Thesis for Master Degree

Microbiological Identification, Antimicrobial Activity and Enhanced Production of Bistroot Strain

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Abstract

Beneficial bacteria, which have been used for medical purpose and for medicine for treating intestinal disorders, include strains of *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, Clostridium butyricum, Lactobacillus sporogenes, Bacillus subtillis, Bacillus polyfermentocus and the like. In particular, *Bacillus polyfermenticus* SCD which is commonly called as Bisroot strain, has been appropriately used for the treatment of long-term intestinal disorders, since the live strains in the form of active endospores can successfully reach the target intestine. The goal of this study were to indentify Bisroot strain microbiologically, show antibacterial spectrum of activity of Bisroot strain against spoilage and pathogenic bacteria, optimize culture media and conditions to get high spore number of Bisroot strain in submerged fermentations, characterize polyfermenticin SCD, a bacteriocin produced from Bisroot strain, and finally obtain the valuable mutant for mass production.

Bisroot strain showed similar identity with *Bacillus subtilis* groúp by API CHB kit, fatty acid and quinone analysis and some conventional methods.

According to flip plate method, Bisroot strain have antimicrobial activity against the most spoilage and pathogenic bacteria tested.

To evaluate the effect of the medium, a batch fermentation using either H5, H7 medium or TSB medium was performed in 5L jar fermenter. The inexpensive optimum medium, H5, allows an increase of the yield of approximately 41% when compared with TSB medium. Pilot plant experiment were also performed for mass production of Bisroot endospores.

Polyfermenticin SCD, a bacterocin produced by Bisroot strain was partially purified by 75% ammonium sulfate precipitation followed by subsequent dialysis. This partially purified bacteriocin showed narrow antibacterial spectrum of activity on sensitive indicator cells was disappeared completely by α-chymotrypsin, which indicates its proteinaceous nature.

Bacteriocin acticity was detected at the early-log growth phase, reached the maxium at the early stationary phase. The bacteriocin exhibited a bactericidal mode of action against *B. subtilis* in phosphate buffer.

Several mutants were obtained using NTG mutagenesis of Bisroot strain. Firstly, growth rate, enzyme production, color of survival colonies were compared with Bisroot parent strain. Secondly, flask fermentations of 3 mutants were accomplished to compare with parent strain. Finally, mutant KD21 were cultivated in the 5L jar fermenter, the productivity and sporulation rate of mutant KD21 was drastically increased when compared with parent strain.

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Summary

As the result of identifying Bisroot Strain by electron microscope, carbon utilization, Isoprenoid and analysis of fungus body fatty acid, it was identified as the bacillus very similar with *B. subtilis.*

The antimicrobial activity of Bisroot Strain was measured with flip plate method, but it showed the antimicrobial activity on every gram positive bacteria except for *Enterococcus faecalis, Staphylococcus aureus, Clostridium perfringens, Bacillus sp., Micrococcus sp.* and *Listeria sp.* and gram negative bacteria showed the antimicrobial effect on every gram negative bacteria used in this study as *Yersinia sp., Vibrio sp., Aeromonas hydrophila, Escherichia. coli, Salmonella sp., Shigella sp.* and *Pseudomonas sp..*

The industrial culture medium and cultivation conditions for producing the active spore by use of Bisroot Strain made optimum.

Keeping pH as 7.0±0.1 from TSB culture medium which is the basic culture medium , the productivity was increased to about 5 times. The optimum industrial medium for producing the active spore was decided as H5 medium, and the productivity against TSB medium was increased to about 1.4 times and the price could be reduced about 10 times, and thus it is thought which it is the competitive medium in the mass production. It was watched which the quantity of total bacillus and active spore was decreased in the latter period of cultivation and it was judged to be affected by the sporelysin which became active in the germination of active spore.

The time of cultivation spore reached to Max. active spore quantities (1.17×10^9 spore/ml) in only 3 days in case of TSB medium, and in case of H5 medium, it reached to Max. active spore quantities (1.17×10^9 spore/ml) within only 4 days.
This was judged as the spore formation was done , about 3% of sugar being entirely wasted to H5 medium, and when it was compared with TSB medium, the increase of about 50% of productivity could be acquired.

The antimicrobial protein produced by Bisroot Strain was named as polyfermenticin SCD and the productivity in the fermenter was considered.

After centrifugal-separating the cultivation liquid acquired from the fermenter , we gained Bacteriocin which partly refined the upper-clear liquid through 75% ammonium sulfate

precipitation way and 2 times of dialysis (MWCO 1,000) and the recovery percentage of Bacteriocin was above 90%.

As the result of considering the antimicrobial effect on the main food decomposition bacillus and pathogenic bacillus, it was judged as the antimicrobial, which was rather narrow in the antimicrobial scope , showing the remarkable effect about 5 species of *Bacillus strain, Clostridium perfringens ATCC 3624, Staphylococcus aureus KCCM 32359 and Micrococcus flavous.*

Also, as the result of examining the action mode, it showed bacteriocidal mode of action.
For the purpose of selecting the excellent variation strain, NTG (100μg/ml) was treated for 30 minutes and then it showed the death of 99-99.9%. As the result of examining its effect by the selection of final 3 strain, total quantity of bacillus reached to the max. total quantity of bacteria (5.5×10^9 CFU/ml) in only 2 days and to the max. active spore quantities (2.7×10^9 spores/ml) also in only 2 days.
This showed which the culture period was reduced about 2 days against the results gained in only 4 days before.
The activity of α-Amylase in Bisroot strain and variation strain reached to max. 3,200AU/ml within only 24 hours.

I. Introduction

1. Definition and Utility of Alive *Bacillus* Medication

The alive *bacillus* medication says the products made for the purpose of improving the physical function by the enzyme secreted by the microbe, eating the microbe fungal body , the organic acid, vitamin and non-toxic antifungal protein.

In these viewpoint, the alive *bacillus* medication can be thought as the microbe which promotes the growth, increases the feed using percentage, prevents the abnormal fermentation of intestines or diarrhea and removes the inhibition factor of nutrition.

In case *E. coli* O-157 which is the sitotoxism bacteria , which caused the large social problems in Korea, Japan and USA in 1996, is given the disinfectant antibiotics, it could be more dangerous by the elution of verotoxin due to the destruction of cell wall of O-157, and thus the normalization of intestines microbe by the administration of alive *bacillus* medication showed the desirable treatment effect.

In these days which the abuse and misuse of antibiotics on the stocks cause the social problems, the precaution of digestive-system diseases by the addition of alive *bacillus* medication gives the economical benefits as well as has the value in the viewpoint of bio safety.

The effect of alive *bacillus* medication on the harmful microbe is shown to control the growth of harmful bacteria by the secretion of acid and antimicrobial materials. Now, *Lactobacillus, Enterococcus, Bifido-bacterium* as the alive *bacillus* medication are mainly studied, but in the alive *bacillus* medication commercially used in the human being or animal, *Lactobacillus, Bacillus, Clostridium, Saccharomyces, Aspergillus* are done as the products under the mixed type.

However, the ability of *Bacillus, Clostridium or Saccharomyces, Aspergillus* as the alive bacillus medication has not been almost judged until now against *Lactobacillus* or *Enterococcus* which has been much studied.

The most important characteristics , which the bacteria as the alive *bacillus* medication must be taken, are which the viability must be large within the intestines of animal as GRAS (Generally Recognized As Safe) microbe.

In the fungus body of microbe, 70-85% of moisture is included, and the main ingredients of dried body is carbohydrate, protein ,nucleic acid, lipid and ash content. Its content is different upon the kind of microbe and becomes changed upon the formation of medium, culture conditions and rearing time.
That is to say, according to C/N percentage of medium ingredient, the content of lipid is largely different and that of nucleic acid is high in the logarithmic phase.

The nucleic acid of fungus ingredients is extracted from the yeast fungus body, and then is used to the manufacturing of nucleotide, but the protein and lipid as being edible and feed-stuff become central and in particular, the ingredient of protein becomes more important in the point of the content's being much and its demand's being much.
Also, the vitamin and inorganic matter are included in the fungus body of microbe and so it heightens the value as the edibility and feed-stuff.

In case of diseases treatment, there are many cases which the purpose required is accomplished by use of living microbe instead of the chemical medicines.
Because, the diseases treatment which is the primary purpose can be accomplished in case the chemical medicines has been used as the treatment medicines, but it gives the abnormal action in other party of physical body and also the caused bacteria has the tolerance to antibiotics, and so there are many cases which it is difficult to be medically treated step by step.
However, if the non-pathogenic alive *bacillus* is used for the diseases treatment, it forms all kinds of breakdown enzyme and then makes the antagonism on the virus, and thus can make up for the poor points of chemical treatment way without having any side effect.

The food which contains the alive *bacillus* medication of L*actobacillus*, *Natto bacillus*, butyric acid bacteria and fermented milk has an effect upon the making-up balance of intestines bacillus, and so promotes the proliferation of intestines useful bacteria and controls that of intestines harmful bacteria, and then improves the intestines bacillus.

As its results, the intestines environment becomes clean and the precaution and recovery of diseases are possible by the antioxidant action, anti-mutation action physiological vital action and increasing of immune system, and also has the big effect upon the aging-control which induces the big interest recently.

2. What is the Bacteriocin ?

The Bacteriocin is the antifungal protein which is noted as the natural & nontoxic antiseptic produced by the microbe.
The existing antibiotics is the secondary metabolism product and on the other hand, it has the characteristics to be the ribosomal translation directly from its own gene, and accordingly, the bio-engineering application by the direct gene operation is easy and as its result, it can be variously reflected to the demand of industrial field.
Also, the molecule consists of the protein, and thus if it is taken to the human, it is dissolved by the protein hydrolysis enzyme of digestive organs, and thus in the point which it is non-toxic to the human and there is no residue, its effect as the natural antiseptic in the food or as the organism controlling agent of fermented food becomes increased.
The Bacteriocin isn't rather inactive in the high temperature, and is the characteristics to have the stability in the wide pH, and is non-toxic, colorless and odorless, and thus can be expected in the wide development for usage as the natural antiseptic which can represent the chemical-composition antiseptic.
That is to say, it can promote the long storage in Gochujang (Korean Hot Pepper Paste), Doenjang (Soybean Paste), Soybean Curd, Lactic-Acid Fermented Food including the advanced storage of fermented milk, fermented alcoholic drink, canned food, cooling & freezing food and also the prevention of acidification and deterioration in the traditional food as like Gimchi, Yakchu (Rice Wine) and Makgeolli (Unrefined Rice Wine), the keeping of freshness in the fish and shell products and storage of fruits and vegetables as Soybean Spouts.

The Bacteriocin produced by the *Lactobacillus* is classified into 4 kinds upon its molecular characteristics by Klaenhammer.

Class I as lantibiotics says the Bacteriocin which contains lanthionine and β-methyllanthionine peculiar produced by the variation after the translation together with cysteine by serine and threonine, and as the representative Bacteriocin, nisin is included.

Class II is included many Bacteriocin which are reported until now as which the molecular weight, which does not contain lanthionine is below 10kDa, and these are rather stable to the heat.

Class III is which the molecular weight is over 30kDa and is the Bacteriosin which is not stable to the heat and

Class IV as the mixed Bacteriocin which the carbohydrate and fatty molecule are connected with Protein molecule includes plantaricin S and lactocin.
The action mode of Class II Bacteriocin is

firstly, the Bacteriocin molecule forms a big transmembrane helix, and this molecule combines with the transporting protein which is already existent in the cell membrane and then changes its structure, and so breaks the substrate features of transporting protein molecule, and causes the random moving of ions into the both sides of cell membrane, and as its result, it breaks the membrane potential difference.

secondly, the Bacteriocin molecule forms transmembrane pore by itself, and thus it causes the breakage of membrane potential difference which was already formed in the both of cell membrane, and so interrupts the energy metabolism of cell and the moving of matter and as its result, the cell becomes fatal and stagnated.
It does not form the complicated tertiary structure due to the small size of molecule and thus has the strong heat stability which it does not lose the molecule activity even in the high temperature of 121℃.

This class II Bacteriocin is placed in the adaptation-experimental stage for the field application in the Industrial Circles of the West, and as already-stated in the above, the cell membrane which becomes weak in the structure by the action of Bacteriocin makes the cell concerned or the spore of cell concerned to be very sensitive to the heat treatment and at last, the cell is given the fatal attack by the Bacteriocin's own action and the heal given from the outside, and accordingly, in case the heat treatment is done at the same time, using the Bacteriocin, it has the merits to reduce the usual required calorie effectively.

Now in Europe, the using of Bacteriocin as like ninsin for the food preservative in the various scope of food industry has been permitted, and it is permitted to the canned food by FDA of USA.
However, in domestic, the Bacteriocin is classified as the antibiotics and so is not legally permitted to be added to the food.
But, nisin is acknowledged to be the nontoxic food additive in many countries of USA and Europe, and our domestic academic and industrial circles make the effort to revise the law and thus it is expected which the safety of Bacteriocin by GRAS (Generally Recognized As Safe) strain is acknowledged soon in Korea and then is added to the food as the natural antiseptic and so will be largely helpful to the national health.

3. Microbiological Identification

The currently & widely-used classification way of cell is that of Bergey Style (Bergey's Manual of Determinative Bacteriology) of USA, that of Krassilinikov (1949) used in USSR and Eastern Europe, and that of Prevot (1961) used in Europe including France and a part of zone in Africa.

Now, Korea follows the classification way of Bergey Style and the handbook of Bergey is which the content allotted and written by the several scholars was edited by Microbiological Academy of USA and it has the merits and on the other hand, has the poor points which there is no unity in the contents.

The Identification of Bacteria is done based on the morphological and physiological characters of bacteria as shown in the following.

I) Morphological Character of Bacteria

1. Shape and Size of Bacteria
2. Moving, Appearance & Position of Flagellum
3. Spore Formation, Shape & Size of Spore
4. Status of Habitation on Agar
5. Appearance when it is cultured in the medium
6. Appearance of Agar Gelatin Stab Culture
7. Appearance of Culture in all sides of agar and 1% Glucose Agar

II) Physiological Character of Bacteria

1. Nutrition Intake Status, that is to say, existence & non-existence of Photosynthesis Capacity, Chemical Composition Autotrophy or Chemical Composition Heterotroph
2. Gram Dyeing
3. Existence & Non-Existence in the fermentability of sugar and acid & gas formation
4. Resolvability of Starch
5. Deoxidation of Nitrate
6. Voges-Proskauer (VP) Test and Methyl Red (MR) Reaction
7. Requirement of Oxygen
8. Existence & Non-existence of Indole Formation
9. Existence & Non-existence of H_2S Formation
10. Existence & Non-existence of Ammonia

11. Existence & Non-existence of Catalase
12. Optimum and Extinct Temperature of Development
13. Anabolism of Citrate
14. Hemolysis
15. Change of Culture Medium at the culture of Litmus Milk Medium
16. mole % of Guanine & Cytosine of DNA

In particular, the content percentage of guanine and cytosine of DNA is noted to be indicated in the handbook of Bergey, the 8th edition.
Also, in the subdivison between the strains, the Serological Reaction & peculiarity on phage and results of homologous analysis of DNA are generalized and then identified.

4. Characteristics of *Bacillus* and Problems of Culture

The *Bacillus* genus is the *bacillus* which forms the spore, and is Gram positive Bacteria and has the filament and much motility.
A part of it has non-motility and there is the Gram Negative Mycetoma.
Generally, the heat-resistance of spore is strong and the resistance to the extreme conditions is strong.
Also, the spore is floated in the water, soil and air, and is the microbe which is widely existent in the Natural Circles.
It is the medium or high-temperature spore bacillus and is positive to aerobic and common anaerobic catalase.
It has the strain strong to the dissolution power of protein and starch and so makes the protein and starch food to be rancid or produces the acid and gas, and gives the peculiar smell and thus turns the food to be worse.
The salt resistance is rather strong and is generally cultivated in 10% salt.

The main *Bacillus* genus which is often detected from the food can be classified into the following 4 class.

a. First Class : It has the widest distribution in the Natural Circles, and can be often shown in the food and in particular, is important as the saprogen of starch food.

It is much existent in the protein food as like the fish jelly, milk & milky food and canned vegetables, and has rather strong saprogenic power.
There are *B. subtilis, B. cereus, B. megaterium, B. pumilis, B. firmus and B. licheniformis.*

b. Second Class : Its mother cell becomes expanded by the formation of spore and makes the big oval and round spore.
It forms rather thin and transparent habitation much on the flat culture medium.
There are *B. circulans, B. alvei, B. brevis, B. laterosporus, B. stearo-thermophilus, and B. coagulans.*

c. Third Class : It is , what is called, *aerobacillus* and has *B. polymyxa and B. macerans.*

The spore formation or the shape of habitation is same as that of Second Class and has the characteristics to form the gas, dissolving the sugar.
It is not distributed in the natural circles or food much.

d. Fourth Class : The cell is expanded by the formation of spore and forms the coccoid-spore in the first stage of cell.

There is *B. pantothenticus, B. sphaericus and B. pasteurii.*
Its distribution is low in the nature, but forms the decay and spot of fish-meat sausage and is detected from the soy-sauce mould.

Of these, *B. Subtilis* of First Class is the representative of *Bacillus* genus and its cell is single or chain and is the Gram Positive or Negative of Peritrichous and is much existent in the hay, soil and all kinds of fermented substrate.

It dissolves the sugar, generates the acid , dissolves the protein and forms the ammonia.

The size of cell is 0.7~0.8x2.0~3.0㎛ and its spore is 0.6~0.8x1.5~1.8㎛ and oval and is formed in the central part of cell commonly within 48 hours.
As there is the strain which secrets a great quantity of protease including the liquefied amylase, it is used to the production of enzyme.

As one of decayed microbe, it becomes the main cause microbe in the rancid of lunch box, and the temperature optimum to the living is 35-37℃ and the spore becomes extinct within only 3 hours in 100℃.

There is the thing which produces the antibiotic of subtilin, subtenolin and bacillomycin, and produces the yeasty-resistance matter having the heat-resistance before & after the formation of spore.
If the soy sauce is made with the soup into which *B. subtilis* is entered, it is said which the fermentation of yeast and contaminant is inhibited.

5. Characteristics of Bisroot Strain

The Bisroot Strain is the spore *bacillus* separated from the air by the microbiological scholar of Japan in 1933, and is informed as *B. polyfermenticus* and secretes 20 kinds of enzyme, and makes the nutrient to be reusable, and composes the vitamin B1,B2 and K and then supplies the nutrients.
Also, it digests and absorbs the carbohydrate, fat and protein which is 3 biggest nutrients of human , and cellulose, and also it makes Typhus *Bacillus*, Paratyphoid *Bacillus*, Dysentery *Bacillus* and *Cholera Bacillus* ,which are the microbe, to be bacteriolytic and thus has the function to control its proliferation.
Moreover, in case it is taken in, it is found which the parenteral infection defense action and the peroral immune function become strengthened, and it reinforces the immune function of host and inhibits the living of intestinal microbe which produces the carcinogen and the cancerogenic promotion matter , and also makes the anti-tumor matter or antimutagen of colon, and inhibits the generation of tumor.

In particular, the *Lactobacillius* or *bifidus bacillus* medication which are currently sold reach to the intestines, but the special coating or capsule products must be thought due to the stomach acid or various enzyme, but this bisroot strain is the bacillus which forms the spore, and thus until when it is reached to the intestines, it does not almost lose the activity and so shows the excellent effect in the treatment of intestines diseases.

The Bisroot strain shows more excellent adaptation to the environment than other *Bacillus* genus, and it appears which the living inhibition on Diarrhea Coliform and *Salmonella* is excellent.

Also, even after it is kept in the temperature of 37℃ until 42 days after the freezing and drying since its culture, the existence is shown to be rather high, and so the possibility as the Alive *Bacillus* Medication has been reported.

After the raw powder of this Bisroot Strain is imported from Mikuni Chemical & Industrial Co., Ltd. of Japan in domestic, the excipient is added to and then the Bisroot medication is made and then it has been sold by Sun chun dang Pharm. Co., Ltd.

6. Culture Way of Microbe

The culture engineering is the science which settles to move the matter found in the experimental room newly and the processing to the industrial production economically within the short period and how it is effectively made with the few raw-materials and the energy in case the bio-engineering products is manufactured under the factory size by use of the biochemical conditions of microbe.

For this purpose, the actual conditions examined in the Engineering Room is quantitatively shown and there are two aspects of Bio Engineering Aspect to be utilized in scale-up and of Mechanical Aspect which is used to the actual industrial device.

6.1. Culture Type

As the culture type, it can be divided into the general aerotropisic ferment and anaerobic ferment representative as alcohol ferment upon the attribute of microbe, but upon the status of medium, it can be classified into the liquid culture and solid culture and into the batch culture and continuous culture.

6.1.1. Liquid & Solid Culture

(A) Liquid Culture

In case of aerotropisic ferment, there is the surface culture and deep culture in the liquid culture.
The surface culture is the aerotropisic fixation culture and makes the surface area of culture liquid in the vessel to be large and then promotes the oxygen movement into the inner of liquid from the surface and so supplies the oxygen to the microbe.

As the traditional example of this culture way , there is the acetic ferment, and the acetic microbe forms the microbe membrane in the liquid surface and then is proliferated, and absorbs the oxygen from the air and the nutritious matter from the liquid, and then is proliferated.

This ferment is used to the vinegar manufacturing at present, but becomes the factor which the oxygen supply dominates the ferment speed, and thus requires a long time in the ferment, and has the problems to be lost into the air of alcohol and the acetic acid, but does not require the power expenses for the mixing and

ventilation as like the deep culture, and thus it can be the beneficial ferment type where the wide area can be easily used.

This way is used to the production of citric acid.
Against the surface culture which the oxygen is dissolved in the liquid by the natural diffusion from the liquid surface, it is the deep culture which the air , which is input to the culture liquid by force from the bottom of fermenter, makes particle by the mixer and promotes the oxygen melting, and then uniformly disperses and cultures the microbe from the culture liquid.

The penicillin is produced by the fixation culture way in the initial period of 1940~1950, but in order to perform the mass production effectively, the deep culture production way using the huge-sized mixing-type fermenter was developed , and now for the production of amino acid and antibiotic, they are mass-produced by the huge-sized fermenter over 100 tons in capacity, and the fermenter having no mixer is used upon the culture purpose.

(B) Solid Culture

Generally, the mold can be lived in the surface of solid substrate of agricultural products having the proper moisture, but the bacteria has the tendency to be lived well in the liquid medium containing the proper nutritious matter as the osmotic pressure within the cell is not high as like the mold.
Therefore, if the agricultural products of grain or the waste given from its processing course is used for the ferment substrate in the status of containing the suitable moisture, it inhibits the living of bacteria and then can preferentially raise the mold.

The example of solid culture using these characteristics is the production of koji in the brewing of Japanese Clear Strained Rice Wine and Soy Sauce using the mold. Also, it is used to the production of various hydrolysis enzyme.
The solid culture has the merits as shown in the following.

① The formation of medium is simple. The main raw-materials is the waste of grain and agricultural products and the water and a small quantity of mineral are added.

② It is used to the culture of mold very much and the pollution prevention by the bacteria is possible.

③ The product in purpose is accumulated in the surface of solid substrate , and the solvent of recovering it is few against the culture of liquid and accordingly the waste water from the processing is low.

④ The oxygen is supplied by the airing mixing which uses the power in the liquid culture, and on the other hand, in the solid culture, the oxygen is directly absorbed from the air in the space of substrate particle and so the power is not specially required.

⑤ The expenses for facilities is rather low and it is beneficial to the small-sized production.

⑥ In the production of feed, the agricultural products and its waste are used and the usable microbe is cultured and can be used for the feed as it stands.
In the liquid culture, the processing of fungus body separation and concentration is required.

On the other hand, in the solid culture, the cooling way is in question in case of mass production, and it requires rather wide area, and it is almost impossible which the control of ferment processing which is possible in the deep culture expects the changing of pH, temperature, quantity of substrate moisture, proliferation quantity of fungus body, and the production quantity of object in the course of culture, and thus it become the poor points which it is difficult to make the control for the effective production.

6.1.2. Batch Culture and Continuous Culture

[A] Periodic Culture

The periodic culture is the way which is generally used in the ferment engineering, and the culture makes continuous until when the constituent of culture medium is reduced upon the progress of culture by use of fermenter and so the main substrate is entirely consumed, and is a kind of closure reaction which proliferates the microbe and accumulates the product in purpose.
The temperature, pH, airing mixing in the culture is adjusted as the living optimum conditions of microbe.

The culture way which properly supplies the substrate, which becomes the limited factor on the proliferation , continuously and uniformly keeps it under the low concentration in the course of periodic culture is called as the additional-type culture. This can be adjusted to the environment suitable to the culture, and thus has the merits as shown in the following.

① In case the substrate (for example, methanol, ethanol, acetic acid etc.) inhibiting the proliferation makes as the raw-materials, the prompt proliferation speed can be kept , adjusting the addition speed and being kept to the low concentration which does make the proliferation inhibition.

② If the substrate concentration of glucose is somewhat high, the substrate can be kept to the low optimum concentration in the production of bread yeast and anti-biotic which the generation of product in purpose is down by the adjusting device of catabolite repression.

③ In case of Amino Acid Ferment using the nutritional requirement mutation strain, the fermented product can be effectively accumulated, adding a small quantity of nutritious matter in this requirement.

[B] Continuous Culture

It says the continuous culture way which continuously eliminates the culture liquid, and pours a same quantity of culture medium and uniformly keeps the phase and environment of microbe.
The continuous culture is started under the purpose of physiological study of microbe, using the characteristics of this culture , but in case of using the regular fermenter,

the activity of microbe that is to say, the proliferation speed and the using speed of carbon substrate are high in the continuous culture against the periodic culture, and also it has the merits which the productivity is advanced and the manpower, electric power and steam are saved, and so is used to the production of feed yeast using the waste liquid of sulfite pulp, production of vinegar, and sewage disposal by the activated sludge.

However, the scope of industrialization is limited and because by a long time of continuous operation, the danger of sundry germs is big and the variation or degeneration of strain can happen and the reaction speed is slow against the chemical reaction and so does not make the big profits and it has the problems of causing much expenses in the segregation of product.

7. Definition of Scale-Up, Expected Problems and Application Standard

7.1. Definition of Scale-Up

The scale-up is the process which assumes the optimum conditions in the size of factory where the industrial mass production is possible , based on the conditions established in the Experimental Room Scale Chemical Reactor, and is that which is established by 10-times-percentage-enlargement generally.

Here it delivers the matter of oxygen and substrate, and is established by the mixing of these substrates.

7.2. Problems expected in Scale-Up

It is almost impossible which the conditions established in the experimental-room-scale is matched with the dynamic conditions of current of scale-up process.
So, the compromise of matter mixing and heat transmission is required.
At first, the characteristics of other scale of system must be similarly made for the successful scale-up.
The fulfillment conditions in the experimental room scale is set as the perfect mixing conditions suitable to the consumption of substrate.

However, it is impossible which these conditions is set equally with that of dynamic current of fluid of scale-up process.

For the purpose of overcoming these defects, the optimum conditions must be set up through the scale-up and scale-down process.

The scale-up process is based upon the experimental assumption up to now as the various factors and the mechanism are complicated.

(1) Height to diameter ratio (H/D) in the fermenter turns changed, and thus the contribution degree of surface aeration becomes decreased very much.

(2) Proliferation in the surface of Fermenter
If the fungus body of microbe is attached to the surface of fermenter, it gets to show other metabolism due to the limit of matter transmission.

(3) The physical conditions can not be equal.

7.3. Standard which can be applied in Scale-Up

In order to settle these problems of Scale-Up, the scale-up process gets to be accomplished upon some following standards.

(1) Power Consumption (Po/V)
(2) Circulation Speed of Liquid within the Fermenter (Q/V)
(3) Stress in the Upper Part of Impeller (NDi)
(4) Reynolds number (NDi2 ρ/μ)
(5) Oxygen Transmission Coefficient per volume (kLa)
(6) Gas Current Speed per volume (V_G/V)
(7) Final Mixing Speed ($\theta\infty$)

8. Importance of *Bacillus* Strain and Strain Development Strategy

The strain of *Bacillus* genus performs the various metabolism action and is informed to be mostly non-pathogenic.
In particular, in case of *B. subtilis*, it was recognized as GRAS (Generally Recognized As Safe) from FDA of USA, it becomes the good data to certify the safety which the Japanese has taken *B. subtilis var. natto* microbe through soybean fermented food natto for a long time.
B. subtilis microbe is the microbe on which the genetic & physiological basic study following that on coliform is done very much.
It has been used as the representative research model of Gram Positive Bacteria since a long time before as it is easy in culture and is not pathogenic as well as holds the important position as the industrial microbe together with the interesting biological conditions as like the formation of spore , which can not been seen in the coliform, and abundant expression percentage.
As about 65 species of *Bacillus* species, its classification is possible and there are many strains which hold the important position as the industrial production strain of many *Bacillus* genus strains as like this, and as the representative product, it can mention the enzyme, antibiotics, spice , food additives and microbe insecticide.
Also, the strains of *B. subtilis, B. brevis* has been actively used as the production host of adventive protein.

The development of *Bacillus* strain can be accomplished, using the various elements, in the several sides. The various application way from enlarging the gene directly related to the product in purpose or promoting the expression to heightening the productivity in the more basic dimension by changing the physiology of host *bacillus*, and in accordance as what is the product in purpose, that is to say, it is enzyme secreted to outside of cell, it is protein accumulated within cell, it is the primary metabolism product of amino acid and vitamin, it is the secondary metabolism product as like the antibiotics or it is adventive protein, the strategy on the development of strain can be different.

In the development way of strain, the main gene related to the productivity through the development of molecular biology together with the traditional development way of selecting the mutation strain which has the characteristics to be wanted, after inspection of ultraviolet rays or handling the mutation organic agent as like NTG is found and then analysed, and then is directly operated and so the more systematic development of strain becomes possible.

9. Strain Development by Mutation

The microbe does the mutation and adaptation in confrontation with the change of environment. That is to say, if the habitation of microbe is watched, it can be rarely seen which the change is surely caused.
There are 2 kinds in this change essentially and the change called as the adaptation is if the conditions make as the original status, it returns to the initial normal type gradually, and that called as the mutation is which the mutated type is owned as it stands even if the conditions make as the original status.

The mutation happens under the natural conditions or under the artificial mutation causing conditions. The natural mutation generally has the low frequency and in the artificial mutation, the mutation source makes acted to any case or suspension liquid of fungus and it heightens the naturally happening mutation percentage to the thousands times.

When the mutation strain is artificially formed, it must pay attention to the appearance of pro-strain to be used, mutation source and selection way of mutation strain.

Firstly, the appearance of pro-strain must pay attention to its living history, proliferation method and physiological character.
The fungus which forms the spore is generally simple in chromosome rather than the nutritious cell and the mutation happens well, and thus it is effective which the spore is handled in the mutation.

Secondly, the thing which is important as the mutation source to be reported is same as shown in Table 1, and the selection of mutation source to be used among these becomes questionable.
The action mode of these mutation agent is each different, and thus the coming frequency and kind of mutation source may be different very much.
Also, the regularities which show the specific mutation (variation) strain following the mutation agent is not found and NTG & EMS are well-informed as which the mutation strain is acquired form the fungus by the high frequency, and other ultraviolet rays, X-ray, γ-ray are often used.
The proper handling conditions of mutation agent is not regular upon the strain and the character which desires to be acquired, but there are many cases to select the conditions of 0.1～1% in the existence percentage.

Thirdly, in the handling of mutation agent, the suspension equivalent to about 10^8 /ml of cell quantity is used and it is questionable how the mutation strain in purpose is selected.

Even in this selection, 2 kinds can be considered, and one is the coming frequency of mutation in purpose, and other is the character of mutation strain to be shown by the mutation in purpose.
If the coming frequency is high, even if the character is inspected by each segment of cell after the handling of mutation agent, the mutation strain in purpose can be selected, but if the frequency is low, this selection way must be thought again.
As this way, the direct way and concentration way are well-informed.
The direct way is which the mutated cell is smeared on the agar and then the habitation makes formed and one & one of each habitation is taken and then the existence & non-existence of mutation is to be considered.
Here, the replica plating method which uses the velvet cloth is used much and for the consideration of nutritious requirement of mutation strain to be acquired, the auxanograph way is convenient.

The concentration way is used when the mutation frequency is very low.
For example, when the nutritious requirement mutation strain used to the ferment of amino acid or nucleic acid is segmented, the cell which is handled by the mutation in the medium containing the minimum nutrient required to the living of cell , that is to say , minimum medium, is added and is cultured, and then the penicillin is added in the suitable period.
In the minimum medium, the cell having no nutritious requirement is proliferated but the cell in which the mutation of nutritious requirement is caused has no requirement matter, and thus is not proliferated.
At this times, the Penicillin is acted only to the cell to be proliferated, and makes it to be extinct, and thus only the cell having no nutritious requirement becomes extinct and the nutritious requirement mutation strain is screened (concentrated).
This way is called as Penicillin Screening Method.

For judging the existence and the non-existence of nutritious requirement, the habitation makes formed on the perfect medium in which the requirement strain and non-requirement strain can be lived together and then the velvet is used in the requirement matter applying minimum medium and no-applying minimum medium, and then is replicated and cultured.

The handling method of mutation organic agent is as shown in the following.

a. Inspection of Ultraviolet Rays

The handling of mutation by Ultraviolet Rays is simple in the performance course and causes the various mutation of deletions, transversions and GC-AT transitions at random , but is not stronger than NTG.
The ultraviolet rays is inspected on the target of microbe cells recovered from the logarithmic phase..
The mold of Neurospora is cultured at 25℃ for 7 days, and then its conidium makes suspended in the saline solution to be about 10^6 /ml of concentration and then moved to 9 cm dia. of pantry dish and has been inspected by its mixing.

For the coliform bacteria or yeast, the fungus body is centrifugal-separated in the culture liquid and is washed 2 times with saline solution, and then is suspended to the concentration of $5x10^{5-8}$ /ml and its 5-10ml is taken and then is handled as before.
It is good to select any case or the conditions of survival percentage 0.1-1% or 0.01 %.

Mostly, the inspection of 10-70 sec. is suitable to this conditions, using 15W ultraviolet rays and being mixed in the distance of 10-40cm.
Also, there is the photo-regeneration conditions in the inspection of Ultraviolet rays, and thus is good to be handled under the yellow or red light of dark room.

b. Nitrogen Mustard Treatment

In Neurospora, the conidium cultured for 7 days makes suspended in 0.2M acetate buffer liquid of pH 6.0 and then is added bis type nitrogen mustard to be 0.1-0.5% and then is treated for 30 minutes, and then is centrifugal-separated, and the upper clear liquid is separated and removed.
The coliform is washed 2 times with a same quantity of saline solution and then makes suspended in saline solution again, after the medication is added and then is acted for 20-30 minutes so as that the final concentration of culture liquid becomes 0.1% and then is centrifugal-separated to 4,000 rpm for 10 minutes.

c. Treatment of Alkylating Drug

The mutation action of mutation organic agent having the Alkyl group of DMS,

MMS is acted to DNA of cell and then it is informed which its phosphoric acid or base part is alkylated.
As its treatment way, the bacteria makes suspended in 0.1M phosphoric acid buffer solution (pH7.2) at the concentration of 10^{6-8}/ml and is added the alkylating drug to be 0.01-0.3M and then is treated for 10 minutes ~ some hours, and then the cell is separated by the centrifugal-separation, and then is washed with the buffer solution and is smeared and cultured on the agar.

d. 4-NQO and NTG Treatment

By Karreman (1962) , it is said which 4-NQG absorbs the adenine of nucleic acid and then as its results, it becomes the reason of mutation which adenine combines with cytosine instead of thymidine.
This 4-NQG is noted as its carcinogenic action is strong.

NTG is informed to be good in the effect among the mutation organic agent and so is widely used.
The logarithmic cell of E. coli K 12 makes suspended in the buffer solution of pH 6.0 at the concentration of $5x10^8$ /ml, and is treated with NTG of 100-1,000μg/ml for10-30 minutes, and then is centrifugal-separated and then the cell is washed, and is put in the medium for about 1 hour and is diluted, smeared and cultured.

e. Treatment of Nitrous Acid

The nitrous acid induces the mutation to Tobacco Mosaic Virus, Bacteria T2 phase and enzyme well.
Generally, the nitrous acid or nitrite is treated in pH 4-5 at the concentration of about 0.02M.

The nitrous acid acted to secede the Amino Group from Base having Amino Group of DNA , that is to say, guanine, cytosine and adenine , and then makes as xanthine, uracil and hypoxanthine and thus induces the mutation.

Table 1. The mechanisms of mutagenesis by important mutagens

Mutagen	Mechanism	Result
Base analogue		
5-Bromouracil	Insertion as T when replication	A-T → G-C
2-Aminopurine	Insertion as A when replication	A-T → G-C
Chemical agent		
Nitrous acid(HNO_2)	Deletion of amine group from A, C	A-T → G-C G-C → A-T
Hydroxylamine(NH_2OH)	React with C	G-C → A-T
Alkylating agent		
Ethylmethane sulfonate	Addition of methyl group to G	G-C → A-T
mitomycin, nitrosoguanidine	cross link DNA chain faulty region excised by DNAase	point mutation and deletion
Acridine, EtBr	Iinsertion between two base pairs	Reading-frame shift
Physical factor		
U.V.	Formation of pyrimidine dimer	Repair error or deletion
Ionizing radiation (X-ray etc.)	Free radical attack on DNA breaking chain	Repair error or deletion

10. Purpose on This Study

The Japanese Manufacturing Company which supplies the Bisroot Strain in the raw-powder has exclusively produced and supplied it through the solid culture method using agar since tens years before.
However, the raw-materials expenses is expensive and each when it is imported, the active spore quantity is not regular and it is the actual conditions which the activity is down very much during its storage.
For the purpose of settling these problems, it requires the establishment of technology which makes the mass production of Bisroot Strain by the liquid culture method and it is expected which the price of raw power makes down and the long-term stable supply will be acquired through it.

In order to reduce the production expenses, the cheap industrial medial is established and the production conditions make optimum to the standard of fermenter.

On the other hand, this study wants to make the microbiological identification of Bisroot strain, perform the examination of antifungal effect on the main decayed bacteria and pathogenic fungus which can be easily generated within the intestines of Bisroot strain and establish the excellence of Bisroot strain.

And up to now, the antifungal effect of Bisroot medication is reported to be based on the various digestive enzymes secreted by Bisroot Strain and so we want to prove the effect of Bisroot Strain Medication more scientifically , confirming the antifungal protein produced by Bisroot Strain.

Finally, we want to separate the high threshold strain through the mutation treatment, and then to enhance the productivity in the fermenter.

II. Experimental Materials and Method

1. Strain

The Bisroot Strain is distributed from the Technical Research Institute of Sunchundang Pharm. Co., Ltd. and the strain makes activated through the several succeeding culture consecutive for 3-4 times.

This Bisroot strain and every strain to be used in this experiment are kept at -70℃ by Glycerol Stock Method and the working culture is used by the succeeding culture once a month.

2. Culture Medium (Agar)

As the medium for the breed fungus , TSB medium is used and for this culture medium, TSB medium and industrial medium are used.
The industrial medium to be used in this study is much used to *Bacillus* genus strain and is the medium remarkable in the spore formation, and is used for the culture of Bisroot Strain.

It is considered which the constituents consisting of the industrial medium must be cheap in price and be rather easily supplied in domestic, and the glucose and corn starch which are the main constituents was supplied from Shin Dong Bang Co., Ltd.

3. Culture Device

The breed fungus used 500ml baffled flask (working volume : 100ml) and this culture is accomplished by use of 5L fermenter (Korea Ferment Device, working volume : 3L).

4. Watching by Electronic Microscope

The electronic microscopic photo was gained by use of FEG-SEM (model S-4200, Hitachi, Japan) and its method is as shown in the following.

That is to say, after the fungus body is centrifugal-separated and gained from the culture liquid of Bispan Fungus , it makes fixed at 4℃ with glutaraldehyde 25% solution, and is washed 3 times with 0.1M phosphoric acid buffer solution (pH 7.0)

and is dried with Ethanol.
After it is spotted with slide glass and is dried, it makes dried for 1 week at the vacuum drier.
It makes Ion-Spatter coating in 200nm at the decompression vaporizer, and is photographed with the electronic microscope and then the photo is gained.

5. Carbon Utilization

The Carbon Utilization of strain is confirmed by use of API 50 CHB kit.

6. Isoprenoid Quinone Analysis

The fungus body of Bisroot Strain was gained and the High Liquid Chromatography (HPLC) was used.
The analysis conditions are as shown in the followings.
That is to say, it is Instrument, Hitach L-5000 LC controller; Pump, Hitach L-6000; Column, YMC-Pack ODS-AM ; Column Temp., 30℃; Eluent Methanol/Isopropyl (3:1, v/v) for Ubiquinone, Methanol/Isopropanol (7:5,v/v) for Menaquinone ; Flow Rate, 1ml/min.

7. Analysis on formation of Fungus Body Fatty Acid

For the experimental fungus body, it was cultured at 30℃ for 24 hours in Trypticase soy agar (TSA) and then only the fungus body was recovered.

After the recovered fungus body about 50mg(wet weight) was moved to telflon-lined screw cap tube (Ø13x100mm,pyrex), the reagent I (Soap-making reagent) 1ml was put and was heated at 100℃ for 30 minutes, and cooled in the room temperature. Here, the reagent II (Methyl Reagent) 2ml was added and was heated for 10 minutes at 80℃ and was rapidly cooled, and then the reagent III of 1.25ml (extraction solvent) is put and then was mixed well for 10 minutes.

After it was kept in the room temperature, if the reaction liquid was separated to 2 layers only the lower-layer-liquid was removed and the reagent IV (base washing) of 3ml was added and is mixed for 5 minutes, and then about 2/3 of upper clear liquid among the reaction liquid was moved to septum-capped sample vial (Ø12x32mm , Alltech Associates, Inc., USA) and then was capping and was used for the sample.

In the analysis of FAMEs, Hewlett Packard series II Gas Chromatograph model 5890A

(Microbial ID., Inc., USA) was used and as the separation column, 25mx0.2mmx0.33㎛ methyl phenyl silicon fused silica capillary column (HP 19091B-102) was used.
For FAMEs profile, Microbial Identification System Software (Microbial ID, Inc., USA) was used and by the comparison with standard calibration solution (Microbial ID., Inc., USA), the identification of peak, retention time, area of peak and area percentage were found.

The conditions of Gas Chromatography used to the analysis is as shown in the followings.

Carrier Gas, Hydrogen; Column Head Pressure, 10psi; Split Ratio, 100:1; Split vent, 50ml/min; Septum Pyrge,5ml/min; FID Nitrogen , 30ml/min; FID Air, 400ml/min; Initial Temperature, 170℃;Program Rate, 5℃/min; Final Temp, 270℃;FID temperature 300℃;Injection Port, 250℃;Injection volumn 2㎕.

8. Culture Condition and Method

The breed fungus was purely separated and the strain kept in TSA Plate was inoculated with platinum needle at TSB medium of 500ml baffled flask, and became shake-culture for 10 hours in 150rpm of mixing speed at 37℃ and then manufactured.

This culture conditions in the flask standard was culture temp. 37℃, initial pH 7.0, mixing speed 140rpm, the culture time for 5 days.
The conditions of this culture in the fermenter was total volume 5L, working volume 3L, culture temperature 37℃, pH 7.0±0.1, mixing speed 500rpm, airing volume 1vvm, and culture time for 4 days.

9. Measurement of Total Strain Quantity

The culture liquid was continuously diluted with 0.1 peptone and then is distributed to the plane medium each 0.1ml and then was cultured for 24 hours at 37℃ and then the colony forming unit was measured and then total strain quantity was calculated.

10. Measurement of Active Spore

The culture liquid was given the heat treatment for 2 hours at 80℃ and the nutritious cell made extinct and was consecutively diluted with germ-free dilution liquid (NaCl 5g/L+peptone 10g/L),and then 0.1ml was distributed to the pantry dish and then TSA soft agar was poured and is mixed well, and then was hardened, and then was overlaid

again.

And then, it was cultured for 24 hours at 37℃ and then the active spore quantity is calculated.

11. Recovery of Active Spore

After the finish of culture, it was centrifugal-separated for 30 minutes at 8,000rpm by use of centrifuge and then the nutritious cell and active spore were recovered.

12. Preparation of Cell-Free supernatant

After the Bisroot Stain was cultured for 6-10 hours at TSB medium, the culture liquid was centrifugal-separated for 15 minutes in 7,000xg and then the upper clear liquid was gained, and then the filtrate acquired by use of 0.22㎛ pore size cellulose acetate filter was adjusted to pH 6.8-7.2 with 2N NaOH solution and then cell-free supernatant for measuring Bacteriosin Activity was prepared.

13. Measuring Method of Antifungal Activity

The antifungal activity of Bisroot Strain was measured by flip plate method and the method is as shown in the followings.

The bisroot strain was cultured for 12 hours at 37℃ in TSB medium and this culture liquid was stricking to the dried TSA plate, and then was cultured for 24 hours again at 37℃.

The bisroot strain turned over the grown plate, and inoculated the target strain 100㎕, cultured in TSB broth for 12 hours at 37℃, to 0.75% soft agar 5ml, and then was mixed (about 10^7 cells), and then was overlaid.
And then, was is cultured for 12-24 hours at 37℃ and then the living-inhibition activity is inspected.

14. Bacteriocin Activity Measuring Method

For the Bacteriocin Activity Measuring Method, the spot-on-lawn method was used. That is to say, Bisroot strain was inoculated to TSB liquid medium (10ml) and was cultured for 6-12 hours at the optimum temperature, and then 5㎕ of cultured upper clear liquid to be acquired by the centrifugal-separation was loaded on the

solid medium and then made dried.

And, it was overlaid with 5ml of soft agar including the target strain 10^7 cells and then was judged with the inhibition ring acquired by the culture of 12 hours again. For the Bacteriosin Activity, the raw-liquid containing the antifungal matter was diluted 2 times consecutively with 0.1M phosphoric acid buffer solution and then each diluted liquid was spotted and then the max. diluted diploid was conversely taken and then calculated.

15. Sensitivity on Heat, pH Stability and Enzyme of Bacteriocin

For the heat stability, the upper clear liquid which is partly purified with Ammonium Sulfate Sedimentation Method was heat-treated for 30 minutes at the thermostat upon the temperature (40-100 121℃), and for pH stability, the partly-purified upper-clear liquid was mixed with each pH buffer solution (pH 2-9) and 1:1 of volume, and then was placed for 4 hours at 4℃, and for the enzyme stability, the purified upper clear liquid and each enzyme were each mixed and then made reacted 1-4 hours under the optimum conditions, and then 0.1M phosphoric acid buffer solution (pH 7.0) was diluted 2 times and each diluted upper clear liquid 5㎕ is spotted to TSA plate in which the target strain was already overlaid, and then the living inhibition ring was each examined.

16. Action Mode of Bacteriocin

In order to measure whether polyfermenticin SCD has bacteriocidal or bacteriostatic effect, the partly-purified polyfermenticin SCD was added to pH 7.0 of phosphoric acid buffer solution in which the contrast bacteria was suspended and then examined.

17. Separation of Best Strain by NTG Mutation Treatment

The strain cultured in LB medium was inoculated to PAB (Antibiotic medium 3) medium and then was cultured at 37℃ up to logarithmic phase (OD_{600}0.7-1.0) under the sufficient airing , and after removing the upper clear liquid by the centrifugal separation, it was washed once with the warm SC (0.15M NaCl, 0.01M sodium citrate pH 7.0)10ml and then NTG was treated for 30 minutes and then was washed with warm PAB medium and then was suitably diluted, and then was cultured in LB Plate medium.

The mutation strain was separated based on α-amylase, appearance, spore formation, and color.

18. Selection of Strain which the activity of enzyme is excellent

In order to select the strain which overproduces α-amylase , the LBS medium which the soluble starch 1.0% was added to LB agar medium was used.
After 100ml of distilled water was dissolved with I_2 14 g and KI 36g, 3 drops of HCl Iodine solution was added 3 drops of HCl and the water and then was made with 1000ml.

Each fungus was inoculated to LBS medium and was cultured for 36 hours at 37℃ and then iodine solution was sprayed on the medium and the strain, which the dyeing was not done around the habitation and the ring was big , was selected as that excellent in the activity.

19. Activity Measuring Method of α-Amylase

The culture liquid cultured in the fermenter was centrifugal-separated at 12,000rpm and the upper clear liquid was diluted 2 times with 0.1M phosphoric acid buffer solution and then was spotted to LBS plate containing 0.1% of soluble starch, and then was cultured for 1 hour at 37℃, and then was measured with iodine coloring method.

III. Experimental Results and Consideration

1. Identification of Bisroot Strain

1.1. Examination by Electronic Microscope

As the result of examining the Bisroot Strain with Electronic Microscope, it showed the form of typical bacillus which the size of nutritious cell was about 1.8-2.0㎛ and that of active spore about 1.5-1.7㎛. (Fig. 1)

1.2. API 50CHB test

As the result of examining the utility of carbon of Bisroot strain, it was identified as the fungus which was very similar with *B. subtilis*. (Table 2)

1.3. Isoprenoid Quinone Analysis

MK-7(menaquinone-7) was detected as the main constituent, and was one of characteristics of *Bacillus* genus. (Fig. 2)

1.4. Analysis on the Formation of Fungus Body Fatty Acid

12-Methyltetradecanoic acid (15:0 ANTEISO) and 13-Methyltetradecanoic acid (15:0 ISO) was analysed as the main constituent, and was one of Characteristics of *Bacillus* genus . (Fig 3.)

As the above-mentioned results, Bisroot strain was identified as the fungus which was very similar with *B. subtilis*.

(a) Vegetative cell

(b) Active endospore

Figure 1. Scanning electron microscopic(SEM) observation of Bisroot strain.

Table 2. Carbohydrate utilization of Bisroot strain

Cabohydrate	Utilization	Cabohydrate	Utilization
Control	−	Esculine	+
Glycerol	+	Salicine	+
Erythritol	−	Cellobiose	+
D−Arabinose	−	Maltose	+
L−Arabinose	+	Lactose	+
Ribose	+	Melibiose	+
D−Xylose	+	Saccharose	+
L−Xylose	−	Trehalose	+
Adonitol	−	Inuline	−
β−Methyl−xyloside	−	Melezitose	−
Galactose	−	D−Raffinose	+
D−Glucose	+	Amidon	+
D−Fructose	+	Glycogene	+
D−Mannos	+	Xylitol	+
L−Sorbose	−	β−Gentiobiose	+
Rhamnose	−	D−Turanose	−
Dulcitol	−	D−Lyxose	−
Inositol	+	D−Tagatose	−
Mannitol	+	D−Fucose	−
Sorbitol	+	L−Fucose	−
α−Methyl−D−mannoside	−	D−Arabitol	−
α−Methyl−D−glucoside	+	L−Arabitol	−
N−Acetyl glucosamine	−	Gluconate	−
Amygdaline	+	2−Keto−gluconate	−
Arbutine	+	5−Keto−gluconate	−
Identification: *B. subtilis*(99.5%)			

+: utilized, −: not utilized

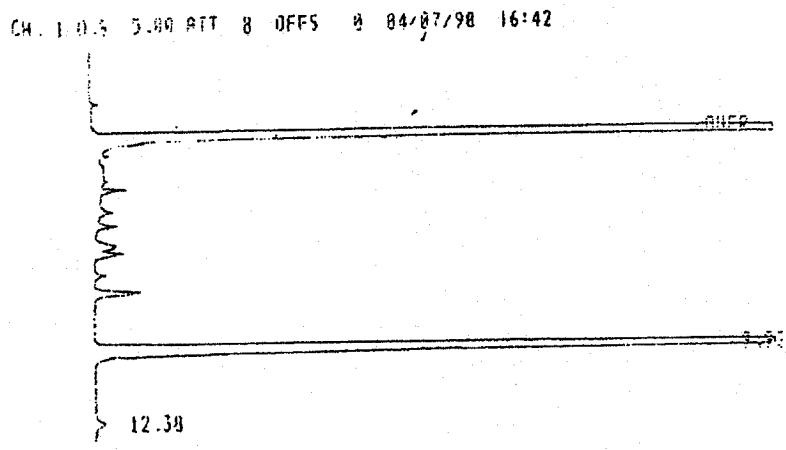

Figure 2. Isoprenoid quinone analysis.

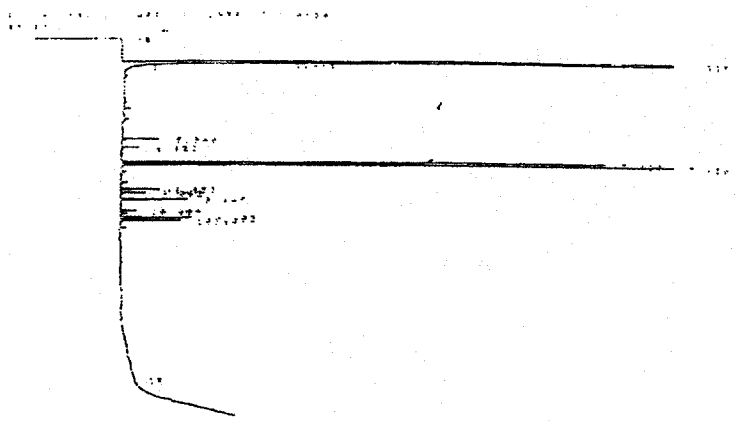

Figure 3. Fatty acid composition analysis.

2. Antimicrobial Effect on the Main Rotten Microbe and Pathogenic Microbe

The target strain to be used for the purpose of measuring the antimicrobial activity by flip plate method which is the method of measuring the indirect living inhibition activity against the grown plate used 9 stains of *Enterococcus faecalis* ATCC 19433 for the Gram Positive Bacteria, and 18 strains of *Yersinia pseudotuberculosis* ATCC 6902 for the Gram Negative Bacteria.

The antimicrobial activity of Bisroot Strain measured with Flip plate method showed the antimicrobial activity on every gram positive bacteria except for *Enterococcus faecalis, Staphylococcus aureus, Clostridium perfringens, Bacillus sp., Micrococcus sp. and Listeria sp., Staphylococcus epidermidis* ATCC 1228, and Gram Negative bacteria showed the antimicrobial effect on *Yersinia sp., Vibrio sp., Aeromonas Hydrophila, Escherichia coli, Salmonella sp., Shigella sp.* and *Pseudomonas sp.* (Table 3)

Based on the inspection results on intestines *bacillus* habitat of Koreans, *Bifidobacterium* is very superior in the infant , in particular , in the mother's milk infant by 10^{10} CFU/g (humid excrement), but in accordance as it gets on years, it is reduced gradually and then it was shown to be fewer than Eubacterium when it becomes older.

Clostridium perfringens which is representative in the harmful fungus was highest in the aged about the detection rate and quantity.
We gained the result which the Bisroot strain showed the activity on *C. perfringens* , and widely indicated the antimicrobial activity on dysenteric virus, staphylococcus and vibrio virus.

This showed which the Bisroot Strain has the remarkable effect in the treatment of intestines diseases.

The Bisroot Strain showed the highest survival rate on the artificial gastric juice, bile acid and pancreatin even in the status of nutritious cell rather than other alive bacillus medication on sale, and the alive inhibition power on the diarrhea causing coliform and salmonella was excellent, and it was reported which the environment adaptation power was excellent.

In case the Bisroot Strain formed the spore, the probability to be reached to the intestines was higher and so it could be mentioned which the activity was also superior.

Table 3. Inhibition of spoilage and pathogenic organisms by Bisroot strain

Spoilage and pathogenic microorganisms	Culture medium[b]	Incubation temp.	Inhibition zone diameter (mm)
Gram positive bacteria			
Enterococcus faecalis ATCC 19433	TSA	37℃	+
Staphylococcus aureus ATCC 25923	TSA	37℃	+
Staphylococcus epidermidis ATCC 12228	TSA	37℃	-
Clostridium perfringens ATCC 3624[a]	TSA	37℃	+
Bacillus cereus ATCC 11778	TSA	37℃	+
Bacillus subtilis ATC 6633	TSA	37℃	+
Micrococcus luteus ATCC 10240	TSA	37℃	+
Listeria monocytogenes ATCC 15313	TSA	30℃	+
Listeria ivanovii ATCC 19119	TSA	30℃	+
Gram negative bacteria			
Yersinia pseudotuberculosis ATCC 6902	TSA	30℃	+
Yersinia enterocolitica ATCC 27729	TSA	30℃	+
Vibrio parahaemolyticus ATCC 17802	TSA	37℃	+
Vibrio vulnificus	TSA	37℃	+
Vibrio cholerae O139	TSA	37℃	+
Aeromonas hydrophila	TSA	37℃	
Escherichia coli ATCC 8739	TSA	37℃	+
Escherichia coli ATCC 29522	TSA	37℃	+
Escherichia coli O157	TSA	37℃	+
Salmonella Typhi	TSA	37℃	+
Salmonella Paratyphi A	TSA	37℃	+
Salmonella Typhimurium	TSA	37℃	+
Salmonella Enteritidis	TSA	37℃	+
Shigella flexneri	TSA	37℃	+
Shigella boydii	TSA	37℃	+
Shigella sonnei	TSA	37℃	+
Pseudomonas syringae ATCC 12855	TSA	30℃	+
Pseudomonas aeruginosa ATCC 15442	TSA	30℃	+

[a]Incubated in anaerobic GasPak jar

[b]TSA, tryptic soy agar

3. Production Medium and Culture Conditions Setting-Up

3.1. Selection of Optimum Industrial Medium in the Flask Scale

As the result of comparing and examining the industrial medium, which the Bisroot Strain was judged to be suitable for the mass production under the cheap price, with TSB medium ,which was the basic medium to be used up to now, in the Flask Scale, H5 Medium and H7 Medium among the industrial medium to be examined were selected as the best medium.

3.2. Increasing of Productivity by pH Adjustment

It was expected which pH was uniformly kept to be 7.0 during the culture period for the mass culture of Bisroot Strain and thus it made the progress of fungus body to be fast and would be able to increase the strain quantity which could be finally gained.

3N H_2 So_4 solution and 3N NaOH solution automatically made supplied and thus pH made to be kept in the scope of 6.9-7.1.

It could be known which the spore quantity as well as Total strain quantity could be increased about 5 times by the culture due to pH Adjustment. (Result Not Submitted)

4. Production in the Fermenter

4.1. Effect Examination in the Optimum Industrial Medium in the Fermenter Scale

As the result of adjusting and culturing H5 and H7 medium , which was judged to be best in the culture of flask, as pH 7.0, H5 medium was more best. (Fig. 4)

4.2. Ferment Process Using the Basic Medium and Optimum Industrial Medium in Ferment Bank Scale

As the result of culturing it by use of TSB medium under the optimum ferment conditions, the max. active spore (1.17×10^9spores/ml) was reached only in 3 days and was somewhat reduced after that times.

As the result of culturing it by use of H5 medium in the optimum conditions, the max. active spore (2.34×10^9spores/ml) was gained only in 4 days and its reason was which the sugar equivalent to about 3% of medium constituents was contained and so total strain quantity of Bistroot strain to be acquired was high but the spore was formed after the sugar was consumed in the ferment process, and thus the time could be mentioned to be taken more.

It was watched which the total strain quantity and the number of active spore were reduced in the latter term of culture, and thus it was judged to be done by the bacteriolysis and these conditions were caused by the various course as like the action of antibiotics matter, protein and enzyme.

The solution enzyme of bacteria cell wall hydrolyzed the peptidoglycan of cell wall in the most case, and caused the bacteriolysis, and was produced by many microbes.

These enzyme are autolysin which acts the cell wall of other bacteria as well as its own cell wall, virolysin to be produced in the cell by the infection of phage, ectoenzyme to be secreted to the outer of cell in a great quantities and sporelysin to be activated in the spore germination.

Here, they are judged to be bacteriolysis by sporelysin. Finally, the case which the active spore numbers exceeded total strain quantities in the latter term of culture was often seen, and it was understood to be the conditions due to spore activation in the course of heat treatment at 80℃ perhaps.

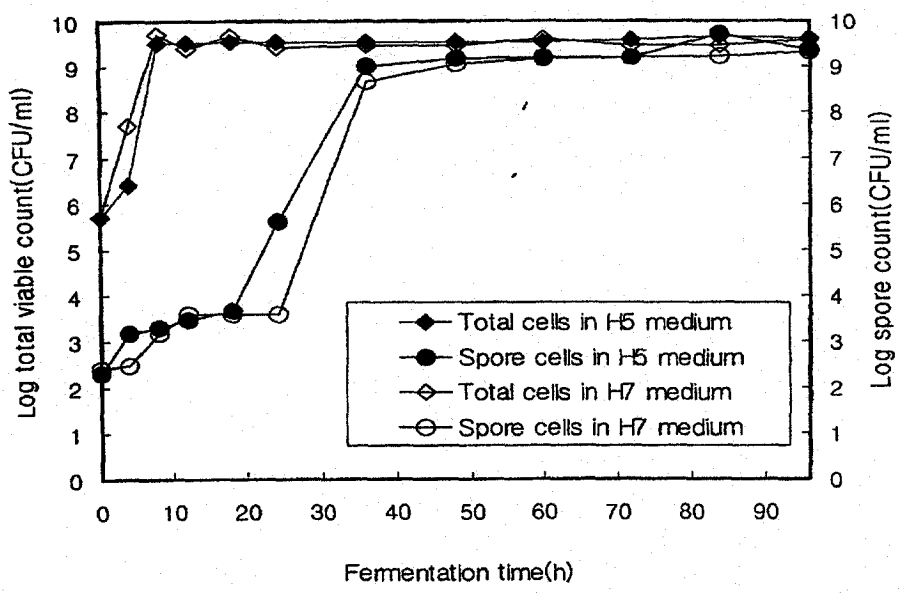

Figure 4. pH-controlled fermentation profiles of Bisroot strain in 5L jar fermenter.

4.3. Comparison of Productivity of Basic Medium and Optimum Industrial Medium in the Fermenter

At the fermenter, the bisroot strain was cultured in TSB medium ,which was the basic medium, and H5 medium, which was the optimum medium, under the optimum conditions and its productivity was compared and examined. (Table 4)

Table 4. Comparation of productivity of culture media in 5L fermenter

Culture medium	Maximum number of active spore (CFU/ml)	Culture time (Day)	Overall productivity (CFU/ml/day)	Increase (%)
TSB	1.2×10^{9}	3	3.9×10^{8}/ml/day	-
H5	2.3×10^{9}	4	5.9×10^{8}/ml/day	50

5. Production at Pilot Plant

The consumption of glucose was entirely done only in about 8 hours and the max. total strain quantity (5.0×10^9spores/ml) was also reached only in about 3 hours. The max. active spores (5.5×10^9spores/ml) was reached only in about 60 hours. The culture time was shortened superior to 96 hours in 5L fermenter and its reason could be mentioned which the culture conditions in the pilot plant was more effective in the transmission of matter. (Result Not Submitted)

6. Bacteriocin Produced by Bistroot Strain

6.1. Production of Bacteriocin in the Fermenter

In the fermenter, the production of Bacteriocin made by the Bisroot Strain was considered.
It was inoculated to baffled flask (working vol. : 30ml) of Bisroot Strain and then was shake-cultured for 8 hours at 37℃ and then was seed cultured. And then, it was inoculated to main medium (3L) as 1%, and then was main cultured.
As the result of confirming the activity, it was reached to the max. activity only within 6 hours, and the activity was sharply reduced from that hour (Fig. 5)

After the culture liquid (1L) acquired by the fermenter was centrifugal-separated, the upper clear liquid made partly-purified through the sedimentation of 75% ammonium sulfate and 2 times of dialyzing (MWCO 1000), and then the Bacteriocin was acquired.
The collection percentage of Bacteriocin was over 90%.

6.2. Antimicrobial Activity on Pathogenic Bacteria

As the result of measuring the antimicrobial activity on Gram Positive and Negative Bacteria in order to inspect the antimicrobial activity of Poly-fermenticin SCD which is the Bacteriocin produced by Bisroot , *C. ferfringens ATCC 36245, B. pumilis, B. subtilis IFO 12113 and B. subtilis ATCC 6633* showed the antimicrobial activity on the partly-purified Bacteriocin and B. cereus and M. flavus showed some antimicrobial activity in cell-free supernatant, and showed the clear antimicrobial activity on the partly-purified Bacteriocin.
S. aureus ATCC 25923, *B. cereus* ATCC 11778 and *L. monocytogenes* ATCC 15313 showed some antimicrobial activity on cell-free supernatant. (Table 5)

It did not indicate the antimicrobial activity on other Gram Positive Bacteria and every Gram Nagative Bacteria, and the antimicrobial activity was not watched about the yeast and mold strain used to the experiment as like *S. cerevisiae KCCM 11201, A oryzae KCCM 11371, A. niger KCCM 11239 and P. chrysogenum KCCM 6933.* (Table 5)

It was reported by Paik which tochicin , which is the Bacteriocin produced by *Bacillus thuringiensis* subsp. *tochigiensis* showed only the strain which is very near to the production strain of Bacteriocin as like *B. thuringiensis and B. cereus,* and there was no antimicrobial effect on *M. luteus, S. aureus, B. subtilis* and every Gram Negative Bacteria and Yeast.

As like Bacteriocin produced in other *Bacillus* genus strain, polyfermenticin SCD showed the narrow antimicrobial scope having the peculiar antimicrobial activity to *Bacillus* genus strain of *C. perfringens* ATCC 36245 and *B. cereus, B. pumilis, B. subtilis* ATCC 6633, *B. subtilis* IFO 12113.
Accordingly, it is considered which it will be able to be used to the part requesting the monovalence limited only to the specific genus, measuring the antimicrobial activity on the various *C. perfringens, Bacillus* sp.

6.3. Sensitivity on Heat & pH Stability of Bacteriocin and All Kinds of Enzyme Handling

The heat stability of Bacteriocin produced by Bisroot Strain showed the remaining activity up to 60℃ and was stable until pH 2-9.
As this result, it could be known which it was rather unstable to the heat, but was very stable in pH.
On the culture upper clear liquid acquired after Bisroot Strain was each cultured for 10 hours, 5 species of protein breakdown enzyme was each treated and the remaining antimicrobial activity on *B. subtilis* ATCC 6633 was confirmed.

When it was considered which the antimicrobial activity,at least, existent on α-chymotrypsin was entirely lost, that antimicrobial matter was certified to be the protein. (Table 6)

6.4. Action Mode

As the result of examining the action mode of Polyfermenticin SCD, as soon as M. flavus , the target strain, was exposed to Bacteriocin, it could be known which the fungus quantity was reduced at once.
As this result, it could be known which polyfermenticin SCD had bactericidal mode of action.

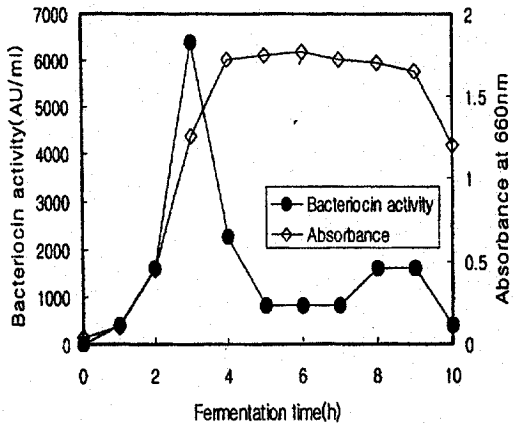

Figure 5. pH-cotrolled polyfermenticin SCD production in jar fermenter.

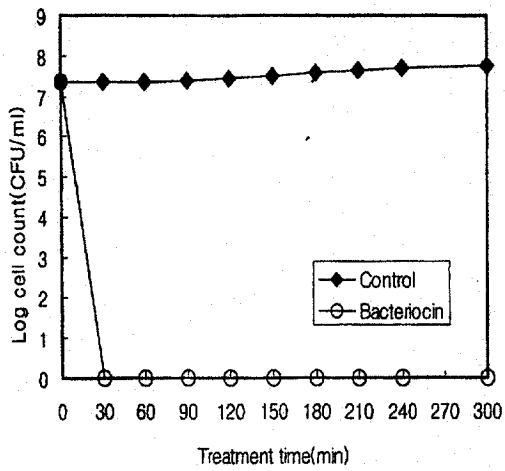

Figure 6. Mode of action by polyfermenticin SCD against *M. flavus*.

Table 5. Inhibition of spoilage and pathogenic organisms by polyfermenticin SCD

Spoilage and pathogenic microorganisms	Culture medium[b]	Incubation temp.	Inhibition	
			Supernatant	Partially purified bacteriocin
Gram positive bacteria				
Enterococcus faecalis ATCC 19433	TSA	37℃	-	-
Staphylococcus aureus ATCC 25923	TSA	37℃	-	+/-
Staphylococcus aureus KCCM 32359	TSA	37℃	-	+
Staphylococcus epidermidis ATCC 12228	TSA	37℃	-	-
Clostridium perfringens ATCC 3624[a]	TSA	37℃	+	+
Bacillus cereus ATCC 11778	TSA	37℃	-	+/-
Bacillus cereus	TSA	37℃	+/-[c]	+
Bacillus pumilis	TSA	37℃	+	+
Bacillus subtilis IFO 12113	TSA	37℃	+	+
Bacillus subtilis ATCC 6633	TSA	37℃	+	+
Micrococcus luteus ATCC 10240	TSA	37℃	-	-
Micrococcus flavus	TSA	37℃	+/-	+
Listeria monocytogenes ATCC 15313	TSA	30℃	-	+/-
Listeria ivanovii ATCC 19119	TSA	30℃	-	-
Gram negative bacteria				
Yersinia pseudotuberculosis ATCC 6902	TSA	30℃	-	-
Yersinia enterocolitica ATCC 27729	TSA	30℃	-	-
Vibrio parahaemolyticus ATCC 17802	TSA	37℃	-	-
Vibrio vulnificus	TSA	37℃	-	-
Vibrio cholerae O139	TSA	37℃	-	-
Aeromonas hydrophila	TSA	37℃	-	-
Escherichia coli ATCC 8739	TSA	37℃	-	-
Escherichia coli ATCC 25922	TSA	37℃	-	-
Escherichia coli KCCM 32386	TSA	37℃	-	-
Escherichia coli JM 109	TSA	37℃	-	-
Escherichia coli O157	TSA	37℃	-	-
Salmonella Typhi	TSA	37℃	-	-
Salmonella Paratyphi A	TSA	37℃	-	-
Salmonella Typhimurium	TSA	37℃	-	-
Salmonella Enteritidis	TSA	37℃	-	-
Shigella flexneri	TSA	37℃	-	-
Shigella boydii	TSA	37℃	-	-
Shigella sonnei	TSA	37℃	-	-
Pseudomonas syringae ATCC 12855	TSA	30℃	-	-
Pseudomonas aeruginosa ATCC 15442	TSA	30℃	-	-
Pseudomonas fluorescens	TSA	30℃	-	-
Pseudomonas putida	TSA	30℃	-	-
Yeast and Molds				
Saccharomyces cerevisiae KCCM 11201	YPD	30℃	-	-
Aspergillus oryzae KCCM 11371	PDA	30℃	-	-
Aspergillus niger KCCM 11239	PDA	30℃	-	-
Penicillium chrysogenum KCCM 6933	PDA	30℃	-	-

[a]Incubated in anaerobic GasPak jar
[b]TSA, trypticase soy agar; YPD, yeast extract peptone dextrose; PDA, potato dextrose agar.
[c]Not clearly inhibited

Table 6. Effect of heat, pH and various enzymes on partially purified polyfermenticin SCD

Treatment (Heat)	Residual activity (AU/ml)	Treatment (pH)	Residual activity (AU/ml)	Treatment (Enzyme)	Residual activity (AU/ml)
Control	12,800	Control	12,800	Control	3,200
40℃	12,800	2	12,800	Protease I	3,200
50℃	6,400	3	12,800	Protease IV	3,200
60℃	800	4	12,800	Protease IX	3,200
70℃	0	5	12,800	Protease XIII	3,200
80℃	0	6	12,800	Proteinase K	0
90℃	0	7	12,800	α-Chymotrypsin	3,200
100℃	0	8	12,800	β-Chymotrypsin	3,200
121℃	0	9	12,800	Trypsin	3,200
				Lipase	3,200
				α-Amylase	800

7. Selection of Best NTG Mutation

7.1. Survival Curve

As the result of examining the extinct percentage by NTG treatment (100㎍/㎖) the inspection time was decided to be 30 minutes in accordance as it was treated for 30 minutes and then showed the extinct percentage of 99-99.9%. (Fig. 7)

7.2. Selection of Mutation

For the selection of mutation, 3000 kinds was primarily selected, based on the size & color of colony and culture speed, and after the tooth pick was done to LBS plate in which 0.1% of starch was contained and then it was cultured for 36 hours, the solution of iodine was sprayed and 40 kinds of strain was secondarily selected , based on the size of transparent pill which was not dyed and then this method was repeated 2 times and 3 strain (mutant KD 21, 35, 63) was selected.

7.3. Culture of Selected Mutation in the Flask

In the culture by TSB medium in the flask of selected mutation, the absorbance of culture liquid and total fungus quantities were sharply reduced since 12 hours of culture. (Results Non-Submitted)
It was shown which it was due to the effect of activated sporelysis , the spore's being formed.
By which the spore formation became difficult due to the shortage of nitrogen required to the formation of sugar, which the medium contains, or of spore and due to which the sufficient conditions of inhalation was not done, it seemed to be more affected by sporelysin.
So, the culture effect in the fermenter was considered by use of H5 medium after that times.

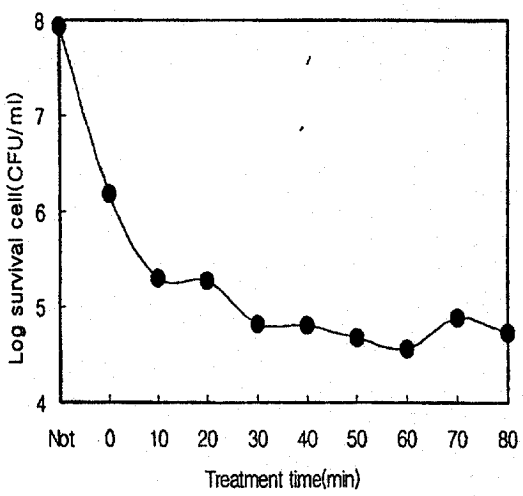

Figure 7. Survival curve of Bisroot strain for NTG mutation.

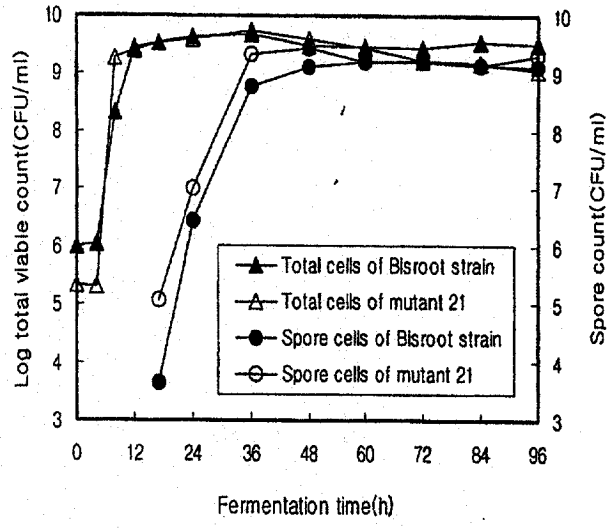

Figure 8. pH-controlled fermentation profiles of Bisroot strain and mutant KD 21 in H5 medium.

8. Overproduction by Best Mutation of Bisroot

The mutation KD 21 , strain to be excellent in total fungus quantities at the flask culture of the selected strain, was cultured by H5 medium in the fermenter.

As its result, total fungus quantities were reached to max. total fungus quantities (5.5 x 10^9CFU/ml) only in 2 days, and the max. active spore quantity (2.7x10^9spores/ml) was also reached only in 2 days.

The culture period was shortened for 2 days against the result acquired only in 4 days before. (Fig 8, Table 7)

In the Bisroot strain and best mutation, the activity of α-Amylase was reached to max. 3,200AU/ml only in 24 hours. (Table 8)

Table 7. Comparison of productivity between Bisroot strain and mutant KD21 in 5L jar fermenter

Strain	Maximum number of active spore (CFU/ml)	Culture time (Day)	Overall productivity (CFU/ml/day)	Increase (%)
Bisroot strain	1.3×10^9	2	6.5×10^8	–
Mutant KD21	2.7×10^9	1.5	1.8×10^9	177

Table 8. Comparison of enzyme activity between Bisroot strain and mutant KD21

Incubation time (h)	α-Amylase activity(AU/ml)	
	Bisroot strain	Mutant KD21
12	1,600	1,600
24	3,200	3,200
36	3,200	3,200

Conclusion

In accordance with this study, the following conclusion could be gained.

1. Microbilogical Identification

   As the result of identifying the Bisroot Strain through electronic microscope, carbon utility, Isoprenoid, fungus body fatty acid analysis, it was identified as the bacteria very similar to *B. subtilis.*

2. Antimicrobial Effect

   The antimicrobial activity of Bisroot Strain measured by Flip plate method was shown on Every Gram Positive Bacteria except for *E. faecalis, S. aureus, C. perfringens, Bacillus sp., Micrococcus sp. and Listeria sp. etc. S. epidermidis* ATCC 12228 and the antimicrobal effect was shown on every Gram Negative Bacteria of *Yersinia sp., Vibrio sp., Aeromonas hydrophila, E. coli, Salmonella* sp., *Shigella* sp. and *Pseudomonas* sp.

3. Culture in the Fermenter and Factory Scale

   The industrial medium and culture conditions for producing the active spore by use of bisroot strain were considered and the separation & purification method of culture liquid made optimum.
   At TSB medium which is the basic medium, pH was kept to be 7.0±0.1 and the productivity made increased about 5 times.
   The optimum industrial medium for producing the active spore was decided as H5 medium and the productivity was increased about 1.4 times against TSB medium which is the basic medium, and the price could be down about 10 times. So, it is thought which it is the competitive medium in the mass production.
   It was watched which the total fungus quantities and the number of active spores were reduced at the latter term of culture and it was judged to be due to the effect by sporelysin which was activated in the germination of active spores.

   In the culture time, it reached to max. active spores (1.17×10^9spores/ml) only in 3 days in case of TSB medium and in case of H5 medium, it reached to the max.

active spores (2.34×10^9spores/ml) only in 4 days. It is judged to be due which about 3% of sugar was entirely consumed in H5 medium and then the formation of spore was established, and when it was compared with TSB medium, the increasing of about 50% of productivity could be gained.

4. Bacteriocin produced by Bisroot Strain

It showed the antimicrobial activity on most food rotten bacteria and pathogenic bacteria used in the experiment, and the antimicrobial activity on 3 species of Bacillus genus strain was confirmed.

As the result of treating 5 species of protein breakdown enzyme based on these results, when it was considered which the antimicrobial activity existent on α-chymotrypsin was lost, that antimicrobial matter was certified to be protein.

The antimicrobial protein produced by the Bisroot strain is called as polyfermenticin SCD and then the productivity in the fermenter was considered.

As centrifugal-separating the culture liquid acquired in the fermenter, the upper clear liquid made partly-purified through the sedimentation of 75% ammonium sulfate and 2 times of dialyzing (MWCO 1000), and then the Bacteriocin was acquired, and the collection percentage of Bacteriocin was over 90%.

As the result of considering the antimicrobial effect on the main food rotten bacteria and pathogenic bacteria , it was judged to be the antimicrobial matter which is rather narrow in the antimicrobial scope, showing the clear effect on 5 species of *Bacillus genus strain, Clostridium perfringens ATCC 3624, Staphylococcus aureus* KCCM 32359, *Micrococcus flavus.*

Also. as the result of examining the mode of action. it showed the mode of bacteriocide which inhibits the growth of bacteria in the inital term of living.

5. Selection and Culture of Best NTG Mutation

After NTG (100μg/ml) was treated for 30 minutes in order to select the best mutation, it showed the extinct rate of 99-99.9% and as the result of considering its effect after the selection of final 3 stains, total fungus quantities reached to the max. total fungus quantities (5.5×10^9CFU/ml) only in 2 days and to max. active spores (2.7×10^9spores/ml) only in 2 days.

The culture period was reduced to 2 days against the results to be acquired only in 4 days before.

About the activity of α-Amylase, the Bisroot strain and mutation were all reached to Max. 3,200AU/ml only in 24 hours.

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