidal chitin was used. Of course, these are extremely preliminary experiments and more studies will be required to work out the possibility of using semisolid fermentation in the large scale production of chitinase.

IX. Chitinase Production in Crab-Protein Chitin Medium

Crab shells contain 30-45% protein material, thus during the deproteinization step in the purification of chitin relatively large amounts of proteinous material is discarded as a waste product. It was thought that this proteinous fraction may serve as a base solution to which chitin can be added for chitinase production. Table 28 presents the results of experiments using this protein fraction together with chitin powder in the production of chitinase by the chitinolytic organisms *Arthrobacter* sp., bacterium BN1, *Streptomyces albus*, *S. griseus* and the mixed culture of *Arthrobacter* sp. with *A. pullulans*. These data seem to be quite similar to those obtained using the normal mineral salts base-chitin medium of Morrisey (Table 28).

X. Chitin Hydrolysis Rate of the Pure Culture of *Arthrobacter* sp. BN2 with *Aureobasidium pullulans*

Table 29 shows the rates at which pure and mixed cultures of *Arthrobacter* sp. BN2 can hydrolyze colloidal chitin during incubation for 4 days in 100 ml medium placed in 1000 ml bottled flasks. The flasks were incubated on a shaker at 25.5 C. This hydrolysis proceeds at a higher rate when *Arthrobacter* sp. BN2 and the chitinolytic fungus F1 were

TABLE 27. Chitinase production by semisolid fermentation.

Organisms	Chitinase Activity (Units)	
	4 days	7 days
Arthrobacter sp. BN2[a]	9.0[b] (9.0)[c]	1[illegible].0 (12.0)
BN2 + *Penicillium* sp.	16.0	17.5
BN2 + *Aspergillus niger*	17.5	17.0
BN2 + *Aureobasidium pullulans*	11.0 (36.0)	20.0 (42.0)
BN2 + *Volvariella volvaceae*	12.0	10.0
Bacterium BN1[a]	8.0	8.0
Streptomyces albus[a]	12.0	10.0
Streptomyces griseus[a]	9.0	14.0

[a] Chitinolytic organisms.

[b] These values correspond to the chitinase activity in powdered chitin.

[c] These values correspond to the chitinase activity in colloidal chitin.

TABLE 28. Chitinase production in crab protein-chitin medium.

Organisms	Chitinase Activity (Units)
Arthrobacter sp.BN2	8.0
Bacterium BN1	4.0
Streptomyces albus	7.0
Streptomyces griseus	11.0
Arthrobacter sp. BN2 + *Aureobasidium pullulans*	38.0

Note: Chitin added at 2 % (w/v). Incubation was at 25.5^{o} C for 4 days.

TABLE 29. Chitin hydrolysis. Rate of *Arthrobacter* sp. BN2 in pure and mixed culture.

Organisms	Rate (μg chitin hydrolyzed per min per ml)
Arthrobacter sp. BN2	40.82
Arthrobacter sp.BN2 + *Aureobasidium pullulans*	64.69
Arthrobacter sp. BN2 + Fungus F2[a]	69.60
Control[b]	-

[a] Fungus F2 is a exocellular chitinase rroducer

[b] No inoculated

Note: Cultures were grown in baffled 1000 ml flasks at 25.5 C for four days. Medium contained 2 % colloidal chitin .

TABLE 30. Survival of *Arthrobacter* sp. BN2 and *Penicillium* sp. in different media.

Media	Survival Time (Hours)			
	Pure culture		Mixed Culture	
	Arthrobacter sp. BN2	*Penicillium* sp.	BN2	*Penicillium* sp.
Chitin agar (broth)	90	5	15	5
Enriched nutrient agar (broth)	3	7	3	90
Soil extract- chitin powder (60% humidity)	90	90	90	7

The media were incubated for 48 h and then stored at 4° C.

grown together than when *Arthrobacter* sp. was grown alone or with *Aureobasidium pullulans*.

XI. Survival of Arthrobacter sp. BN2 in Chitin Medium

As is illustrated in Table 30, *Arthrobacter* sp. BN2 died off rapidly when it was maintained in an enriched nutrient medium either alone or in mixed culture with *Penicillium* sp. On the other hand, *Arthrobacter* sp. BN2 was able to survive for considerable time in soil extract-chitin powder medium both in pure and mixed culture with *Penicillium* sp.; it remains viable during the 3 month period that it was checked. However, *Penicillium* sp. remained viable in soil-extract-chitin powder only without *Arthrobacter* sp. BN2. Thus, soil extract-chitin powder medium (60% relative humidity) may be useful in the conservation of chitinolytic organisms. Also, it is apparent that chitinolytic organisms may control the growth of other organisms in natural soil environments.

DISCUSSION

Several workers have selected chitinolytic organisms using pure microbial isolates directly from microbial culture collections (Chigaleichik, 1976 ; Clarke and Tracey, 1956; Tiunova et al., 1971; and Tracey, 1955b) while others have selected chitinolytic microorganisms from soils, seawater or marine muds and by means of the enrichment technique (Reynolds, 1954; Benton, 1935; Campbell and Williams, 1951; Brisou et al., 1964; and Zobell and Rittemberg, 1938; Benecke, 1905; Rammelberg, 1931). Most of these authors recommend incubating the flasks for at least 4 weeks before discarding them while those who investigate chitinolytic activity with pure cultures on agar plates or under shaking conditions incubate flasks or plates for 4 to 10 days.

Our results from the screening for chitinolytic organisms in soil clearly show that the direct plating technique is much quicker and more efficient, giving larger numbers of chitinolytic organisms than the enrichment technique. In addition, the direct plating technique is less tedious, less time consuming, and involves the use of less laboratory material than the enrichment technique. Most of the chitinolytic organisms obtained during this study needed up to 4 days to show their chitinase-producing property when the direct plating technique was employed, while most of the chitinolytic organisms obtained with the enrichment technique needed about 2 weeks of incubation to show turbidity (growth) and at least 5 additional days to show their chitinolytic capacity on chitin plates. Hence, when searching for highly efficient chitin-degrading isolates the direct plating technique may be the best choice.

It is not surprising that most of the chitinolytic cultures isolated were members of the Actinomycetales, since chitin agar, the medium used to detect chitinase activity by the direct plating technique, has demonstrated superior selectivity to any other media for isolating actinomycetes from water and soil (Kuznetsov and Yangulova, 1970; Lingappa and Lockwood, 1962; Sykes and Skynner, 1973; and Hsu and Lockwood, 1975). The selectivity of chitin agar is based on the near universal ability of actinomycetes to hydrolyze this carbohydrate, unlike most bacteria and fungi. Jeauniaux (1955) reported that 98 of 100 isolates of Streptomyces tested produced chitinase. Noveroske (1959) showed that all 38 isolates of Streptomyces tested utilized colloidal chitin as a sole carbon and nitrogen source in chitin agar. Hsu and Lockwood (1975) demonstrated that more than 85% of actinomycetes growing on colloidal chitin agar developed chitinolytic activity. Tiunova et al. (1971) found that 70 out of 100 strains of actinomycetes were able to synthesize and release chitinase into the medium.

On the other hand, the frequency of chitinase production in microorganisms as a whole is lower than that of actinomycetes. Tominaga and Tsujisaka (1976a) detected 16 chitinolytic organisms among 170 tested. Chigaleichik et al. (1976) found eight chitinolytic strains among 50 different bacterial cultures tested. The fungal strains isolated were relatively few, and several explanations for this have been postulated. Fewer fungi than actinomyces produce exochitinases. Monreal and Reese (1968) reported 7 chitinolytic fungal cultures from 70 which were tested. Tiunova et al. (1973b) found 16 chitinolytic organisms out of 44 fungal cultures. Also, the chitin medium used here may not be nutritionally

adequate for the normal growth of chitinolytic fungi. Moreover, fungi usually grow more slowly than bacteria and actinomycetes, especially in agar media, compared to bacteria and actinomycetes. It must be noted, in addition, that chitinase activity was examined in the agar plates only during the first 7 days of growth. The relative population of chitinolytic bacteria, actinomycetes, and fungi per gram of soil cannot be discussed without regarding other environmental factors such as temperature, pH, relative humidity, type, etc. of the soil samples. Jensen (1932), Skinner and Dravis (1937), Veldkamp (1955), Okafor (1966), and Gray and Bell (1963) have directed ecological studies of microorganisms thriving on chitin buried in soil samples from different areas with regard to the effects of environmental factors using the direct microscopic technique or the dilution method. At least it can be concluded that chitinolytic microbes seem to be relatively abundant in soil. Indeed, this follows if one recalls that chitin is one of the most abundant naturally occurring polymers in soil and waters. Jensen (1932), Veldkamp (1955), and Okafor (1966) report numbers that range from 10^5 to 10^8 cells per gram soil which are in accordance with those reported here.

As demonstrated on chitin agar, the 56 chitinolytic organisms obtained during this study displayed a wide range of chitinase activity. The intensity of chitinase activity as judged from the width of the zone of clarification (in mm) around the colonies covers values from 0.5 to 5.5 mm. The chitinase activity was measured during the first 7 days of growth.

Reynolds (1954), using the enrichment technique and after plating

out his positive (showing growth) enrichments on chitin agar, was able to isolate 41 pure cultures of chitinolytic organisms. He then selected the most active organisms based on the percent of chitin degraded from the medium at different intervals during growth on shaking conditions. It appears that this screening procedure is quite complicated since it involves a series of steps including alkalinization of cultures, use of precisely weighed amounts of chitin powder for each flask, sterilization of flasks after adequate incubation, collection of the alkali-insoluble residues. The residues are then washed, dried, ignited for 8 h at 650 C, and reweighed for ash. Monreal and Reese (1969) selected the most active chitinolytic organisms among 70 fungi and 30 true bacteria and actinomycetes by growing these organisms in shaking conditions and determining the maximum chitinase activity by measuring the reducing sugar produced during the enzyme assay. Tiunova et al. (1973, 1971) studied the chitinase production of 46 strains of several genera of fungi, 36 strains of true bacteria and 100 strains of actinomycetes by the agar block method (Dingle et al., 1953). This process consists of transferring agar blocks (10 mm in diameter) obtained from plates with 7-10 day old cultures into cuvettes of plexiglass which are then filled with a suspension (0.1 - 0.5%) of the test substrate (crab shells or colloidal chitin). They detected chitinolytic areas with radius from 2 to 5.5 mm for actinomycetes, 0.5 to 3.5 mm for fungi, and 0.5 mm for bacteria. Comparing the results of Tiunova et al. (1973, 1971) with data reported here with respect to chitinolytic activity on chitin plates, there is a good correlation in relation to actinomycetes and fungi but

apparently not with bacteria. However, it must be recognized that differences exist between the agar block method and the methods used in this study.

The optimum maximum and minimum growth temperatures obtained for Arthrobacter sp. BN2 on chitin medium coincide exactly with that reported for the genus Arthrobacter in the eighth edition of Bergey's Manual (1975). No data were found in the literature concerning the maximum, minimum, and optimum growth temperature or growth rate of any Arthrobacter sp. on chitin-containing media. It has been observed that the cell yield of Arthrobacter sp. BN2 varied only slightly when grown in Morrisey medium whether lacking chitin, containing different concentrations of colloidal or powdered chitin, or on enriched nutrient agar. However, the isolate remained viable for a long time in chitin-containing media while it died off within 3 days in non-chitin media such as an enriched nutrient medium and Brain hearth infusion medium, in which never grown.

Generally, the optimum temperature profiles for growth and chitinase biosynthesis of the isolate Arthrobacter sp. BN2 almost coincide with each other. This leads us to believe that chitinase production is closely correlated with the capacity of cells to grow. This is not surprising if chitinase synthesis is seen as a process directly associated with the metabolic processes responsible for providing essential carbon and nitrogen intermediates to the cell. These intermediates rise from the hydrolysis of chitin since it is present in the culture medium as a unique carbon and nitrogen source. Consequently, the rate of chitinase biosynthesis and chitinase activity may be limiting the magnitude of the growth rate and viability of the cells. The intrinsic regulatory

processes of the cell, in turn, are controlling the rate of chitinase biosynthesis in harmony with the regulation of the biosynthesis of the other enzymatic systems involved in the complete metabolization of chitin.

It is interesting to note that significant chitinase production is observed at temperatures between 18 and 24.5 C while total growth and growth rates are relatively low at that temperature range. At these low temperatures, the metabolic processes might still be working but at lower rates than at the optimum temperatures (25.5 - 33.5 C), thus chitinase may be produced in order to allow cells to degrade chitin and make suitable intermediates needed to sustain the normal metabolic processes. Moreover, chitinase may be synthesized by those cells that have lost their capacity to multiply, but capable of proceeding with vital metabolic processes involved directly with survival, so that the proportion of chitinase biosynthesis to the number of viable cells at those low temperatures appears large. Okafor (1965) has reported that the rate of chitin decomposition and the frequency of organisms in buried chitin in soil are well correlated, but the rate of chitin decomposition and the frequency of chitinolytic organisms are higher at 10 C than at 29 C. Therefore, chitinolytic organisms whose normal habitat is the soil (Arthrobacter sp. BN2 was obtained from soil) give better yields of chitinase at the soil temperatures, under natural conditions. This might be a result of an evolutionary adaptation of mesophylic chitinolytic organisms to the soil conditions that furnish them with the capacity to thrive efficiently in an environment where the competition between organisms is at a maximum.

On the other hand, chitinase production is relatively low even though cells apparently multiply at an optimum when grown at temperatures higher than 25.5 C. However, the opitmum growth rate was observed at these temperatures only at the early incubation times (24 h) and thereafter the growth rate dropped abruptly.

No report was found in the literature concerning the optimum temperature for chitinase biosynthesis in Arthrobacter. Monreal and Reese (1968) determined the enzyme yields of three mesophilic chitinolytic organisms, Serratia marcesccens, Enterobacter liquefaciens, and Aspergillus fumigatus at three temperatures (25 C, 30 C and 35 C), and were able to demonstrate that the yields of enzyme were higher at 30 C than at 25 C or 35 C. Arthrobacter sp. BN2, a mesophile as well, yielded higher amounts of enzyme at 25.5 C than at any other temperature of incubation tested. Chigaleichik et al. (1976b), studying the influence of 30 and 35 C temperatures on the growth and biosynthesis of chitinase of Achromobacter liquefaciens 301, under conditions of periodic cultivation in a fermentor, have found that enzyme biosynthesis paralleled growth and development of the cells and that chitinase synthesis was higher at 30 than at 35 C.

In reference to the effect of pH on growth and chitinase biosynthesis of Arthrobacter sp. BN2, it was found that the optimum pH levels for its maximum biomass yield, maximum growth rate, and maximum chitinase production fell between 6.0 and 7.0. It appears then, that growth and chitinase biosynthesis of Arthrobacter sp. BN2 are affected by the pH of the culture medium to a similar degree. Chigaleichik et al. (1976b),

studying the influence of pH on the growth and synthesis of chitinase in *Achromobacter liquefaciens*, were able to demonstrate that a weakly alkaline pH value of the buffered medium promoted an increase in chitinase production, yet with a slightly acidic initial pH, the culture was less active, and at the more acidic pH of 5.5, it did not synthesize chitinase at all. They also observed that maximum chitinase production was reached at pH values of 7.0 or 8.0 depending upon the type of culture medium used. In addition, they demonstrated that the beginning of exponential growth was accompanied by acidification of the medium, subsequently, however, the pH of the medium would increase to 8.0 - 8.8. The same phenomenon was observed with *Arthrobacter* sp. BN2, with the final pH of the medium, after 96 h of incubation, larger than the initial one (about 6.4). On the other hand, Morrisey *et al.* (1976), using *Arthrobacter* sp. P35, observed that the pH of the medium fell even though they used a phosphate buffer with a high ionic strength. Their final pH values nevertheless were never lower than 5.65. The discrepancy between Morrisey's *et al.* (1976) data and those presented here may be due to differences in the type of buffer employed, the composition of the culture media, and the strains themselves. Indeed, Morrisey *et al.* used media that contain other carbon sources besides chitin. Finally, Monreal and Reese (1968) observed that the optimum pH for enzyme production was about 7.5 for *Serratia marcescens*, 7.0 for *Enterobacter liquefaciens*, and 4.5 for *Aspergillus fumigatus*.

Taking into account the optimum incubation time for chitinase biosynthesis of *Arthrobacter* sp. BN2, generally around 4 days, it is in

very close agreement with those reported for other relatively active chitinolytic organisms with slight differences ascribed to cultivation conditions and species differences. Maximum concentration of extracellular chitinase was reached on the fourth day of growth for *Arthrobacter* sp. P35 (Morrisey *et al.*, 1976), on the sixth day for *Serratia marcescens* QMB1455 (Monreal and Reese, 1969), on the fourth day for *Streptomyces* sp. 2B (Skujins *et al.*, 1970), on the sixth day for *Streptomyces* sp. C-10 (Reynolds, 1954), on the fourth day for *Actinomyces* (*S.*) *kurssanovi* (Tiunova *et al.*, 1976a), on the fourth day for 44 *Streptomyces* isolates (Tiunova *et al.*, 1976b), on the fifth day for *Achromobacter liquefaciens*, *Serratia marcescens* B63 and *S. marcescens* B68 (Chigaleichik *et al.*, 1976b), on the twelveth day for *Streptomyces albidoflavus*, and between the seventh and nineth day for the fungus *Chytriomyces hyalinus* (Reisert, 1972).

It is interesting to note that once the peak chitinase activity was reached by *Arthrobacter* sp. BN2 it fell sharply thereafter although the culture continued to increase in total mass. The same phenomenon has been reported by Monreal and Reese (1969), Skujins *et al.* (1970) Sundaraj and Bhat (1972), Reisert (1972), Tiunova *et al.* (1976b, 1976c), Reynolds (1954) and Tracey (1955). The sharp decrease in chitinase activity after reaching maximum production could be explained by both inactivation of the enzyme already present in the medium and a rapid repression of its synthesis. Inactivation of extracellular enzymes has been reported to be a very common process in bacteria (Switzer, 1977). On the other hand, Morrisey *et al.* (1976) have reported that the maximum activity for *Arthrobacter* sp. P35,

once reached, was maintained for 7 days. In the case of the Arthrobacter sp. P35 used by Morrisey et al. (1976), the rate of chitinase production might be limited only by the rate of uptake of the hydrolytic products, so that the maximum enzyme production probably results from a steady state condition, where only the repression control is playing a role, not enzymatic inactivation.

The observation that the optimum incubation time for growth rate, biomass yield and chitinase biosynthesis are distinct from one another for Arthrobacter sp. BN2 seems to reflect a common pattern encountered among microorganisms with the capacity to synthesize hydrolases. Hydrolases specific for large macromolecules as the sole C source are synthesized gradually once the organism reaches its exponential phase. Generally, chitin media contains very low amounts of glucose or yeast extract to aid the organism beginning growth. It should be mentioned that while chitin is present in the culture medium, a positive growth rate was observed, otherwise the progressive accumulation of cell biomass stops and death is rapidly apparent.

The results in this study showing that chitinase biosynthesis increases in both pure and mixed culture conditions of Arthrobacter sp. BN2 as the total concentration of chitin in the medium is increased agrees with results of Monreal and Reese (1969) for S. marcescens QMB1455, Enterobacter liquefaciens CDC 214951 and Aspergillus fumigatus QM45. They used chitin concentrations of 2 and 3%. However, our data only correlates partially with those of Morrisey et al. (1976) for Arthrobacter sp. P35. They demonstrated that when the concentration of chitin was raised from .25% to 1.0% an increase in chitinase

production was observed, but in raising the chitin concentration to 2% a dramatic decrease in chitinase production was detected. This difference between the two Arthrobacter strains can be explained by enzyme synthesis regulation differences.

Among the mineral salts tested, Fe and Co enhance chitinase production in Arthrobacter sp. BN2. This may indicate that these metal ions are playing a primary role either in the biosynthesis of the enzyme or integrating the active enzyme as cofactors. It is important to note that in order to avoid secondary effects of the metal ions during the enzyme assay, the culture supernatants were dialyzed against distilled water at 4 C for 24 h. The literature contains only reports about the effects of mineral salts on chitinase activity, not on chitinase biosynthesis. For instance, Skujins et al. (1970) have found that Co and Zn in concentrations below 0.001 M had a slight activation effect of chitinase in Streptomyces sp. 2B, and Tominaga and Tsujisaka (1976) have shown that Ba, Ni, Co, and St slightly activate the chitinase system in Streptomyces orientalis.

The data from the pure culture of Arthrobacter sp. BN2 and mixed culture with A. pullulans indicate that the concentration and type of metal ion can either stimulate, inhibit or not affect chitinase production, yet the effects are quite similar for both pure and mixed cultures, at least, at the Morrisey's concentrations. The effects of the mineral salts at the concentrations 3 mg/100 ml and 15 mg/100 ml only were tested in the mixed cultures. It is interesting to observe that Ca and B at all three concentrations used, and Mg and Fe at the

Morrisey's concentrations, favored chitinase biosynthesis in mixed culture. How these metal ions promote a better expression of chitinase biosynthesis in mixed culture is difficult to explain and its elucidation will need in depth studies at the molecular level. These ions, perhaps are exercising their respective influence by chelation reactions, precipitation of noxious by-products, or by direct participation in the production of the active enzyme. For instance, Skujins *et al.* (1970) have found that four atoms of Ca are bound to each molecule of chitinase produced by *Streptomyces* sp. 3C. Moreover, Ca ions appear to have a role in determining the electrokinetic potential of the colloidal substrate particles thus increasing the enzymatic reaction rate (Bangham-Dawson, 1962; Kurioca and Liu, 1967). On the other hand, activities of *Lycoperdon* sp. and *Aphanomyces astaci* chitinases are not affected by the loss of Ca (Tracey, 1955; Unestam, 1968).

To ascertain whether or not the effects on chitinase production by the addition of minerals alts to the complete medium are the result of a direct effect on growth and subsequent chitinase production, differential rates (Monod Rate) were calculated. The differential rate values obtained, clearly showed that differences on chitinase production were due to differences in the enzyme biosynthesis process rather than to differences in growth rate or to total cell yields (differences in cell densities). However, one cannot completely rule out the possibility of a direct role the ions may have on growth or other metabolic processes of the organism since this has not been investigated.

The addition of all mineral salts to the complete basal medium

did not alter the level of chitinase synthesis as observed in the complete base medium alone. This may be explained as a result of competitive and/or non-competitive inhibition between the ions favoring chitinase production and those unfavorable to it, in such a way that the net result would be an insignificant or total absence of the individual effects of the metal ions on chitinase biosynthesis.

Apparently there exists a controversy concerning the enhancement of chitinase biosynthesis obtained by removal of yeast extract from the complete basal medium. It should be remembered that yeast extract was added at a very low concentration (0.1%) to serve as a growth initiator. Reynolds (1954) had shown that a number of chitinolytic bacteria which could grow with chitin as a sole carbon and nitrogen source were much more effective in decomposing the polysaccaride when yeast extract was present. At the same time, he also has shown that the chitinolytic Streptomyces sp. C10 did not respond to yeast extract supplementation. If a dense, exponentially growing culture is used as an inoculum, the role of yeast extract will be obviated and chitinase synthesis induction will occur much earlier during growth. Another explanation could be that if yeast extract is removed from the complete base medium then glucose, which is still present in the medium, will participate as the initiator of growth, and be consumed at a faster rate than it would be if added together with yeast extract. Glucose is a recognized repressor of chitinase synthesis (Monreal and Reese, 1962; Morrisey et al., 1976). Its faster consumption will liberate the organism from glucose repression at an earlier time. Still, with the removal

of yeast extract and a normal glucose consumption, derepression may result anyhow since chitin will be the only nitrogen source for the organism. This type of derepression, in the presence of glucose but in the absence of a nitrogen source, has been recognized in several degradative systems such as hystidase (Hut) and proline oxidase systems (Prival and Magasanik, 1971). However, Chigaleichik et al.(1976b),using a medium which contains demineralized crab shells, phosphates, Mg_2SO_4, yeast extract and tap water, have found that the addition of peptone to the medium has a positive effect on chitinase synthesis by Achromobacter liquefaciens and that a mixture of amino-acids or casein hydrolysate did not replace peptone. The circumstances in the report of Chigaleichik et al., are quite different to those of this study. They did not use pure chitin as substrate, and consequently,peptone may be inhibiting the effects of toxic products present in the demineralyzed crab shells, by means of chelation reactions or other unknown mechanisms. It may also act as a buffer to avoid a rapid increase of the pH of the medium. When crab shells partially purified by demineralization were used, the pH of the culture medium always increased rapidly, perhaps as a consequence of rapid production of potent anions during the degradation of the protein fraction from the crab shells.

The fact that chitinase production of Arthrobacter sp. BN2 both in pure and mixed culture with A. pullulans approached very similar values regardless of the size of the inoculum, can be explained if the growth rate of the chitinolytic culture is taken into account. It has been shown that with an initial inoculum of 0.1 ml to 5 ml of an Arthrobacter sp. BN2 culture containing about 3×10^5 cell/ml

and a growth rate of 0.87, stationary phase is reached within 24 h of incubation. After 96 h of growth, when significant levels of chitinase production are detected, the numbers of cells likewise involved in chitinase synthesis may be very close even though each flask received a different inoculum. In fact, the total cell numbers after 96 h of growth were found to be very much similar in all flasks. In the mixed culture, Aureobasidium pullulans does not alter or at least not at very significant degree, the normal growth rate of Arthrobacter sp. BN2 since the chitinase production under these conditions also reached similar values at different inoculum sizes of Arthrobacter sp. BN2. Therefore, given the relatively high growth rate of Arthrobacter sp. BN2, after 96 h of incubation the culture will be in stationary phase, regardless of inoculum size and, subsequently, the potential for chitinase production is the same.

Since chitin in crab and crayfish, like in all arthropodal shells, is complexed with (possibly covalently bounded to) an equal or smaller amount of protein, it is protected more from direct enzymatic hydrolysis than chitin in purified form. Thus, purified chitin is expected to be better a substrate and inducer of chitinase biosynthesis. Although the concentration, texture and complexity of the chitin from arthropodal exoskeleton depend on the sex, age, and molt stage as well as the species, it has been determined that crab shells contain 12 - 30% of chitin (Spindler-Barth, 1976), while crayfish contains from 9.1 to 16.3% of chitin (Stain and Murphy, 1976). Therefore, the larger amounts of and, perhaps, less complexed chitin present in crab shells

may account for the higher chitinase production obtained using crab shells than crayfish shells. Chigaleichik et al. (1976b) have observed very low chitinase production using crab shells and comparable levels on demineralized crab shells and native (powdered) pure chitin for three isolates, Serratia marcescens B63, S. marcescens B68, and Achromobacter liquefaciens 301. Tiunova et al. (1976b) have found that the chitinase production by 44 strains of Streptomyces was not less and sometimes greater if demineralized crab shells were used instead of native chitin. Morrisey et al. (1976) have observed that grinding chitin flakes in a Wiley mill or adding Tween 80 to the culture medium substantially increased chitinase production by Arthrobacter sp. P35. They also found that production of chitinase with 16 mesh milled chitin was greater than with colloidal chitin. They claimed that the greater enzyme synthesis probably resulted from increased surface area of the flakes by milling and/or the addition of Tween 80, and, therefore it was more accessible to enzymatic attack. In this study, chitin 24 mesh was always used. Morrisey et al. (1976) obtained slightly lower chitinase synthesis with 24 mesh chitin than 16 meshed. They attribute it to the tendency of the finer particles to adhere to the walls of the flasks which could be overcome by the addition of Tween 80. Tiunova et al. (1976b) observed that the enzyme catalyzing the degradation of ground chitin begins to accumulate in the medium earlier than the enzyme decomposing colloidal chitin (24 h of fermentation in comparison with 36 h). Yet after 48 h of growth, the enzyme activity for native chitin decreased while the activity using colloidal chitin

continued to increase for additional 66 h of fermentation. They confirmed, with these observations, the presence of a special enzyme (CHl), necessary for the cleavage of native chitin. Monreal and Reese (1968) have obtained low chitinase yield on mushroom chitin and on beetle (Tribolium) chitin, a better yield on shrimp chitin, but by far the best yield was on commercial milled chitin and colloidal chitin for the isolate S. marcescens QMB1455.

The advantages of shaking cultivation over static conditions of growth are evident in high cell yields, or rapid and large accumulation of desired end-products. The results obtained in this study with regard to the use of shaking cultivation for chitinase production by Arthrobacter sp. BN2 in both pure and mixed culture, are in keeping with the benefits stated above. Most of the studies regarding chitinase production have been carried out under shaking growth conditions (Tiunova et al., 1976b, Monreal and Reese, 1968, Morrisey et al., 1976, etc.). It is interesting to note that Reynolds (1954) demonstrated that with static incubation the amount of chitin breakdown after a month was only a small fraction of that obtained with shaken flasks of two Streptomyces and a true bacterium in 5 days of growth.

On the basis of preliminary results presented here, semisolid fermentation offers some promise for large-scale production of chitinase, especially when using the mixed culture system and colloidal chitin as a substrate. Semisolid fermentation is an advantageous method in the production of microbial enzymes or secondary metabolites because of the less complicated methods required for isolation and purification of the

desired from that fermentation (Johnson, 1971: Hesseltine, 1972).

The similar enzyme yields obtained using Morrisey's medium and the alkali-soluble crab protein chitin medium suggest that the latter may be promising for use of a material that otherwise is a residue from the purification of chitin. As has already been mentioned, demineralized crab shell, thus, chitin-protein complex, has been proved to be an adequate substrate for chitinase production by several microbial cultures (Chigaleichik et al., 1976b; Tiunova et al., 1976b).

When the rate of chitin hydrolysis was calculated for the isolate of Arthrobacter sp. BN2 in pure and mixed culture with A. pullulans or or the fungus F1 (an extracellular chitinase producer), Arthrobacter sp. BN2 alone would be able to hydrolyse about 1.4 g chitin/day/100 ml medium, and in mixed culture, 8.8 and 10 g chitin/day/100 ml medium, respectively. This is under shaking conditions, with 2% colloidal chitin as a substrate, in 100 ml medium in 1000 ml flasks, incubation at 25 C and at the maximum chitinase activity. Reynolds (1954), testing 41 different isolates for their ability to decompose powdered chitin (0.5% w/v) in agitated culture, found that the amount of chitin decomposed ranged between 10 and 97% of the starting material after 15 days of incubation. Therefore, there appears to be a great variation between cultures with reference to their ability to degrade chitin.

Regarding the maximum chitinase activity, the rate obtained for Arthrobacter sp. BN2 (12 units) is close to those reported for Streptomyces albidoflavus (about 15 units, Tracey, 1955), Streptomyces kursanovi (8.88 units , Tiunova et al., 1976 a), Achromobacter liquefaciens

(12.2 units, Chigaleichik, 1976b), and Aspergillus niger (8.33 units, Otakara, 1962). Relatively lower maximum chitinase activities have been shown, as well, such as, for Arthrobacter sp. P35 (3.0 units, Morrisey et al., 1976), Enterobacter liquefaciens (1.54 units, Monreal and Reese, 1969), Serratia marcescens (1.42 units), Aspergillus fumigatus (1.46 units), and Cytophaga johnsonii (5.10 units) (Sundarraj and Bhat, 1972). It has to be recognized that most of the enzyme assays in these reports differ from each other on the methods employed; thus, the data presented in the original reports have been converted to the units of chitinase production as presented in this study. In addition, other accessory differences may be a consequence of heterogeneous culture conditions and individual characteristics of the diverse strains. However, the maximum chitinase synthesis observed in the mixed culture of Arthrobacter sp. BN2 with several molds is quite larger than any reported in the literature for single cultures.

Several procedures and techniques have been proposed to improve both chitinase biosynthesis and enzyme activity. These improvements have mainly included modifications of the environment, culture medium or substrate, or conditions under which organisms are grown. For instance, Morrisey et al. (1976) found that addition of Tween 80 and grinding of the chitin flakes readily increase the enzyme yield. Chigaleichik et al. (1976b) have observed that enzyme production not only is plausible but also at a high rate when continuous cultivation was used. Tiunova et al. (1976) detected that the highest chitinase activities of Streptomyces kurssanovii can be obtained when veronal

buffer is employed. Nakagama et al. (1966) showed that by dialyzing the culture fluid against a phosphate buffer before determining chitinase activity, caused an increase in the level of enzyme activity. Skujins et al. (1970) have shown the importance of Ca ions for the maximum activity of chitinase of Streptomyces sp. However, all these procedures have led only to relatively low increases in chitinase production and activity.

The large enhancement in chitinase biosynthesis achieved by growing the chitinolytic Arthrobacter sp. BN2 in mixed culture with some molds is considered the most outstanding finding of the data presented here. When chitin was used at the concentration of 2% in the culture medium, the biosynthesis of chitinase was triggered about 13 times by Aspergillus niger after 8 days of growth, 12 times by Penicillium sp. after 4 days of growth, and 8 times by Phaneochaetae chrysosporium and Aureobasisdium pullulans after 4 days of growth.

Using a chitin concentration of 3%, and adding a large inoculum of mold after incubating Arthrobacter sp. BN2 for 24 h result in the optimum conditions found for the maximum enhancement of chitinase synthesis in the mixed culture of Arthrobacter sp. BN2 with A. pullulans. It is important also to note that the degree of enhancement varied according to the source and purity of the chitin substrate; chitinase biosynthesis was enhanced 9.33 times when demineralized crab shells were employed, and 8.08 times with pure powdered chitin.

The most likely and simplest explanation for such a great enhancement of chitinase biosynthesis in mixed culture, was thought to be that of the mold efficiently "scavenging" N-acetyl glucosamine thus avoiding

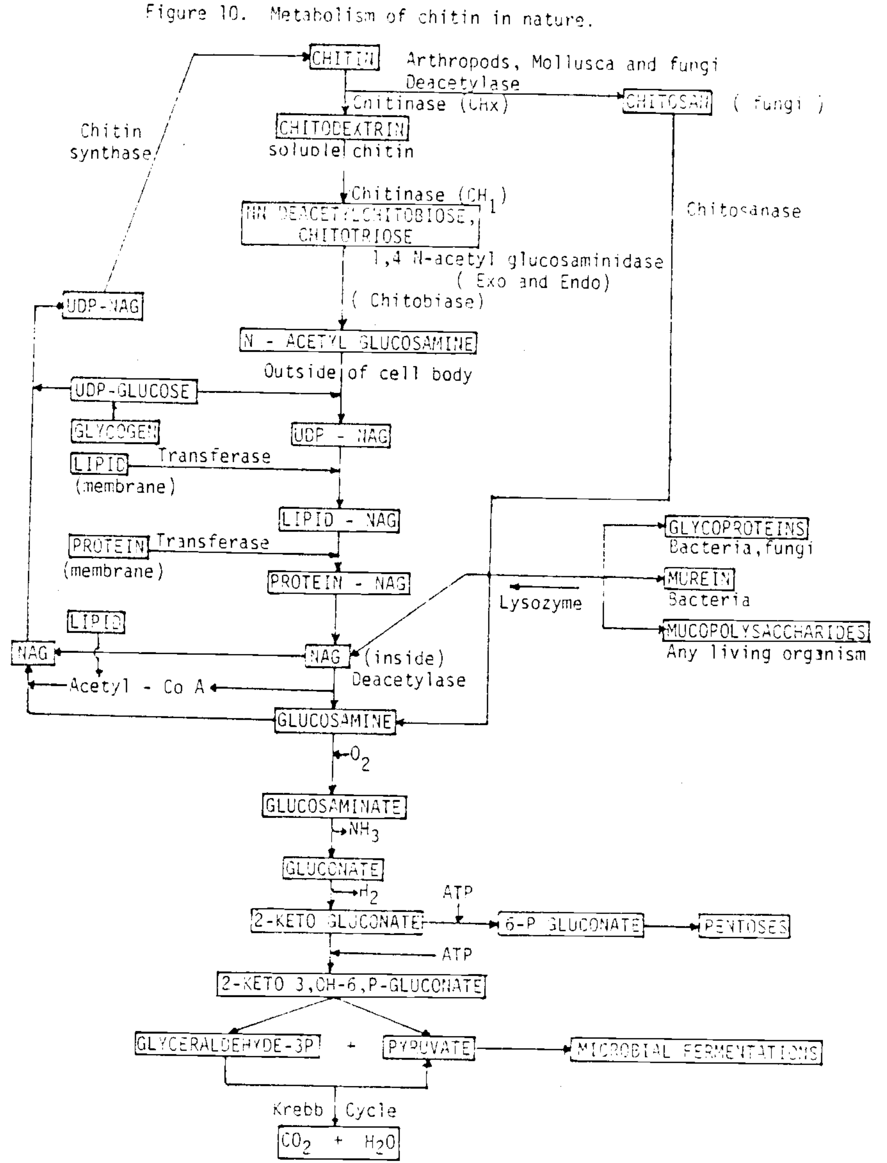
its accumulation in the fluid medium which normally acts as a repressive control of chitinase synthesis once NAG reaches the interior of the cells. However, no lucid mechanisms could be deduced when the NAG uptake was determined both in pure and mixed cultures using NAG as a unique carbon and nitrogen source. Nevertheless, this hypotehsis cannot be ruled out if one realizes that the results may be different when the molds are grown in NAG medium with a mixed culture from those grown in chitin medium. For example, since chitinase synthesis is not observed in the NAG medium, it might be possible that, in the presence of chitin, the chitinolytic bacterium produces chitinase at normal rate which will disrupt the cell wall of the fungal partner: When the fungus rebuilds its cell wall, its chitin synthesis mechanisms are accelerated and, consequently, the fungus will increase the uptake of NAG from the medium (Iten and Matile, 1970; Cohen, 1974; Christias and Baker, 1967). In addition, chitin synthesis and thus NAG uptake may be also increased when chitinase acts as a natural excitation of germination or apical growth of the mold, as has been shown for some species by Middlebrok and Preston (1972) and Ortega et al. (1974). There is also the possibility that chitinases, and other hydrolytic enzymes of Arthrobacter sp. BN2 may produce or induce fungal autolysis with subsequent fungal chitinase liberation. Lysis of fungal cells by chitinolytic organisms has been proved extensively (Tanaka et al., 1970; Janzen and Wessels, 1970; Tanaka and Phaff, 1965; Berthe et al., 1976; De Vries and Wessels, 1972; Jackson and Gay, 1976; Mitchell and Alexander, 1961; Wessels and Marchant, 1974; and Lloyd

et al., 1965). Chitinases have also proved to be instrumental in the release of wall-bound enzymes (Chang and Trevithick, 1972). Since it is recognized that most of fungal chitinases are intracellular, and perhaps wall-bound, bacterial chitinase may provoke the release of the fungal chitinases resulting in a higher chitinase concentration in mixed culture conditions. Pegg and Vessey (1973) and Vessey and Pegg (1973), for example, have not only proved that *Lycopersicon esculentum* (tomato) and the fungus *Verticillium albo-atrum* are chitinase producers, but also that when tomato plants were infected with *V. alboatrum*, chitinase synthesis increases significantly in both tomato and fungus.

The reasons for enhanced chitinase production in the mixed culture system studied here must still be worked out. It will be necessary to investigate the possibility of chitinase liberation from the fungal partner, and the identification purification and study of its properties. It must be noted that the enhancement of chitinase biosynthesis in the mixed culture was dependent on the presence of the active mold, although chitinase production was also found using fungal mycelia as carbon and nitrogen sources instead of chitin.

Moreover, new approaches for improving chitinase yield from bacterial isolates can be pursued through genetic treatments, such as, artificial or spontaneous mutations or recombination techniques in order to select a super producer line. This will also enable one to study the kinetics of chitinase synthesis itself, as in the mixed

Figure 10. Metabolism of chitin in nature.



culture system presented in this study.

Finally, what could be an integrated metabolic pathway of the chitin metabolism in nature is presented in Figure 10.

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