LIPASE PRODUCING PSYCHROPHILIC MICROORGANISM ISOLATED FROM ANTARCTICA

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ABSTRACT

A psychrophilic microorganism (PI12) producing a cold-adapted lipase upon growth at low temperature (5°C) was isolated from Antarctic sea water. PI12 was screened for production of lipase by using medium which contained nutrient agar and lipase substrate, tributyrin, palm oil and olive oil, at 5°C. In the preliminary studies, lipase activity was determined by the titration method. PI 12 grew in nutrient broth containing 1% (i/i) tributyrin and 0.1% (i/i) Tween 20, and 0.5% (i/i) olive oil and 0.1% (i/i) Tween 20. Lipase assay was carried out at 5°C and room temperature (24±2°C) at pH 7. The lipase activities in both tributyrin and olive oil broth media at 5°C were 0.75 U/ml and 0.017 U/ml, and the specific activities were 7.894 U/mg and 0.163 U/mg respectively. For the reaction at room temperature, the lipase activities in both tributyrin and olive oil broth media were 0.9 U/ml and 0.183 U/ml whereas the specific activities were 9.474 U/mg and 1.763 U/mg respectively. The molecular weight of PI12 lipase was also estimated by natrium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE).

INTRODUCTION

Most microorganisms isolated from cold environments are either psychrophilic or psychrotolerant (psychrotrophic). Psychrotolerants grow well at temperatures close to the freezing point of water, but have fastest growth rates above 20°C (up to 40°C), whereas psychrophilic organisms grow fastest at temperature of 15°C or lower, but cannot survive at temperature above 20°C (Madigan *et al.*, 2003). "Psychro' microorganisms are cold-adapted and exhibit properties distinctly different form other thermal classes, such mesophiles and

thermophiles. Psychrophilic organisms and their products have potential applications in a broad range of industrial, agricultural and medical processes. In order for growth to occur in low temperature environments, all cellular components from membranes and transport systems to intracellular solutes, protein and nucleic acids must adapt to the cold (Cavicchioli *et al.*, 2002). Psychrophilic produces enzymes that can function in cold environment and simply denature at high temperature. The cold-active enzymes have a more flexible structure to undergo the conformational changes necessary for catalysis with a lower energy demand (Arpigny *et al.*, 1997). Cold-active enzymes have activities at low environment temperature and do not denature at this low temperature. Enzymes from organisms which adapted to cold environment will retain their activities at the low temperature. Studies on psychrophilic microorganisms that inhabit cold environment are carried out for the economic value of their cold-active properties for novel industrial applications.

Lipases or glycerol ester hydrolases (EC 3.1.1.3) are hydrolases acting on the carboxyl ester bonds present in acylglycerols to liberate fatty acids and glycerols. Lipases are enzymes that extremely versatile because they can catalyse the large number of reaction, such as hydrolysis, esterification and transesterification of triglycerides. They resemble esterases, but differ markedly from them in their ability to act water-insoluble esters (Choo *et al.*, 1998). Lipases are produced by a wide range of microorganisms, including bacteria, fungi and yeast (Dalmau *et. al*, 2000). In recent years, research on microbial lipases has increased because of their potential application in novel biotechnological processes. A variety of mesophilic and thermophilic microbial lipases have been found. However, studies on cold-active lipase is also carried out because it has the molecular characteristics of resistance and functional at low temperature. Lipase producing psychrophilic microorganisms was isolated and reported to have

catalytic activities at low temperatures (Rashid *et al.*, 2001; Lee and Rhee, 1992; Mayordomo *et al.*, 2000 and Christen and Marshall, 1984).

The objectives of this work were to isolate cold-active lipase producing psychrophilic microorganism, to screen the extracellular lipase by using specific agar medium for lipase activity and determine the activity of lipase by quantitative method as well as the molecular weight of the lipase from the psychrophilic microorganism.

MATERIALS AND METHODS

Strains and culture media

The microorganism used in this study was a psychrophilic strain, isolated from Antarctic water sample on nutrient agar plates (pH 7) and named as PI12. In the previous study, PI12 was reported to have high similarity to *Devosia neptuniae* in Proteobacteria kingdom, Alpha subdivision, Rhizobiales order and Hyphomicrobiaceae family (Goh, 2003).

For screening of lipase activity, the nutrient agar medium (5.0g of peptone, 3.0g of beef extract and 12.0g of agar in 1L dH_2O) was supplemented with tributyrin medium (1% v/v), or palm oil medium (0.5% v/v and a drop of detergent) or Rhodomine B medium (1% v/v olive oil and 10ml of Rhodomine B stock 0.01% w/v). The preparation of culture media was done at pH 7.0 and the mixture was well homogenized by a mixer. Cultures were incubated for 7-10 days for tributyrin medium and 7-14 days for palm oil and Rhodomine B media at 5°C.

Growth of bacteria in broth media was also carried out. The nutrient broth (5.0g/L of peptone and 3.0g/L of beef extract) was supplemented with 1% v/v tributyrin and 0.1% Tween 20, or 0.5% v/v olive oil and 0.1% Tween 20. For growth in broth media, the colonies from

nutrient agar plates were inoculated into the tributyrin and olive oil broth media (50ml each) in the 250ml Erlenmeyer flask. The cultures were then incubated at 5°C with the shaking rate of 150rpm for 14 days.

Determination of growth curve of PI12

Pi 12 was cultured in 50ml of nutrient broth in 250ml Erlenmeyer flask and incubated at 5°C. The optical density (OD) reading was recorded at 540nm for each day of incubation and the growth curve was plotted.

Screening of lipase activity.

The secreted lipolytic activity was detected through the formation of clear halos around the colonies in three kinds of agar plates as mentioned above at 5°C. Lipase production on Rhodomine B medium was monitored by fluorescence with UV light at 350nm. Screening for an extracellular lipase was also carried out by using the supernatant (crude enzyme extract) on tributyrin agar plate. This method was carried out to confirm the lipase produced by PI12 strain was an extracellular enzyme. The crude enzyme from the supernatant was pipetted into the holes on tributyrin agar. The agar plates were incubated at 5°C and room temperature (24±2°C). Then the surrounding holes were observed. The supernatant that contained lipase enzyme would hydrolyse the substrate and produced halo zone surrounding the holes on the agar plates.

Preparation of crude enzyme extract

The cultures from tributyrin broth medium were centrifuged at 6000rpm for 15 minutes at 5°C. This process would allow the separation of cell pellet and supernatant for lipase activity

determination. The supernatant consisted of crude enzyme extract was stored at -20°C for further experiment.

Assays of lipase activity

Protein determination was carried out as described by Bradford (1976) with bovine serum albumin as the standard. Lipase activity was assayed by the quantitative titration method as described by Arima *et al.*, (1972) with polyvinyl alcohol and olive oil (3:1) emulsion as a substrate. In the standard assay conditions, the assay medium was prepared by adding 5.0ml of the olive oil-polyvinyl emulsion to 4.0ml of 0.2M phosphate buffer (pH7). The mixture was incubated for 10 minutes at 5°C. Then 1.0ml of crude enzyme solution was added into the assay medium and the mixture was incubated for 30 minutes at 5°C. The lipase reaction was terminated with the addition of 20ml of acetone-ethanol mixture (1:1 v/v) into the reaction mixture. The amount of free fatty acids liberated during the incubation was titrated with 0.05M NaOH and 1.0% phenolphthalein as the indicator. One unit of lipase activity is defined as the amount of enzyme required to liberate 1μmol of fatty acid per ml per minute under the specified conditions (such as incubation temperature etc.)

SDS polyacrylamide gel electrophoresis

The sodium/natrium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out to estimate the molecular weight of the PI12 crude lipase. The SDS-PAGE was done in accordance with Laemmli (1970). The gel system included a resolving gel (12% acrylamide) and stacking gel (4% acrylamide). The gel was stained with 0.2% Coomasie Blue R-250 after electrophoresis. The protein molecular weight marker (10-160kDa), *Prestained Protein*

Ladder (Fermentas) was used as reference markers. The distance of each protein band and the line marker (bromofenol), R_f value was measured. The molecular weight of protein sample was determined from the standard curve of log molecular weight of standard proteins versus the R_f values of proteins.

RESULTS AND DISCUSSION

Isolation of psychrophilic microorganism from the growth temperature profile

PI12 was sub-cultured at two different growth temperatures, 5°C and room temperature (24±2°C). After 5 days incubation, the colonies only grew from the culture that was incubated at low temperature (5°C), whereas the plate cultured at room temperature did not show any growth even after two weeks incubation (Figure 1).

Figure 1: Plate A shows the pure colonies of isolate PI12 on nutrient agar at 5°C.
Plate B shows no growth of PI12 at room temperature (24±2°C).

Figure 2: PI12 cocobacillus cells under the light microscope.

Temperature is one of the four major environment factors (temperature, pH, water and oxygen) that affect the growth of microorganisms (Madigan *et al.*, 2003). Psychrophiles are very sensitive to the changes of temperature, owing to the facts that all components of the psychrophilic cell from membranes and transport systems to intracellular solutes such as protein and nucleic acid must be suitably adapted (Cavicchioli *et al.*, 2002). As a result of changing of

environment temperature would influence the entire microorganism metabolism and stop the growth. The psychrophilic would only grow and survive at certain range of temperature, maximum growth temperature below 20°C and minimum growth temperature at 0°C or lower.

Growth of PI12 and production of lipase

The growth curve was plotted to determine growth of PI12 in tributyrin and olive oil broth media (Figure 3). From the graph, lag phase was not detected. However, the exponential/logarithmic and stationary phases were very obvious.

Figure 3: Graph of PI12 growth curve in nutrient broth medium at 5°C.

The growth or cell biomass reached maximum at day 15 (late logarithmic phase). At this phase, the growth of PI12 was maximum and then stopped for dividing at the stationary phase. The observation of growth rates for PI12 strain during the logarithmic growth phase is in accordance with the concept that the production of enzymes is advantageous to the microorganism only when nutrients become limiting, i.e. during the late logarithmic phase or early stationary phase. In this case, the high amount of cells produced more enzymes in the oil medium and the oil was hydrolysed to provide enough carbon sources for metabolism of the cell. Therefore, 14 days of incubation time was chosen to culture PI12 in the tributyrin and olive oil media for the optimum lipase activity.

The psychrophilic isolate was cultured on the tributyrin agar for screening of lipase activity. Clear hydrolisis zone surrounding the bacteria colonies was detected after 7 days of

incubation at 5°C (Figure 4). The halos indicated the hydrolysis activity of lipolytic enzyme on tributyrin sustbrate. The hydrolisis zone was bigger with the increase of incubation time.

Figure 4: Hydrolisis zone surrounding the PI12 colonies on tributyrin agar plate after 7 days of incubation at 5°C.

Tributyrin consists of short chain fatty acids, butyric acid ($C_{4:0}$) and dissolved quite easily in water as compared to triolein, palm oil and oilve oil as lipase substrates. It was usually used for screening of lipase and determination of lipase activity by titration (Brockerhoff and Jensen, 1974). However, the halo zone surrounding colonies may also due to the esterase activity, another lipolytic enzyme that is able to hydrolyse tributyrin. Therefore further screening of lipase was carried out by using the longer fatty acid chains substrates such as triolein, olive oil and palm oil because esterase only reacts with those short chain fatty acid substrates.

For Rhodomine B agar plate, PI12 colonies showed orange flourescent colour under the ultra-violet (UV) light (Figure 5). The olive oil in Rhodomine B agar acted as substrate for the reaction of lipase, and the Rhodomine B reacted with the fatty acid that has been released from the hydrolysis reaction to produce orange flourescent colour. It was found that PI12 colonies showed the flourescent colour but there was no flourescent halo detected surrounding the colonies. This is in accordance with the report by Kouker and Jaeger, 1987 who worked with *Staphylococcus aureus*. The production of lipase from the bacteria was observed from the flourescent *S. aureus* colonies. When the incubation time was increased, the orange flourescent zone was observed surrounding the colonies. The low concentarion of Rhodomine B was reported not to produce fluorescent hydrolysis zone (Gupta *et al.*, 2003), and the zones were only observed under the UV light with the existence of high lipase activity. Therefore, the flourescent

zone was not observed in the experiment may be due to the low concentartion of PI12 lipase acivity.

Figure 5: PI12 colonies on nutrient agar with Rhodomine B showed orange flourescent colour under the UV light.

The lipase activity was also detected from the broth media. After 14 days of incubation with shaking at 5°C, the colour of both tributyrin and olive oil broth media have changed from the yellowish white to clear medium. Figure 6 showed the different of medium colour after incubation at 5°C for 14 days. The clear medium indicated that lipase was produced and the enzyme hydrolysed the oil substrat in the medium.

Figure 6: PI12 cultured in the nutrient broth medium consisted of tributyrin (1.0% v/v) and Tween 20 (0.1% v/v). Flask A is the PI12 culture medium after 14 days of incubation at 5°C and flask B is the control medium, without PI12.

In the experiment, the broth containing the substrates for lipase activity (tributyrin, olive oil and palm oil) was incubated for 14 days because the production of enzymes from the microorganisms occured when the nutrient became limiting, ie at the late logarithmic phase or early stationary phase (Lee and Rhee, 1993). At this point, the subtrates in broth media induced PI12 to produce lipase. When the carbon source in the nutrient broth has finished, PI12 produced an extracellular lipase to hydrolyse the substrates if it has the gene for the lipase synthesis. The hydrolysed substrates provided carbon sources for the growth and other metabolisms activities in the cells.

The reaction medium was added with Tween 20 as an emulsifier to increase lipase activity at the oil-water surface. Tween 20 was reported to increase the lipase acivity of *Aspergillus nidulans* (Mayordomo *et al.*, 2000). The change of medium colour from yellowish-white to clear medium in tributyrin and olive oil broth media indicated that both substrates were used by PI12 with the aid of lipase acitivty that was produced. There was a growth of PI12 in palm oil medium but no lipase activity was detected from the medium. This medium was not a good inducer in lipase production because the palm oil was observed to became semi-solid form at 5°C.

In the experiment for screening of extracellular lipase, the supernatant from tributyrin and olive oil broth media containing the crude enzymes were transfered into the holes on tributyrin and olive oil agar plates to detect the existence of extracellular lipase activity. Halo zones were appeared surrounding the holes of both agar plates after 1 day incubation at 5°C and the room temperature (Figure 7). The diameter of hydrolysis zone increased with the increase of incubation time (Figure 8).

Figure 7: Hydrolysis zone formed surrounding the holes on tributyrin agar that contained the crude enzyme (from olive oil culture medium) at 5°C (plate A) and room temperature, 24°C±2°C (plate B) after 1 day of incubation.

Figure 8: Hydrolysis zone formed surrounding the holes on tributyrin agar that contained the crude enzyme (from olive oil culture medium) at 5°C (plate A) and room temperature, 24°C±2°C (plate B) after 6 days incubation.

Determination of lipase activity

The standard curve for protein determination by Bradford method (1976) with bovine serum albumin, BSA (20-200µg/ml) as the standard and wavelength 595nm was plotted (Figure

9). The determination of protein content by Bradford assay was easier and faster because it did not involve a lots of preparation steps as compared to the Lowry method (Lowry *et al.*, 1951). Therefore, the protein assay will be more accurate.

Figure 9: Standard curve of BSA for protein determination.

The concentration of secreted protein from the cell was determined by using the standard curve (Figure 9). The concentration of protein in the supernatant of tributyrin and oilve oil medium was 0.095mg/ml and 0.104mg/ml respectively. The protein concentration produced was quite low (only a small amount of protein was secreted from PI12). This may be due to the denaturation of certain proteins in the supernatant preparation process or from the assay itself.

Lipase activity in supernatant cultures at 5°C and room temperature (24°C ±2°C) was measured by the titration method using olive oil-polyvinyl alcohol emulsion as a subtrate and phenolphthalein as the titration marker. The volume of NaOH used to neutralise acid was recorded to determine the lipase activity. The oilve oil substrate will only be hydrolysed in the present of lipase activity produced by PI12. One unit of activity was defined as the amount of enzyme needed to release 1µmol of fatty acid per min.

Figure 10: Lipase assay medium with olive oil-polyvinyl alcohol emulsion as substrate. Flask A is the medium before the reaction and flask B is the medium after 30 minutes of lipase assay.

PI12 lipase activity from tributyrin broth medium at 5°C was 0.750U/ml and the specific activity was 7.894U/mg (Table 1). The lipase activity was lower in the olive oil broth medium (0.017U/ml) with the specific activity of 0.163U/mg. For the reaction at room temperature, the

activities of lipase from both tributyrin and olive oil medium were 0.900U/ml and 0.183U/ml, respectively, wherease the specific activities were 9.474U/mg and 1.763U/mg respectively (Table 1).

Table 1: Lipase activities after 30 minutes reaction at 5°C and room temperature (pH 7).

It was found that the activity of lipase was higher at the room temperature as compared to activity at lower temperature (5°C) for both types of culture. Ezymes extracted from Antarctica species usually have the specific activities in the range of 0-30°C (Arpigny *et al.*, 1997). The rates of chemical and enzymatic reactions are effected by the temperature factor. The decrease of temperature (10°C) will decrease enzyme activity with the factor of 2 or 3 (Arpigny *et. al.*, 1997). The activity of lipase was higher in the tributyrin medium than those of olive oil medium. This was because the tributyrin medium provided more reaction surface for the lipase activity.

The production and activity of lipase are depended on a few parameters such as temperature (Arpigny *et al.*, 1997; Choo *et al.*, 1998), pH (Brockerhoff and Jensen, 1974), lipid source (Temerler *et al.*, 2001), protease inhibition, and inocolum size. PI12 was a psychrophilic microorganism and the activity of lipase from PI12 was detected at both cold and room temperatures. PI12 was not survived at room temperature, but the lipase produced retained the activity at the room temperature. The psychrophilic microorganis produced cold-active enzymes, however the enzymes may have optimum activity at the higher or lower temperature than the growth optimum temperature. Brockerhoff and Jensen (1974) reported that lipase has a wide range of temperature for its activity, -20°C – 65°C and most enzymes have activities at 30°C – 45°C (Jensen, 1983). Sheridan *et al.*, (2000) was also reported that the optimal temperature for

enzyme activity is usually not the same as the growth temperature for the organism and enzymes are often found to have optimal temperatures of activity that are 11-20°C higher than the optimal growth temperature.

Determination of lipase molecular weight by SDS-PAGE

The molecular weight of lipase from PI12 was judged by SDS polyacrylamide gel electrophoresis using the standard curve of log molecular weight of standard proteins.

Figure 11: SDS-PAGE analysis.
Lane 1, 2 and 3 – crude enzyme from tributyrin broth at 14 days of incubation.
Lane 4 and 5 – lipase standard.
Lane 6, 7 and 8 – crude enzyme from oilve oil broth at 14 days of incubation.
Lane 9 – protein marker (*Prestained Protein Ladder – Fermentas*).

Figure 11 shows the two protein bands, whose molecular weight about 61 kDa and 66 kDa were observed from the supernantant of crude enzyme extracts (tributyrin medium, lane 1-2 and olive oil medium, lane 6-8). However, the bands for lane 1-3 were not very clear. The supernatant sample was used for SDS-PAGE because the ezyme produced was the extracellular lipase. The two bands indicated two types of protein in the supernatant and one of the protein would be the lipase produced from PI12. The bands were not very clear may be due to the low concentration of protein in the supernatant. The molecular weight of lipase was determined based on the R_f values of protein bands and the standard curve of log molecular weight of standard protein. The commercial lipase was also used as standard in SDS-PAGE analysis.

From the literature, molecular weight of lipase for most bacteria such as *Pseudomonas aeruginosa* (Chihara-Siomi *et al.*, 1992; Chartrain *et al.*, 1993), *Pseudomonas putida* (Lee and

Rhee, 1993), *Pseudomonas* sp. (Choo *et al.*, 1998; Yang *et al.*, 2000; Rashid *et. al.*, 2003) was between 29kDa – 45kDa. However, there were also larger molecular weight of lipase isolated from different microbes. The molecular weight of the two bands detected in the experiment was close to those commercial lipase. The result showed that one of the band may be was the lipase enzyme. The further lipase purification process would need to be carried out to confirm the molecular weight of PI12 lipase. The other approach that can be done is the Native PAGE analysis. The gel containing protein bands can be cut and place on the tributyrin agar for zymogram analysis.

CONCLUSION

Psychrophilic microorganism, PI12 was successfully isolated from Antarctica sea water after 5 days of incubation at 5°C. PI12 was detected to produce an extracellular lipase activity by showing the clear hydrolysis zones on the lipase specific media. Results showed that the activity of lipase from PI12 at room temperature (24°C±2°C) was higher than those at 5°C. The estimated molecular weight of PI12 lipase was either 61kDa or 66kDa.

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Broth medium	Temp.	[b] Volume of NaOH (ml)	Lipase activity (U/ml)	Conc. of protein (mg/ml)	Specific activity (U/ mg)
Tributyrin	5°C	4.5	0.750	0.095	7.894
	24°C	5.4	0.900		9.474
Olive oil	5°C	0.1	0.017	0.104	0.163
	24°C	1.1	0.183		1.763

[b] = average of two readings

Table 1: Lipase activities after 30 minutes reaction at 5°C and room temperature (pH 7).

Figure 1: Plate A shows the pure colonies of isolate PI12 on nutrient agar at 5°C.

Plate B shows no growth of PI12 at room temperature (24±2°C).

Figure 2: PI12 cocobacillus cells under the light microscope.

Figure 3: Graph of PI12 growth curve in nutrient broth medium at 5°C.

Figure 4: Hydrolisis zone surrounding the PI12 colonies on tributyrin agar plate after 7 days of incubation at 5°C.

Figure 5: PI12 colonies on nutrient agar with rhodomine B showed orange flourescent colour under the UV light.

Figure 6: PI12 cultured in the nutrient broth medium consisted of 1.0% tributyrin v/v and 0.1% Tween 20 v/v. Flask A is the PI12 culture medium after 14 days of incubation at 5°C and flask B is the control medium without PI12.

Figure 7: Hydrolysis zone formed surrounding the holes on tributyrin agar that contained the crude enzyme (from olive oil culture medium) at 5°C (plate A) and room temperature, 24°C±2°C (plate B) after 1 day of incubation.

Figure 8: Hydrolysis zone formed surrounding the holes on tributyrin agar that contained the crude enzyme (from olive oil culture medium) at 5°C (plate A) and room temperature, 24°C±2°C (plate B) after 6 days of incubation.

Figure 9: Standard curve of BSA for protein determination.

Figure 10: Lipase assay medium with olive oil-polyvinyl alcohol emulsion as substrate. Flask A is the medium before the reaction and flask B is the medium after 30 minutes of lipase assay.

Figure 11: SDS-PAGE analysis.
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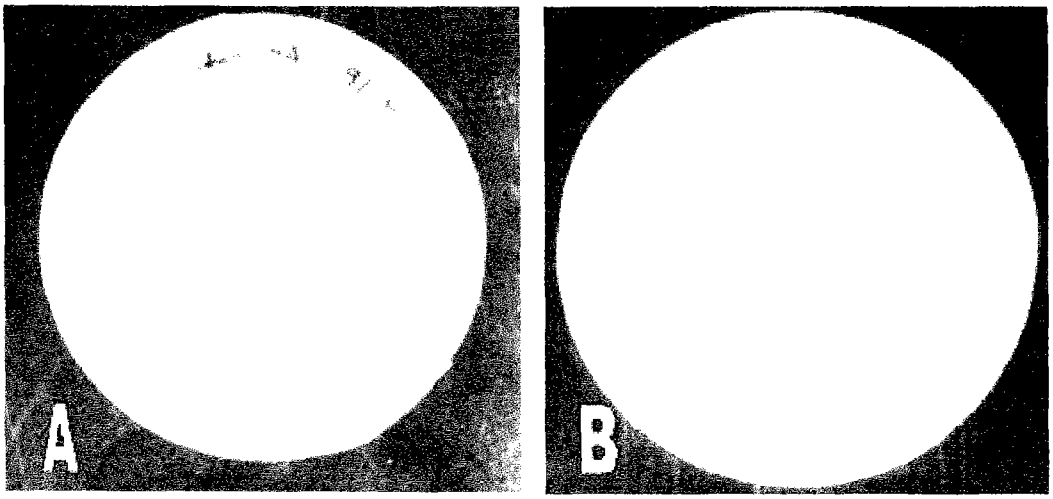
Lane 9 – protein marker (*Prestained Protein Ladder – Fermentas*)



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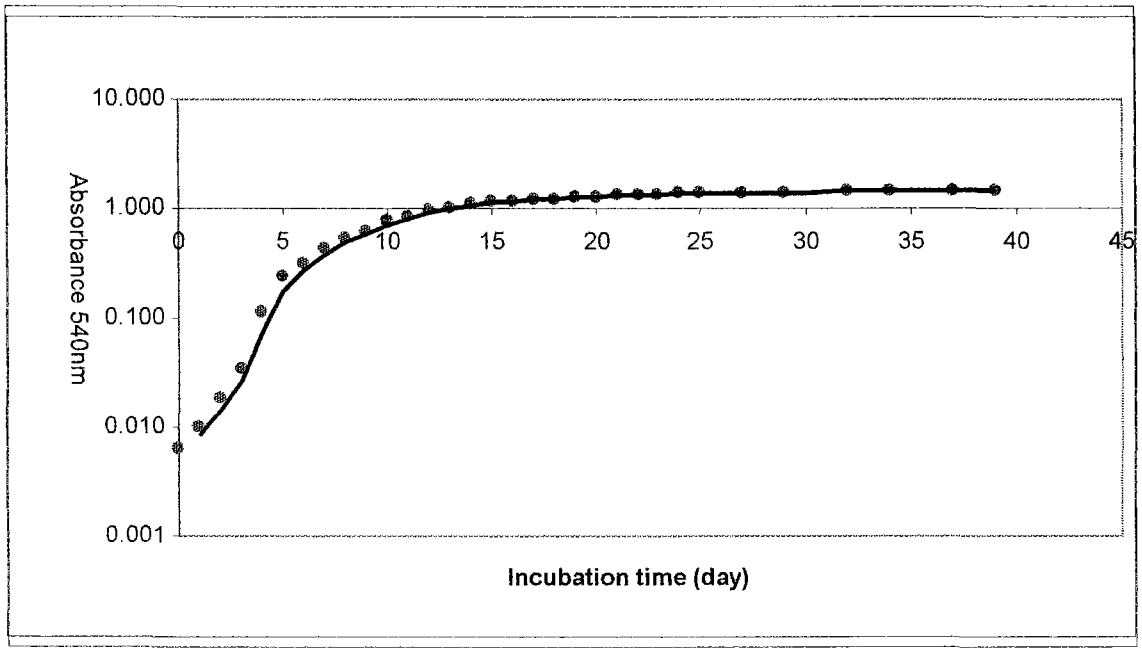
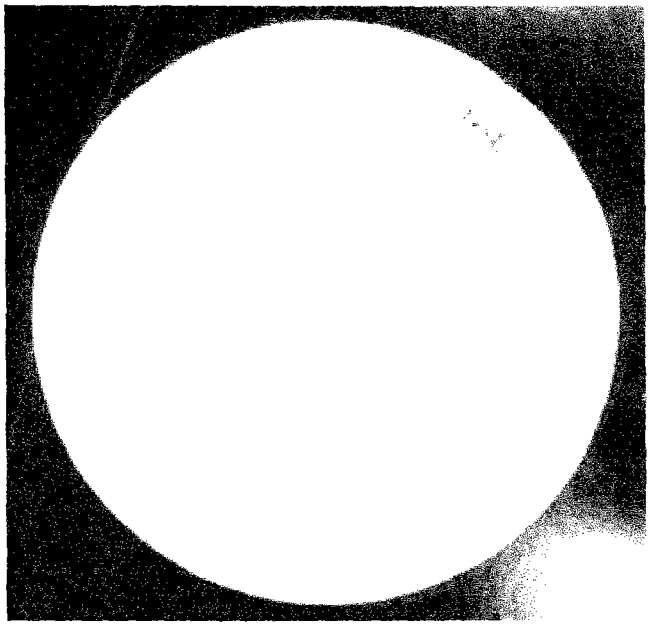


Figure 3: Graph of PI12 growth curve in nutrient broth medium at 5°C.



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