MINISTRY OF EDUCATION & TRAINING
NHA TRANG UNIVERSITY

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STUDY ON HYDROLYSIS OF SEAWEED WASTE (*Gracilaria verrucosa)* BY CELLULASE ENZYMES FROM BACTERIA TO APPLY IN FEED PRODUCTION FOR UNISEXUAL TILAPIA

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LIST OF PUBLICATIONS

1. Le Huong Thuy (2009), "Research on biotechnology applications to utilize waste from agar production for producing animal feed", *Science Conference on Review the implement and results of Agricultural- Fishery Biotechnology Projects, phase 2007-2008*, Ministry of Agriculture and Rural Development, pages 193-195.

2. Vo Hoai Bac, Le Huong Thuy, Le Thi Lan Oanh (2010), "Screening microorganisms cellulase used in hydrolysis of agar waste", *Bulletin of Research Institute of Marine Fishery, No. 15*, pages 20 - 24.

3. Le Huong Thuy, Vo Hoai Bac, Le Thi Lan Oanh (2011), "Optimization of conditions for extracellular cellulase yielding from 2 strains of Bacillus subtilis B-505 and Bacillus lichenformis Li in industrial fermentation environment ", *Bulletin of Research Institute of Marine Fishery, No. 19*, pages 20-25.

4. Le Huong Thuy (2011), "Research on application of hydrolyzed seaweed waste in feed production for tilapia", *Bulletin of Research Institute of Marine Fishery, No. 22*, pages 19 -26.

5. Huong Thuy Le, Vo Hoai Bac (2011), "Research on screening and optimization of culture conditions of cellulase microorganisms strains used for hydrolysis of agar waste", *Science and Technology Journal of Agriculture and Rural Development*, pages 140-148.

6. Le Huong Thuy (2013), "Research on application of biotechnology to utilize agar watste for production of animal feed", *Science and Technology Journal of Agriculture and Rural Development*, pages 275-284.

INTRODUCTION

1. Background

Seaweed is a type of material that consists of natural substances of high value and is used in many fields of industry, medicine and food such as agar, carrageenan.... In processing technology, the production technology of agar from seaweed is more researched that due to agar is used in many fields of life ... In the world, it is around 7.000 - 10.000 tons of agar products annually. In Vietnam, fresh seaweed production is around 3.000 tons / year, which is largely used to produce agar and carrageenan. Every year, our country produced about 500 tons of agar. In the processing of agar, the amount of organic waste discharged into the environment is very high at around 6-8 tons of waste/1 ton of agar, so the estimated amount of waste from seaweed processing that is annually around 7000-9000 tons. Seaweed waste contains a large amount of organic material thus when they exists outside they will be decomposed that causes stench and reduce human health. Therefore, the research of treatment for processing and getting more profit from seaweed waste is necessary to reduce environmental pollution and utilize organic waste efficiently.

Agar waste often contains proteins, minerals originated from the sea such as iodine, phosphate and trace elements that is useful for the growth of farming animals. Especially, this waste consists of a large amount of cellulose – the indigestible ingredients for animals. Thus, study of technology for fish feed production from seaweed waste will bring in economic benefits because of this effective waste using. It is more important that this will help to minimize the risk of environmental pollution. In besides, It also saves costs of waste treatment for agar processing. Thus, the thesis is necessary in our actual production activities.

With the practical requirements outlined above, we have chosen thesis topic "Study on hydrolysis of seaweed wastes *(Gracilaria verrucosa)* by cellulase enzymes from bacteria to apply in the production of feed for tilapia".

2. Practical significance of the thesis

Seaweed waste from agar production technologies are often richly organic cellulose that is very difficult to decompose. Todays, seaweed waste has processed to produce animal feeds by several hydrolysis methods in alkali or acidification. However, the processing of cellulose by physical and chemiscal methods are often complicate,

expensive and polluting the environment. while the treatment of organic waste containing cellulose by microbial technology, especially the use of cellulase enzyme from microbial extracellular would have advantages in technical, economic and environmental aspects. Hydrolysis of cellulose from seaweed waste will help animals digest and easily absorb protein, glucide, mineral elements in the waste after the production of agar. The success of the research will be scientific basis for the utilization of seaweed waste in feed production of aquatic animals.

3. Objective

Process of applied biotechnology using agar-processing waste to produce materials for animal feed production, contributing more effective usage of waste for feeds and less environmental pollution.

4. Reseach Content

1. Identify the basic components of agar waste.

2. Screening strains of cellulase bacteria used in hydrolysis of seaweed waste (Gracilaria verrucosa).

3. Investigate hydrolysis of seaweed (Gracilaria verrucosa)by cellulase enzymes from microbes.

4. Experiment of using hydrolysis products in feed production for unisexual tilapia in commercial stage.

5. **The Novelty of the thesis**

Investigation on 17 strains of microorganisms capable of yielding extracellular enzyme cellulase has been surveyed and screened by Institute of Biotechnology and University of Science and Technology to get 02 strains of extracellular Cx enzymes cellulase microorganisms with the highest active: *Bacillus lichenformis* (Li) and *Bacillus subtilis*-B-505 VTCC to cellulase hydrolys seaweed waste used to produce animal feed, contribute to environmental protection, reduce waste disposal costs and provide valuable feed source for livestock.

6. The layout of the thesis

Thesis consists of 131 pages, including three chapters, 33 tables, 24 figures, 19 charts, and 146 references (40 in Vietnamese, 102 in English, 4 in web).

CHAPTER 1
OVERVIEW

In developed countries, livestock and poultry are bred by industrial methods requiring large amounts of feeds with high quality, stability, high absorption and low cost. Thus, it is strategic to utilise waste resources for producing of low cost feed for livestock. Lots of countries use agricultural by-products as a source of supply of cellulose in animal feed production. Agricultural by-product can be from crops such as rice straw, corn stalks, stems peanuts, sugarcane tops, bagasse, grass, cassava pulp, ... This raw of by-product is normally poor nutrient, high cellulose, low digestibility when used as animal feed.

The amount of agar output in the world is estimated at about 7,000 to 10,000 tons per year. According to the statistic data in 1992, quatities of red seaweed in the world is 1,256,981 tons (FAO statistic Year book of Fishery, 1992), amount of agar production and consumption in 1984 was 6685 tons (Coppen, 1989). The technology of Agar production is make to high organic waste about 6 tons /1 ton of product. It will cause serious environmental pollution and loss the scrap sources if they are not spent.

According to the literatures and investigations , the current state of waste treatment at the agar production factories, it show that this treatment received less attention. Especially, seaweed waste is organic waste that contains a high amount of water, decomposable and rot organic material causing sour odor and toxic to humans. So this is an urgent matter and should be settled as soon as possible.

The composition and the level of substances of agar depend on the technology of agar production, but it generally contains 15 to 20% as dry substance, with moisture of 75- 80%. The dried composition in seaweed waste contains 40-50% of organic compounds, mainly cellulose (raw fibers), 4-5% of nutrients and proteins; 50-60% of inorganic compounds, including about 55% of inorganic insoluble salt and many microquantity elements. [10]

Many studies show that the majority of red seaweed protein exists in the form of compounds with glucide. Therefore, it is to use effectively protein in seaweed for breeding that seaweed waste need to be chemically treated or biotechnology is used by

fermentation, resolution and destroy the out-links of glucide groups with protein molecule of -protein complex. Therefore, protease enzyme in animals can digest and absorb protein with other useful components.

The number of kinds of microorganisms joined in the biosynthesis of cellulase enzymes are plentiful in natural conditions. They belong to filamentous fungi, actinomycetes, bacteria and in some cases, the scientists also found out the yeast joining this resolution process. The microorganism has cellulase activity that is significant and has wide application range in practice and daily life.

Haiphong is home of the agar production factories. There are about of 40 factories of all sizes with total estimated agar production of 500 tons/year, revenue of about 60 billion and offering thousands of jobs for workers and waste disposal is unsolvable problems for agar factory. These wastes are handled mostly by digging holes to bury or compost as fertilizer for crops causing the stench, pollution in factory and residential areas. People are living near the waste dump and administration who are very concerned about the disposal of the above waste. Utilizing them as a source of raw materials for the production of animal feed has big significance, economic and environmental protection.

CHAPTER 2
MATERIALS AND METHODS

2.1. Materials

2.1.1. Seaweed waste

Agar waste was obtained from agar production factory of Hoang Yen Manufacturing & Trading Company Limited located at Lam Ha Ward, Kien An District, Hai Phong City. After the cooking process to extract agar, agar waste was discharged through the frame filter stages. Random samples were taken in different cooking time and then pressed to reduce water and stored at cold temperature.

2.1.2. Microorganisms capable of cellulase synthesis

Microorganisms capable of cellulase synthesis were collected from the breed collection of the Institute of Biotechnology, University of Natural Science and Technology. Microorganisms culture medium was collected from collection of laboratories of the University of Natural Sciences and Biotechnology Institute.

2.1.3. Tilapia breeding

2.2. Methods

2.2.1. Methods of analysis

- Determination of protein content in accordance with ISO 4328-1: 2007 (ISO 5983-1: 2005);

- Determination of water according to ISO 4326: 2001 (ISO 6596: 1999);

- Determine the ash content in accordance with ISO 4327: 2007 (ISO 5984: 2002);

- Determination of cellulose according to AOAC No 973.09.1997/4329: 2007 (ISO 6865: 2000);

- Determine the lipid content according to TCVN 4331: 2001;

- Determination of toxic mold under HPLC 3.19-05-2/CL1/ST;

- Identify NaCl according to TCVN 4330: 1986;

- Determining Calcium TCVN 1526: 2007;

- Determination of Phosphorus according to TCVN 1525 - 2001;

- Analysis of metal elements on an atomic absorption spectrophotometer.

2.2.2. The method of obtaining and determining cellulose activity

2.2.2.1. Method of obtaining crude cellulose enzyme

2.2.2.2. Determining the activity of cellulose enzymes by inverted sugar method

2.2.3.3. Method of determining the reliability of cellulose enzymes by time preserved

2.2.2.4. Assessment of cellulose activity by electrophoretic method

2.2.2.5. Method of determining the relative activity of hydrolysis agar waste

2.2.3 Method of culture and assessment of cellulose yielding activity of microorganism

2.2.3.1. Microorganism culture method

2.2.3.2. Assessment of bacterial growth

2.2.3.3. Evaluation method of cellulose yielding activity of microorganisms

2.2.3.4. Method of determining the optimal pH of cellulose enzyme

2.2.3.5. Method of determining the optimum temperature of cellulose enzyme

2.2.3.6. Optimize culturing condition for 02 selected strains of bacteria B505 and Li.

Select culture medium: determine the growth and secretion of extracellular cellulase enzyme capabilities of Li and B505 strains on some substrates. Determine the optimum culturing time, temperature, pH for the growth and extracellular cellulase secretion of bacterial.

2.2.4. Experiment arrangement method

2.2.4.1. General experiment arrangement method

2.2.4.2. Optimized experiment arrangement for seaweed waste hydrolysis by the cellulose enzyme

2.4.4.3. Experimental testing arrangement of using seaweed waste hydrolysis products in tilapia feed

2.2.4.3. Method for determination of feed ration formula

2.2.4.4. Method of experimental testing arrangement for feed added with seaweed waste hydrolysis in tilapia culture.

2.2.4.5. Method for determining tilapia culture parameters

2.3. Chemistry and intrumentation

2.3.1. Chemistry

Carboxymethyl cellulose (CMC), Glucose, Peptones, meat extract, starch, yeast extract, malt glue, $MgSO_4$, $CaCl_2$, K_2HPO_4, $NaHCO_3$, NaCl, ... the chemicals imported by reputable firms in the world as Sigma, Merck, Bio-Canada, ...

2.3.2. Instrumentation

The equipment mainly consists of : Incubation cabinet, Oven, Stirring hydrolysis device, Centrifuge, pH meter, UV Vis Spectrophotometer, Grinders, Crushers, Feed bolus compressor ….

Laboratory: studies were conducted at the laboratory of the Research Department of Post-Harvest Technology - Research Institute Marine Product and National Core Laboratory of Gene Technology - Institute of Biotechnology, with high precision modern equipments to ensure implementation of project.

2.4. Statistic analysis

T-test standard was used for analysis of statistical data, Design Expert software version 6.0 (DX6), was used to process for optimize data, Excell and K-graph software was used for analysis and graphing [1], [4].

CHAPTER 3
RESEARCH RESULTS AND DISCUSSION

3.1. Survey and evaluation of quantity and basic chemmical components of seaweed waste after agar production

Solid waste generated from the agar production process is quite huge, average about 9 ÷ 10 tons of waste/1 ton of agar, the main component of this waste is cellulose seaweed waste. The quality of gold seaweed is affected by the climatic conditions in the different regions, therefore the quality of seaweed wastes is also affected.

Analyzing results showed that seaweed waste had the protein content of 3,26 ± 0,11*%;* cellulose content of 74,26 ± 4,68*%;* ash content of 12,28 ± 3,77 %. The results also indicated that in agar waste the levels of the trace mineral elements are complete and elemental content of heavy metals (As, Pb, Cd, Hg...) are below the allowed levels in feed according to EU standards. In addition to traditional feeds, the role of the protein, vitamins, amino acids and minerals would have great sense to the growth, especially, the quality of commercial product is the top matter nowaday.

The addition of each individual minerals encounters many difficulties and complex. Minerals, especially trace minerals, is required with a very small amount, so it is difficult to quantify and easily inaccurate. The lack of minerals and vitamins supplements on the diet will reduce the resistance and arises many diseases. Adding waste from manufacturing seaweed agar to animal feed shall ensure nutritional quality and open up a new road in searching for raw materials for feed processing plant.

3.2. Screening micro bacteria yielding for cellulase enzymes with high activity

17 strains of microorganisms capable of extracellular cellulase enzymes yealding (6 strains of bacteria, 1 strains of actinomycetes, 3 strains of yeast fungi and 2 strains of filamentous fungi and and 5 strains of un-named microorganisms), surveyed by the Institute of Biotechnology and the University of Science and Technology, was screened to find out a few strains that had strongest capability of extracellular cellulase enzymes yielding. The microorganisms was cultured in their specific environment and supplemented with the CMC substrate with the aim of increasing the ability to secrete cellulase enzymes as described in the methods section. CMC substrates is the best substance to determine Cx enzyme. CMC is a specific substrate for cellulase, using 1%

CMC substrates on agar plates and in inverted sugars reaction solution by DNSA solution to affirm cellulase activity of this microorganism strains

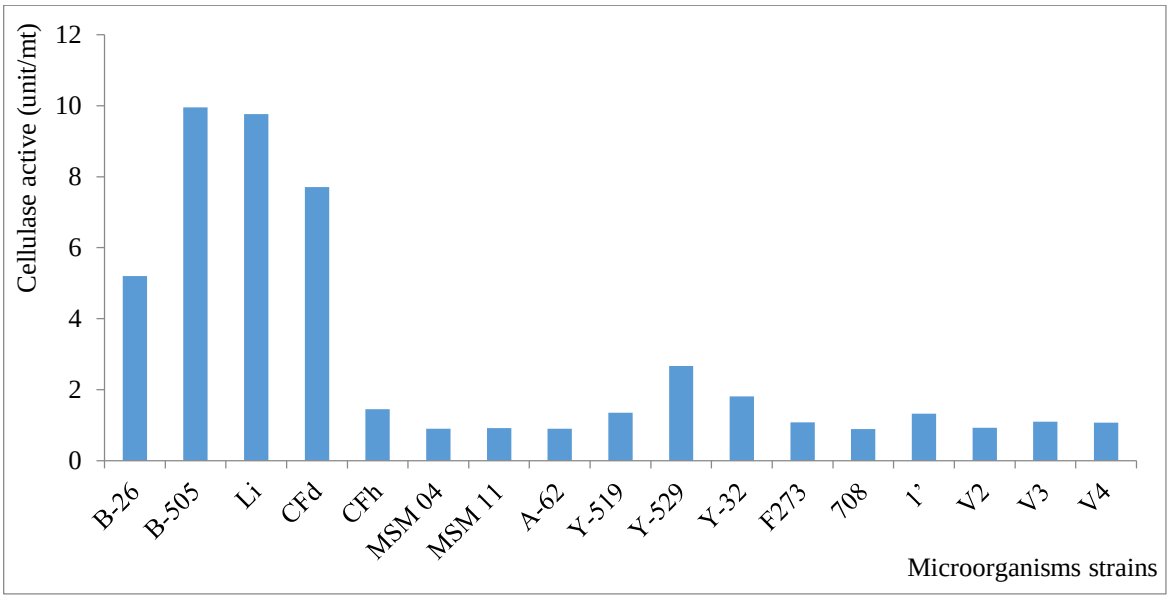

Figure 3.1. Cellulase enzyme activity of 17 tested strains of microorganisms

Results obtained 4 strains of Li, B26, B505 and CFd obtained stronger cellulase enzyme activity. Li and B505 strains are twicethat have the strongest cellulase yielding ability with the activity to nearly 10 units/ml, 6-7 times higher than other strains. Enzyme activity was quantified by inveted sugar method. Four strains (B26, B505, Li and CFd) made powerful cellulase yealding that are bacteria group, where B26, B505 and Li strains have named genus Baccillus.

Effect of temperature was investigated on the extracellular cellulase enzyme activity of 4 strains Li, B26, B505, CFdD . at pH =5.5 and temperature (50-60 ^{0}C) two extracellular cellulase enzymes of Li and B505 strains had strongest Cx activity.

3.2.2. Determine conditions for the culturing of cellulase bacteria

3.2.2.1. Effect of pH on cellulase enzyme activity of 4 strains of bacteria (Li, B505, B26 and CFd)

pH influence on the ability of cellulase yealding is shown in Figure 3.2

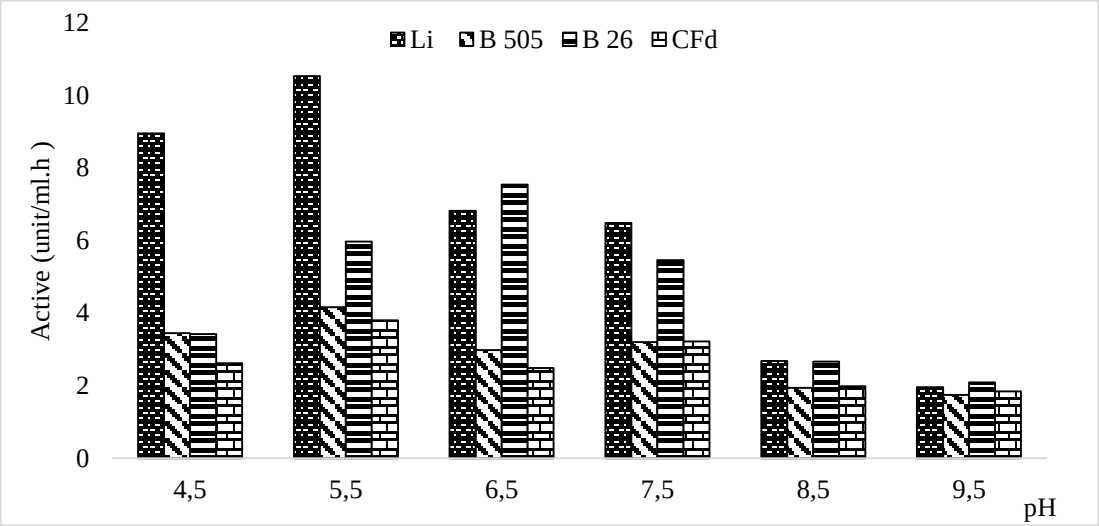

Figure 3.2. Effect of pH on cellulase enzyme activity of 4 strains of bacteria (Li, B505, B26 and CFd)

Figure 3.2 shows the effect of pH on cellulase enzyme activity of four strains (Li, B505, B 26, CFd), it indicates that extracellular cellulase enzyme of Li B505 and CFd strains have the highest active at pH = 5.5; for only extracellular cellulase enzyme of B26 strains shows the highest activity at pH = 6.5. Thus extracellular cellulase enzyme activity of the 4 strains above reach at the strongest activities at close to neutral pH (pH = 5.5 ÷ 6.5).

3.2.2.2. Effect of temperature on the cellulase enzyme yielding activity of 4 strains (Li, B505, B26 and CFd)

Figure 3.3 shows that the extracellular cellulase enzyme of Li strain has highest activity at 50 ^{0}C temperature, extracellular cellulase enzymes of B505, B26 and CFd strains has highest activity at temperature at 60 ^{0}C. Extracellular enzymes cellulase of 4 screened strains of microorganisms has the strongest activity in the temperature range from 50-60 ^{0}C, this temperature range is very suitable for the processing of agar pulp because this temperature does not cause loss of active of multi-trace minerals, protein and amino acid in seaweed waste used for animal feed.

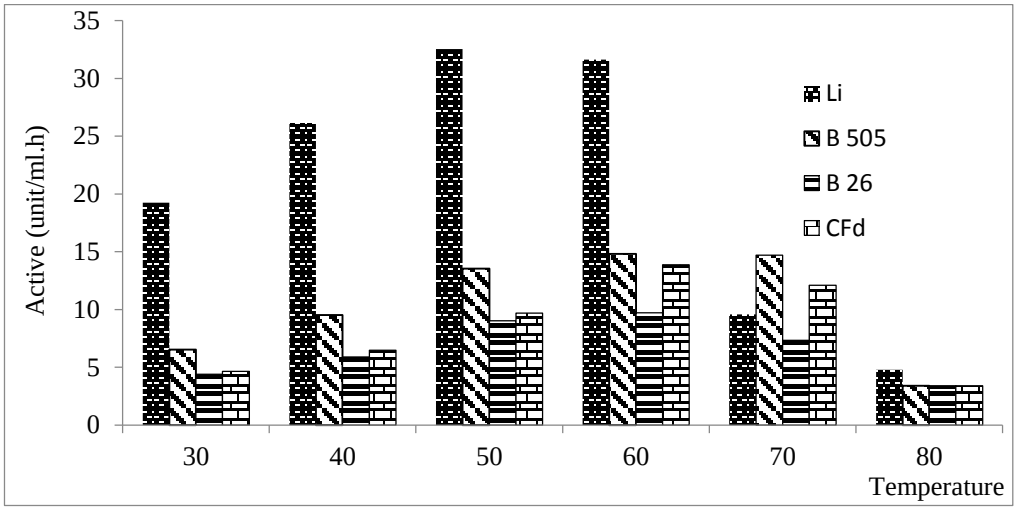

Figure 3.3. Effect of temperature on the extracellular cellulase enzyme yielding activity of 4 strains (Li, B505, B26 and CFD)

effect of temperature on the of extracellular cellulase enzyme activity of four strains Lichenformis bacillus (Li), Subtilis bacillus VTCC-B-505, Subtilis bacillus VTCC-B-26 and CFd) shows that at pH = 5.5 and temperature (50-60 ^{0}C), extracellular cellulase enzymes of Li and B505 strains have strongest Cx activity.

3.3. Study on getting crude cellulase enzyme from B. subtilis VTCC – B – 505 and B. lichenformis (Li) bacteria and application for hydrolysis of seaweed waste (G. verrucosa) after agar production

3.3.1. Determine conditions for the cellulase enzymes breeding process using B505 and Li strains.

3.3.1.1. Selection of culture medium.

Investigation 2 lots of 6 cellulase bacteria culture experiments with different culture environments: soybean, rice, fish sauce, mineral-rich environment and comparison with laboratory environment (MT1, MT1', MT2, MT2', MT3, MT4, MT5, MT6). After culturing, assessment of the growth and cellulose biosynthesis was done by measuring optical density (OD) of the culture solution by a colorimetric spectrophotometer at a wavelength of 620nm. Then cellulase activity was determined by agar diffusion method with CMC substrates: 0.2 ml of crude enzyme solution was added to the agar disc with a diameter of 10mm and incubated at 30^{0}C for 24 hours, then coated lugol solution on disc, measured non staining ring around the agar disc.

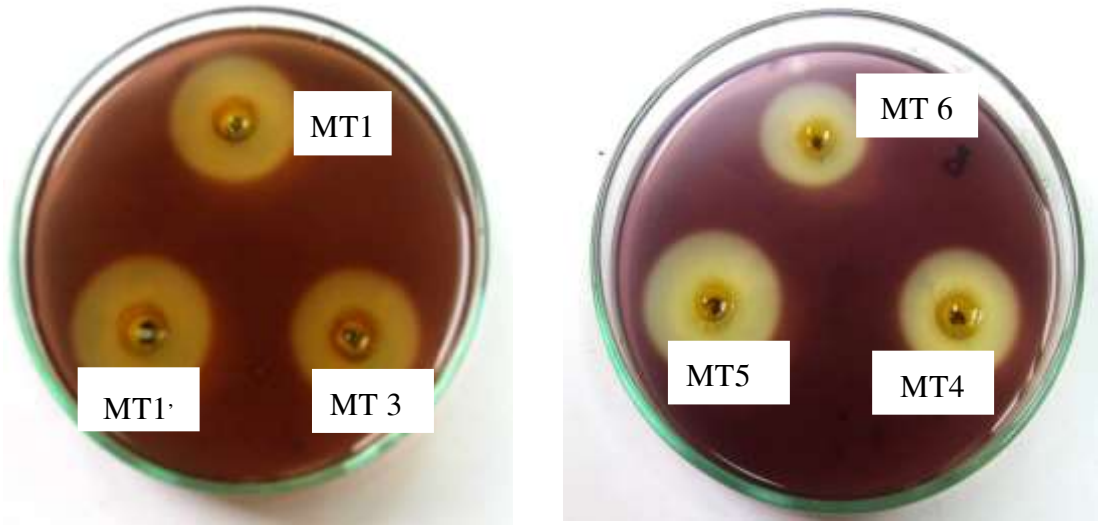

Figure 3.4. Photos of cellulase hydrolysis circle of B505 strains cultured in different environments

Figure 3.5. Photos of cellulase hydrolysis circle of Li strains cultured in different environments

+ Cellulase activity of subtilis B. VTCC-B-505 strain: Figure 4 shows that in MT1' and MT5 nutrition environments, B505 strains had the highest capable of cellulase biosynthesis and growing and of with hydrolysis circle diameter at 0.9 cm, 1.02 times higher than the MT1; 1,125 times higher than MT3; and 1.37 times higher than MT4; 1,153 times higher than MT6. Mixed medium of various components for bacterial culturing can meet the requirements for nutrients such as C source, N source, vitamins, minerals. MT1' environment (2g of CMC; 0.2g of $CaCl_2$; 2g of NaCl; 1g of Cao meat, 1 liter of distilled water) requires high cost because of getting C source and N sources

from synthetic environment. MT5 culture medium (2g of CMC; 2g of soybean powder, 2g of rice flour; 0.4g of NH4Cl, 0.6g of KH2PO4, 1g of K2HPO4; 1 liter of distilled water) gets C source and N sources from the natural environment such as soybean powder and rice flour so it is cheaper. Therefore for large-scale production, culture MT5: (2g of CMC; 2g of soybean powder, 2g of rice flour; 0.4g of NH4Cl, 0.6g of KH2PO4, 1g of K2HPO4; 1 liter of distilled water) is more economic than MT1'.

+ Cellulase activity of lichenformis B. (Li): Figure 5 shows that in MT5 (2g of CMC; 2g of soybean powder, 2g of rice powder, 0.4g of NH4Cl, 0.6g of KH2PO4; 1g of K2HPO4, 1 liter of distilled water) nutrient environment the Li strains had the highest capable of cellulase biosynthesis and growing with hydrolysis circle of 1.12cm in diameter, 1.24 times higher than MT2; 1.27 times higher than MT2' and MT4,; and 1.33 times higher than MT3 and MT6.

Therefore, MT5 is suitable environment for both Li and B505 strains, based on that the project was conducted to determine the optimal culture conditions of Subtilis bacillus VTCC-B-505 and Lichenformis bacillus (Li).

3.3.1.2. Determine the optimal incubation temperature

Temperature is one of the important factors affecting the growth and development of bacteria. So experiments were conducted to survey the extracellular cellulase secretion and growth of B505 and Li strains at temperatures of 25, 30, 35, 40, 45 and 50 ^{0}C.

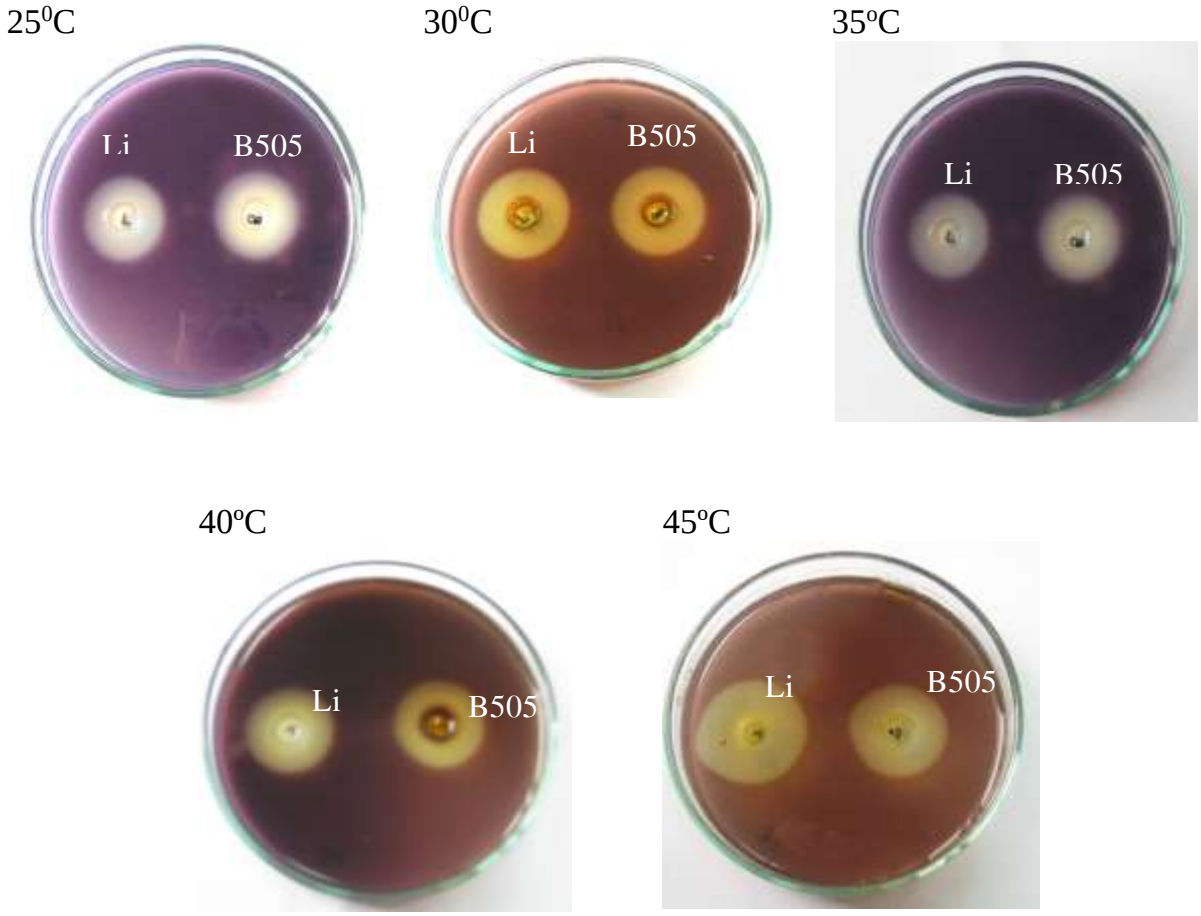

Figure 3.6. The growth and activity circle of Li and B505 strains at different temperatures

The temperature of environment influences strongly on the growth of microorganisms. In fact, microorganisms are normally unicellular organisms, so they are very sensitive to temperature variation. They often transform along with the variation of the environmental temperature. *Result above shows that the suitable temperature for cellulase secretion and growth of B505 strains is 30 ^{0}C and suitable temperature for cellulase yielding, growth and development of Li bacterial, born is 45 ^{0}C.*

3.3.1.3. Determining optimum culture pH

pH is also one of important factors affecting the growth and development of bacteria. So we have studied and evaluated effect of pH on the growth and secretion of extracellular cellulase. pH has marked influence on the growth of microorganisms. Every microorganism has a particular pH range for growth and a given pH for the best growth. Most bacteria and protozoa prefer neutral pH for 5.5 to 8.0. *Figure 3.7 show that the ability of extracellular cellulase secretion of B505 and Li strains was*

significantly influenced by pH variation. B505 strains cultured at pH 7.0 had the strongest cellulase secretion while Li strains had the strongest cellulase secretion when cultured at pH 5.0.

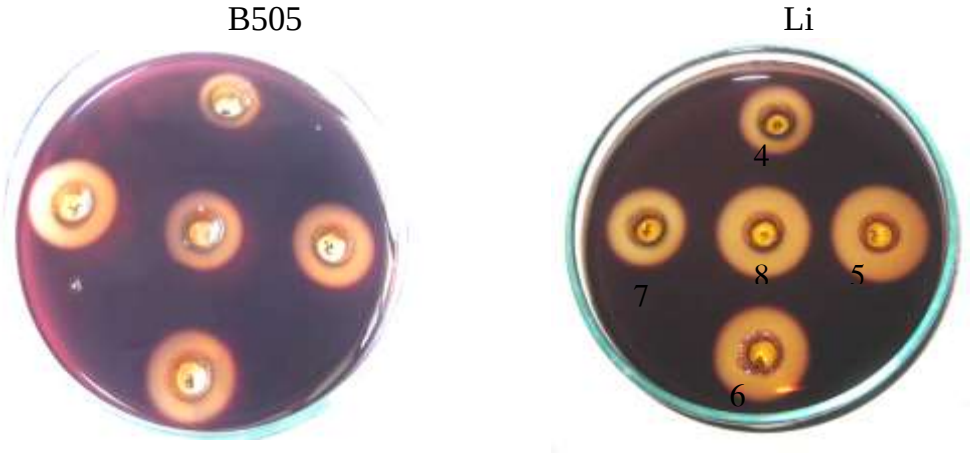

Figure 3.7. Cellulase activity circle of B505 and Li strains cultured at different pH.

3.3.1.4. Optimal culturing time.

As pH, temperature, culturing time is also to affect the enzyme secretion and growth of bacteria. In the process of culturing the active of subtilis B. VTCC-B-505 and lichenformis B. (Li) to be monitored and identified in the specific timeline.

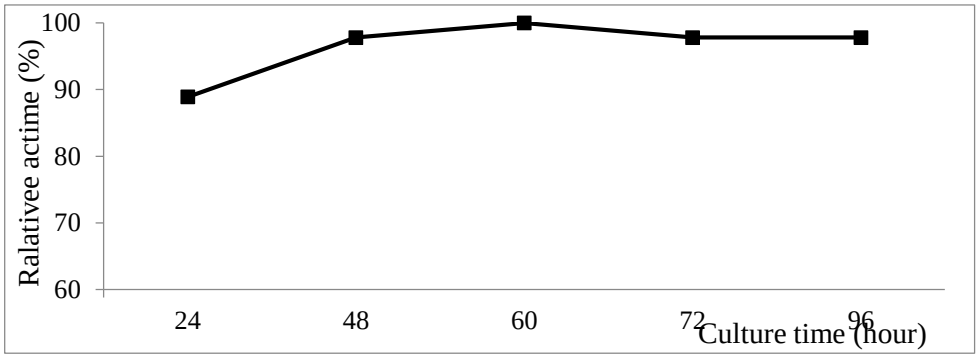

Figure 3. 8. Extracellular cellulase activity of Li strains over cultured time

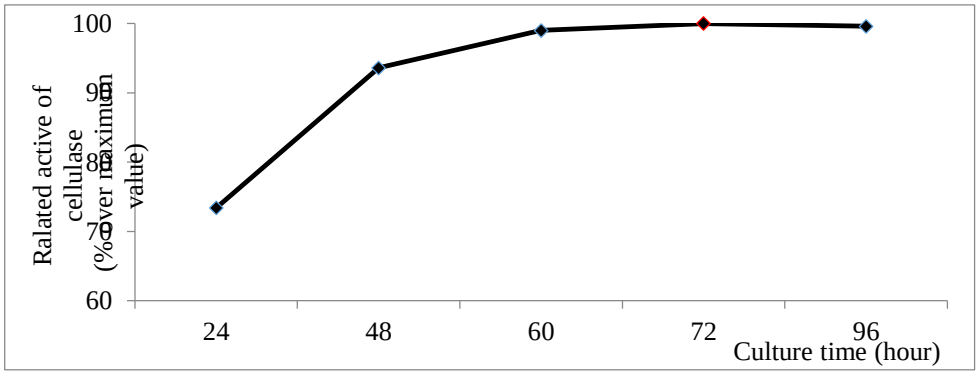

Figure 3.9. Extracellular cellulase activity of B505 strains over cultured time

Figures 3.8 and 3.9, illustrated Li strain has the strongest extracellular cellulase secretion ability after 60 hours of culturing and after 60 hours of B505 strains culturing, crude extracellular CMCase enzyme obtained had activity reaching 99% compared to the extracellular CMCase enzyme obtained after 72 hour of culture. Therefore, 60 hours of culturing the B505 strains to get enzymes brings back the most economic efficiency and saves production costs.

The results were recorded as follows: the optimal conditions for cellulase secretion and growth are activity of subtilis bacillus 2-B-505 and VTCC and lichenformis (Li) bacillus; environment MT5; pH 5.0-7.0; temperature 30 – 45 ^{0}C, time 60 minutes.

3.3.2. Recommend raw cellulose obtaining process

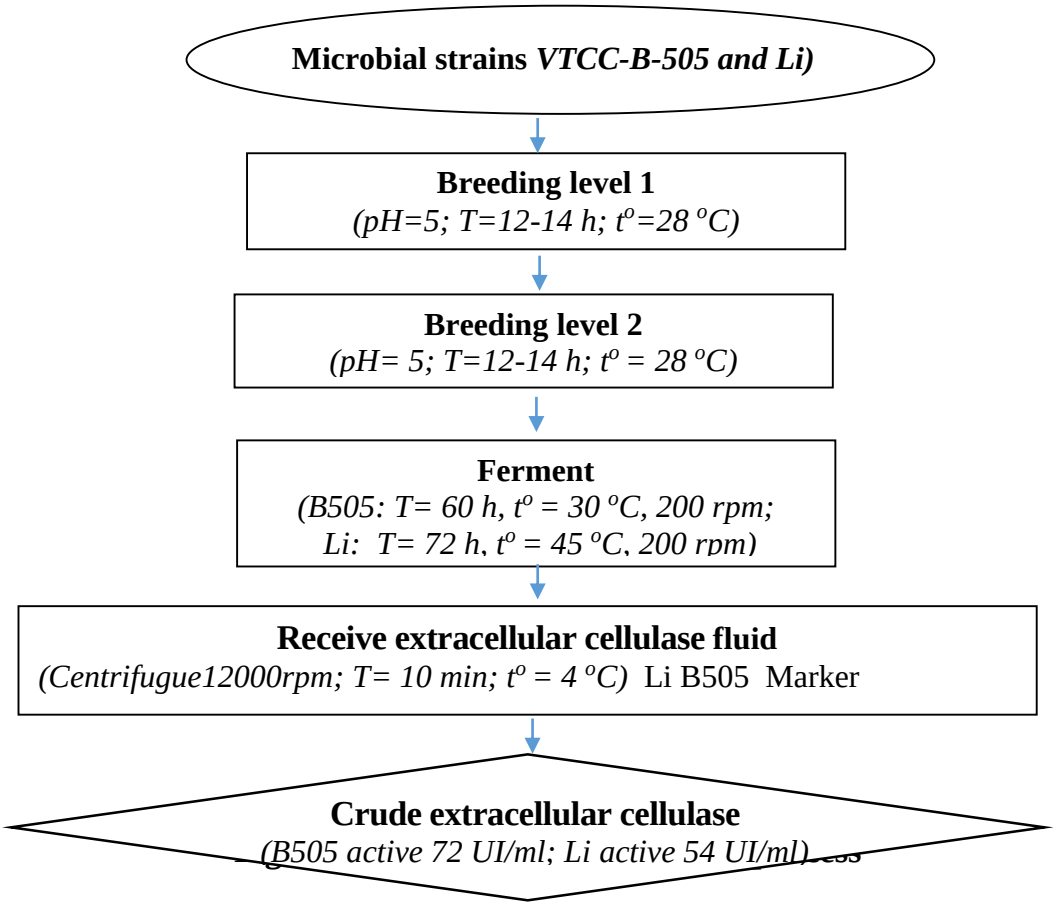

Figure 3.10. Shows that the volume of fish in all six formulas increased.

3.3.3. Comparison and evaluation of the cellulose activity of Li and B505 with cellulose made from other microorganisms.

extracellular cellulase activity of Li and B505 strains was Compared with extracellular enzymes of *Tricoderma* và *Aspergillus* strains and cellulase enzymes of T3 Bacillus strains given by the Institute of Biotechnology by methods to determine cellulase activity on the CMC substrates contained environment (optimal environment to detect Cx enzyme) and the cell environment added with paper pulp (good environment for C1 enzyme activity).

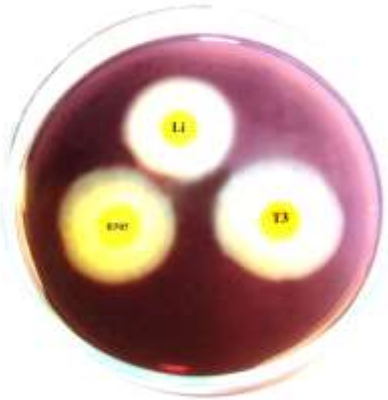

Figure 3.11. Hydrolysis ring diameter of cellulose from *Li, B505 and T3* on agar plates with 0.2% CMC substrate

Figure 3.12. Hydrolysis ring diameter of cellulose from *Tricoderma* on agar plates with 0.2% CMC substrate

Figure 3.13. Cellulase activity of *Aspergillus* from on agar plates with 0.2% CMC substrate

Figure 3.14. Cellulase activity of *Trichoderma* and *Aspergillus* cultured in different environments on agar plates containing 0.2% paper pulp substrate

Where: Sample 1: Cellulase from *A. niger* cultured in the Cell environment; Sample 2: Cellulase from *T. konigii* cultured in the Cell environment;

Sample 3: Cellulase from *A. niger* cultured in the Zapek environment; Sample 4: Cellulase from *T. konigii* cultured in the Zapek environment;

Sample 5: Cellulase from *T. konigii* cultured in their own specific environment; Sample 6: Cellulase form *A. niger* cultureed in their own specific environment.

Figure 3.15. Cellulase hydrolysis ring diameter from Li, B505 and T3 on agar plates with 0.2% pulp substrate

• Cellulase activity of 5 microorganism strains on agar plates with 0.2% CMC substrate

Figure 3.12 and 3.13 show that *Aspergillus niger* and *Tricoderma konigii* strains, even cultured in the optimal environmental conditions for the fungus, but CMC hydrolysis substrate cellulase activity (Cx) was weaker B505, Li and T3. Cx enzym activity of B505 strains was roughly equivalent to Cx active of T3 strains (strains of cellulase yielding Bacillus has been used in the prodution of the Institute of Biotechnology). Therefore, B505 Li 2 strain secreted extracellular cellulase Cx enzyme much stronger than the extracellular cellulase of *Tricoderma* và *Aspergillus* strains.

• Cellulase activity of 5 microorganism strains on agar plates with 0.2% paper pulp substrate

Figure 3.14 and 3.15 show that *Tricoderma konigii* strains cultured in the Zapek environment provided the strongest hydrolysis pulp extracellular cellulase activity. However, B505 and Li strains also provide nearly equivelent hydrolysis pulp extracellular cellulase as *Tricoderma konigii* strains. The data collected is consistent with the results of other authors, cellulase of fungal strains typically have higher C1 activity, but lower Cx activity as compared to cellulase of the bacteria.

Thus, T3, B505 and Li strains have the highest capability of CMC substrates and pulp hydrolisis. Bacterial culturing to obtain enzymes are usually simple and less expensive than culturing the fungus strains. In the group of bacteria, the strains of the *Baccillus* genus always dominate (87%) when applied in waste treatment, because these strains are thermophilic bacteria and create spores, therefore they are able to survive and grow as well in the waste pile at high temperatures.

Producing 1 liter of crude enzyme product in liquid form required by the research costs 40,000 ÷ 45,000 dong. Meanwhile, crude cellulase product produced by Biology Development Company at 186 Ngo Gia Tu Street, Hanoi cost 250,000 dong/1 liter of product. Imported cellulase product cost is very high, which is not feasible for agar hydrolysis on a large scale. The studies to get cellulase enzymes from microorganism help the project to hold the initiative in producing enzyme with lower production cost than commercial enzymes manufactured in the country.

The results showed that cellulase of B505 and Li strains had higher Cx and C1 activity than cellulase from Aspergillus niger and Tricoderma konigii strains, not less than cellulase of T3 strains produced by Biotechnology Institute. So we can use each strains of B505 or Li separately, or use both strains simultaneously to obtain cellulase for hydrolyzing agar waste with high efficiency. The studies to receive cellulase enzymes capable of hydrolyzing waste from B505 agar and Li strains help to hold the initiative in producing enzyme with lower production cost than commercial enzymes manufactured in the country.

3.3.4. Study on hydrolyzing seaweed waste after agar production by cellulase enzyme from the two strains B 505 and Li

3.3.4.1. Optimize the hydrolysis of seaweed waste by cellulase bacteria

Affecting zone of factors (enzyme concentration over substrates, hydrolysis time, pH, temperature) were found out that Based on exploratory experiments to hydrolysis, we conducted the optimisation by means of response surface, used the Box-Behnken model design in hydrolysis of agar waste with boundary parameters as follows: concentration of the enzyme to substrate (1% - 3%), hydrolysis time (24 - 48 hours), the temperature is 40 – 60 ^{0}C, fixed factors: pH = 6.5.

The obtained results show that there is the relationship of second derivative between the total sugar content (mg/kg) and the hydrolysis time (hours), the concentration of enzyme to substrate (%) and hydrolysis temperature (^{0}C) ($P_{F\ lack\ of\ fit}$ = 0.8081).

$$Y = 2.77 + 0.15\ X_1 + 0.21X_2 + 0.058X_3 - 1.12X_1^2 - 0.13X_2^2 + 0.071\ X_3^2 - 0.11X_1X_2 + 0.078\ X_1X_3 - 0.068\ X_2X_3$$

Where:

Y: the total sugar content (mg/kg)

X_1: hydrolysis time (hours)

X_2: ratio of enzyme to substrate (%)

X_3: Temperature (^{0}C).

Statistical processing results showed that in the range of studying two factors affected the total sugar content (mg/kg) including the hydrolysis time (p=0.0137) and the concentration of the enzyme to substrate (p=0.0026), if hydrolysis time and enzyme concentration increased the total sugar content (mg/kg) increased. Statistical results also showed that hydrolysis temperatures did not significantly affect the hydrolysis (p = 0.2538), in other words, research in the temperature range from 40-60 ^{0}C, hydrolysis temperature did not increase the total sugar content (mg/g).

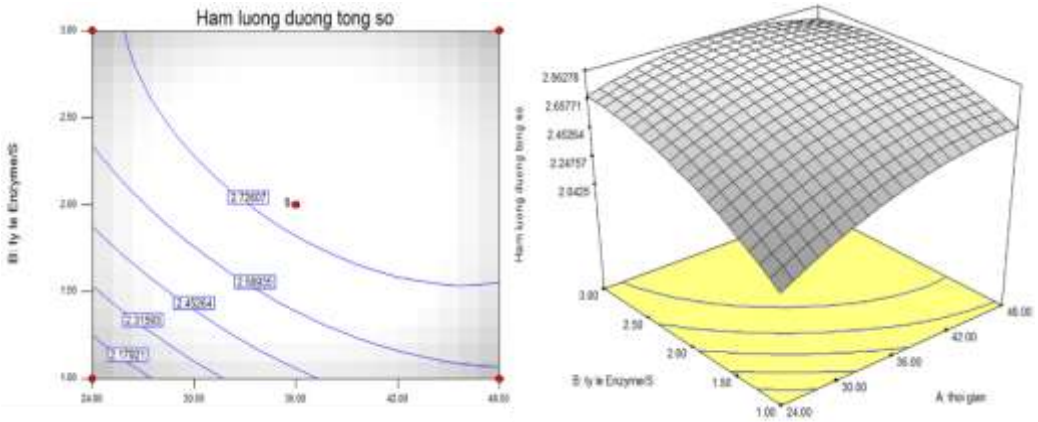

Figure 3.16. Contours and response surface (3D) of the derivative between total sugar content (mg/kg) and hydrolysis time (hours) and concentration of enzyme to substrate (%).

The prediction results of the optimal area for the total sugar content (mg/g) are shown in Figure 3.16. The results indicated that an optimal area and total sugar content (mg /g) was 2.98 (mg/g).

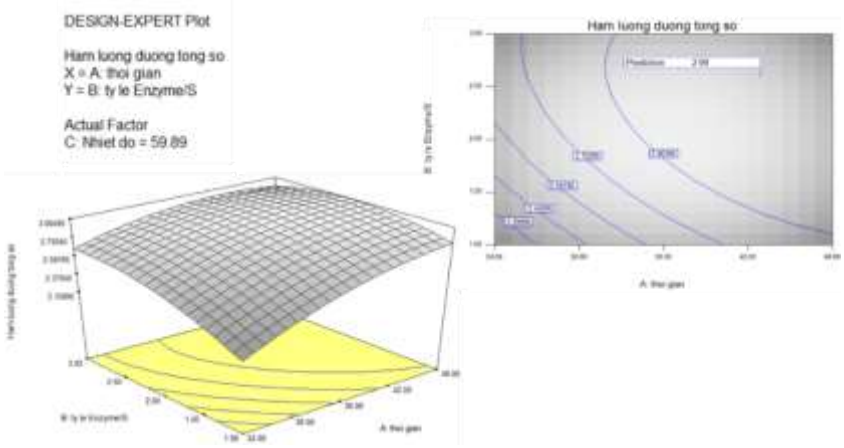

Figure 3.17. Contours and response surface (3D) predicting the derivative between total sugar content (mg/kg) and optimal hydrolysis time (hours) and concentration of enzyme to substrate (%).

The obtained results indicate that there is a compatibility between theory and experiment. The total sugar content was 2.68 ± 0.09 (%) when hydrolysis of agar waste by crude enzyme at temperature 60 ^{0}C, pH = 5.5, hydrolysis time of 42 hours, ratio of enzyme to substrate of 2.6%. Organoleptic evaluation results showed that agar pulp was soft, crushed if lightly squeezed with fingers, light brown colour and smell of seaweed. The protein hydrolysis product obtained from agar waste can be used to mix other material in feed production. The optimal selected environment is temperature 60 ^{0}C, pH = 5.5, hydrolysis time àf 42 hours, the rate of enzyme to substrate 2.6%.

3.3.4.2. Recommend the process of hydrolysis of seaweed waste by cellulase from bacteria

From the results above of the project to draw the manufacturing process of hydrolyzed seaweed product in form of powder as follows:

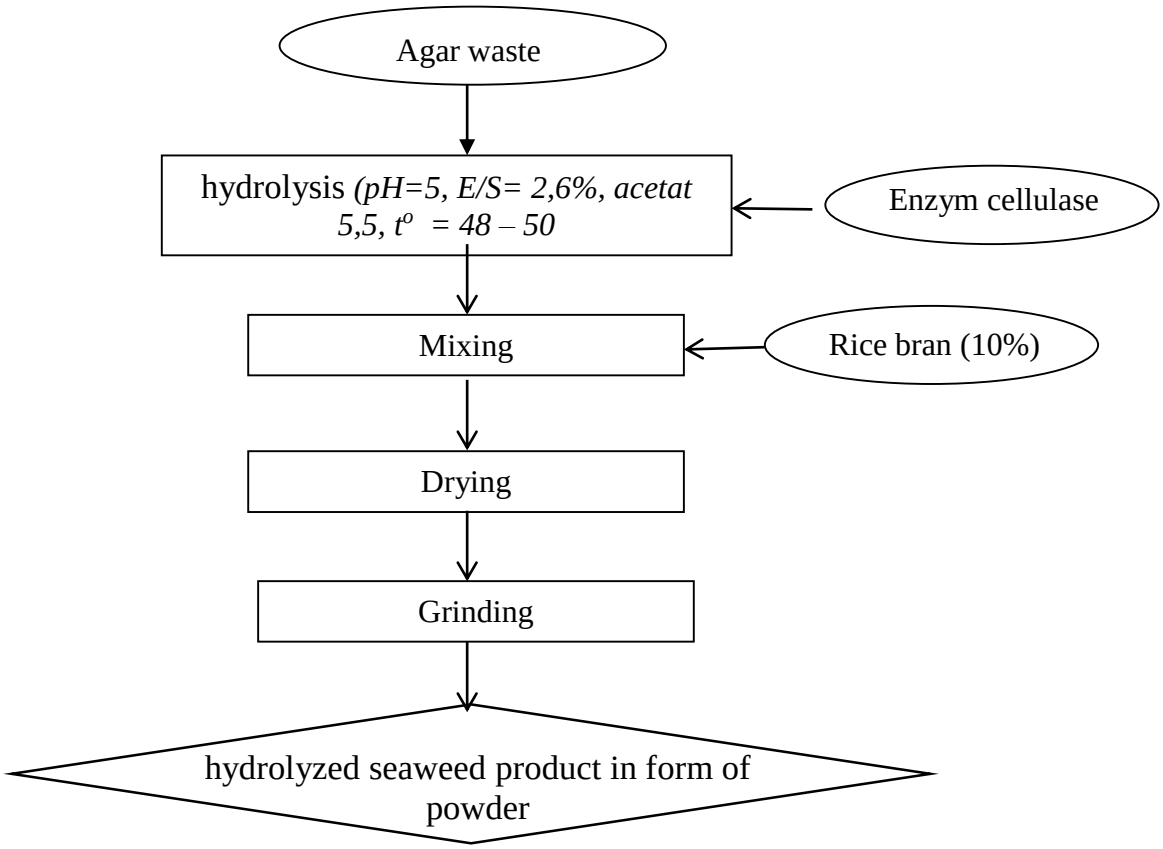

Figure 3.18. Processes of hydrolysis of seeweed waste by cellulase from bacteria

3.4. Trial use of hydrolyzed seaweed waste in feeds for Tilapia

3.4.1. Construction of feed recipe for tilapia

Basing on biological characteristics and nutritional needs at different growing stages of tilapia, basing on the needs of protein and lipid as standards to use appropriate formula, calculation appropriate rate energy and protein demands for tilapia feed.

From choice of materials mentioned above, determination chemical composition of mixing materials and used WUFFF DA software to compute the nutritional components of mixing formula for tilapia feed.

3.4. 2. Recommend production processes for tilapia feed

Process flow chart

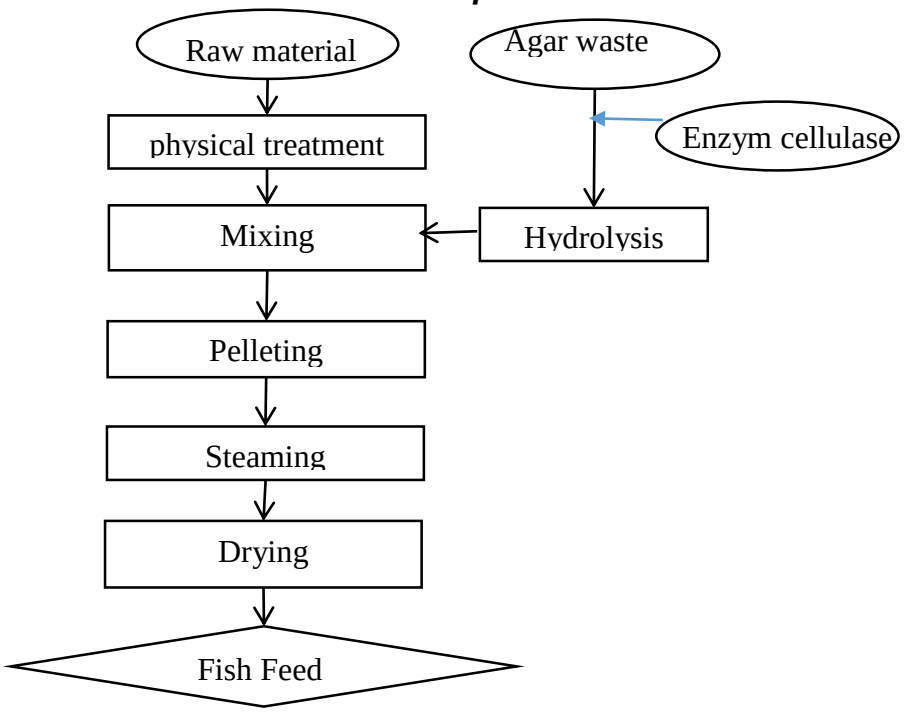

Figure 3.19. Production processes for tilapia feed

3.4.3. Results of trial farming of tilapia

3.4.3. Effect of feed formula on the growth of tilapia

Tilapia was cultured in the 18 tanks on sizing 3m x 2m x 1m with 6 kinds of experimental feed supplemented with hydrolyzied agar waste with mixing ratio in turn is 0%, 5%, 10%, 15%, 20%, 25% and protein is 25% corresponding to 6 expreriments. Each experiment was repeated 3 times. Number of experiments are respectively 18 experimental units (18 culturing tanks). Culturing density is 4 fish/m^2. The average weight of fish at the start of the experiment was 205.9 ± 4.3 g/fish. Fish are fed twice daily at 8:00 and 16:00 during experimental period. Daily feed ratiodependon the culturing stage: from 40 – 100 g/fish was 5% of fish weight, 100 - 150 g/fish was 4% of fish weight, 150 – 200 g/fish was 3% of fish weight. *Feeding ration was adjusted according to the fish growth by capturing and weighing 10 random individual fishs to calculate average weight of individual fish then estimating total weight in the experiments.*

The growth rate of the fish weight after 80 days of culture are presented in Figure 19÷20.

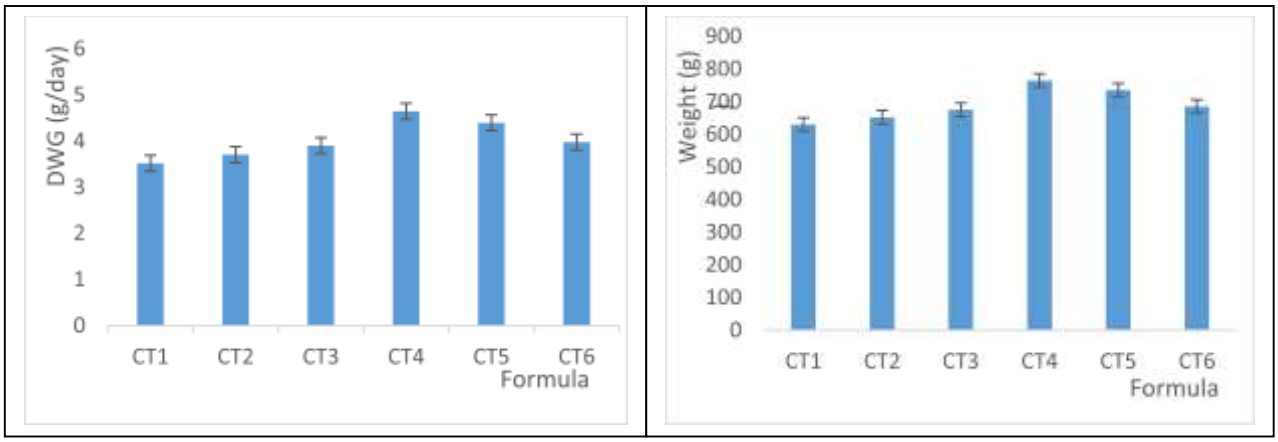

Figure 3.20. Shows that the volume of fish in all six formulas increased.

The fish average weight in all culturing tanks was from 652 to 765 g. The feed formula has 25% of protein content with hydrolyzed seaweed waste mixing ratio of 15%/weight giving the best growth with the highest weight of 765 ± 4.2 g and best growth of 4.65 ± 0.03 g/day. Feed has 25% protein (Pr25) with a control formula not mixing with hydrolyzed seaweed waste brought to lower growth rate during the experiments, the average growth was 3.52 ± 0.04 g/day.

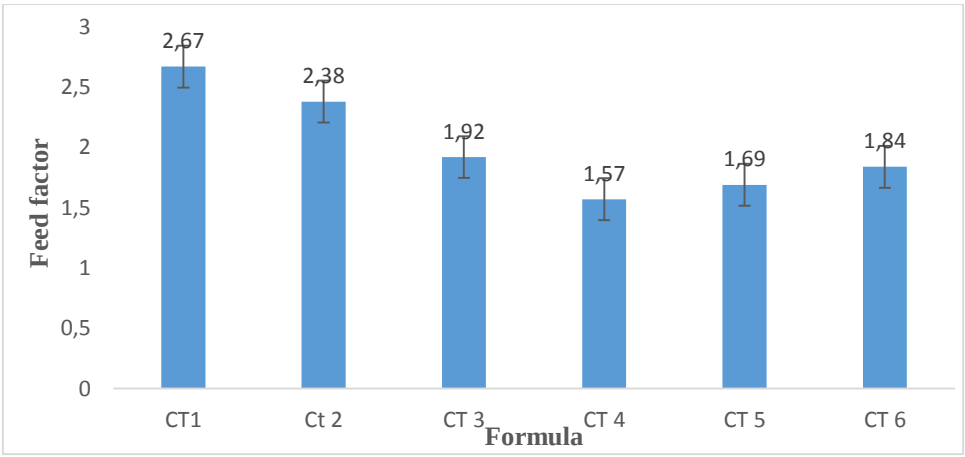

Figure 3.21. Feed factor of culturing experiment and feed formula

The survival rate and feed factor of fish after the culture in the control experiment (CT1) with no hydrolyzed seaweed waste was 76.4 and 2.67 %, respectively. while other experiments using feeds with additional mixing of hydrolyzed seaweed waste, feed factor was from 1.57 to 2.4 and survival rate was from 80.6% - 88.5%. Compared to some former results of other authors, this survival rate is much higher.

CONCLUSIONS AND PERSPECTIVE

1. CONCLUSIONS

The obtained results above allow us to draw some conclusions as follows:

1) The investigation and evaluation of the amount and composition of seaweed waste after agar production from seaweed processingin Hai Phong and some provinces showed that, annual agar production enterprises in Hai Phong discharge 3,500 tons of seaweed waste that contains proteins at content of 3,26 ± 0,11*%;* cellulose content of 74,26 ± 4,68*%;* ash content of 12,28 ± 3,77 % and heavy metals content under the permission of the Ministry of Health.

2) Screening of cellulase enzyme fertility 17 microorganisms in the microorganisms museum of Biotechnology Institute under the Vietnam Academy of Science and Technology and the University of Natural Sciences under the National University discovered that 4 strains of *B. subtilis VTCC-B-505 (B505), B. lichenformis* (Li), B26 and CFD were capable of extracellular cellulase enzyme fertility with high activity.

3) Optimized the culture conditions for those 2 strains and determined the optimal conditions for growth and cellulase yielding of *B. subtilis VTCC-B-505 and B. lichenformis (Li)* bacteria: environment MT5 (2g of CMC; 2g of soybean powder, 2g of rice powder; 0.4g of NH4Cl, 0.6g of NH_4Cl, 1g of KH_2PO_4; 1 liter of distilled water); pH at 5-7; temperature at 30 – 45 ^{0}C, time is 60 minutes. In the selected environment, *B. substilis* VTCC-B-505 strains was capable of yielding cellulase with activity of 72 IU/ml and *B. lichenformis* Li strains was capable of yielding cellulase with activity of 54 IU/ml.

4) Experiment of optimizing hydrolysis of agar waste by cellulase product from *B. substilis VTCC-B-505 and B lichenformis Li* bacteria and determination of optimal conditions for hydrolysis of seaweed waste after agar production: proper temperature is 60 ^{0}C, proper pH is 5.5, the optimal hydrolysis time is 42 hours, the rate of enzyme to substrate is 2.6%. Hydrolyzed seaweed waste product in form of powder is fine and not sticky, smell of dried seaweed, has 4.55% of protein, 23.12% of crude fiber and 12.97% of moisture.

5) Mixed Food with hydrolyzed agar waste was used for tilapia feeding trials with 06 feed formula and found that tilapia culturing had a high survival rate of 80.6% - 88. %, feed ratio was 1.57 to 1.94.

2. Perspective

1) Expand the cellulase production by strains of *B. subtilis VTCC 2-B-505 (B505) and B. lichenformis (Li)* on a large scale to obtain cellulase enzymes for use in industrial hydrolysis of seaweed watse after agar production.

2) Trials of Processingon feed production using formula of the thesis to culture tilapia and some freshwater fish species in industrial scale.