The Efficiency of Different Enzyme Cocktail Conditions in Primary Cell Line Establishment

ประสิทธิภาพของเอ็นไซม์ต่างชนิดในการเพาะเลี้ยงเซลล์ปฐมภูมิ

Jiriya Phasasuk (จิริยา ภาษาสุข)* Dr.Sanya Roysommuti (ดร.สัญญา ร้อยสมมุติ)**

Dr.Paradee Auvichayapat (ดร.ภารดี เอื้อวิชญาแพทย์)** Dr.Wichittra Tassaneeyakul (ดร.วิจิตรา ทัศนียกุล)***

Amnard Kitkhuandee (อำนาจ กิจควรดี)**** Dr.Tidarut Boonmars (ดร.ธิดารัตน์ บุญมาศ) *****

Dr.Wiyada Punjaruk (ดร.วิยะดา ปัญจรัก)******

ABSTRACT

The objectives of this study are study effects of different enzyme cocktail conditions on quality of cell line establishment. Brain tumor specimens were proceeded for cell dissociation using 4 different enzyme cocktail conditions: 1) collagenase 2) collagenase + DNase type I 3) collagenase + hyaluronidase 4) collagenase + DNase type I + hyaluronidase. All 4 conditions provided similar good cell disaggregation with some clump cells. The percentages of live cells after cell dissociation are not different from 4 conditions. Before 96 hours, cells from 4 conditions reached maximum of cell attachment. However, less than half of seeding cells from the condition using 3 enzymes could attach the flask surface. Only 2 conditions (collagenase and collagenase + hyaluronidase) could reach 80% confluency within 5 days after establishment. Therefore, using single enzyme is effective enough for primary brain tumor cell line establishment and also provides a more economic solution.

บทคัดย่อ

การศึกษานี้ต้องการศึกษาถึงประสิทธิภาพของเอ็นไซม์ในแต่ละสภาวะในการสร้างเซลล์เพาะเลี้ยงเนื้องอกสมอง โดยใช้เอ็นไซม์ 4 สภาวะ ดังนี้1) collagenase 2) collagenase + Dnase I 3) collagenase +hyaluronidase และ 4) collagenase + Dnase I + hyaluronidase ภายหลังการศึกษา พบว่า ทุกสภาวะสามารถแยกเซลล์เป็นเซลล์เดี่ยวได้ดีพอๆ กัน มีเซลล์ที่ เกาะเป็นกลุ่มเพียงเล็กน้อยเมื่อนับสัดส่วนของเซลล์ที่มีชีวิตพบว่า ไม่แตกต่างกันในทุกสภาวะ ในการศึกษาเกี่ยวกับความ สามารถของเซลล์ในการเกาะพื้นผิวภาชนะที่ใช้เลี้ยง พบว่า เซลล์จากทุกสภาวะสามารถเกาะได้จำนวนสูงสุดภายใน 96 ชั่วโมง หลังจากใส่เซลล์ลงไป แต่เซลล์ที่ได้จากการใช้ 3 เอ็นไซม์สามารถเกาะได้จำนวนเพียงครึ่งเดียวของเซลล์ที่ใส่ลงไปนอกจากนี้มีเพียงเซลล์ที่สกัดด้วย collagenase และ collagenase ร่วมกับ hyaluronidase เท่านั้นที่สามารถเจริญเติบโตได้ถึงร้อยละ 80 ของพื้นที่ผิวที่เพาะเลี้ยงภายใน 5 วันหลังจากใส่เซลล์ลงไป ดังนั้น จะเห็นได้ว่า การใช้เอ็นไซม์เพียงชนิดเดียว ในการสร้างเซลล์เพาะเลี้ยงก็มีประสิทธิภาพเพียงพอและยังเป็นการประหยัดงบประมาณด้วย

Key Words: Primary cell line establishment, Cell dissociation, Pediatric brain tumors

คำสำคัญ: สร้างเซลล์เพาะเลี้ยง การแยกเซลล์ เนื้องอกสมองในเด็ก

* *Student, Master of Science Program in Neurosciences, Department of Physiology, Faculty of Medicine, Khon Kaen University*

** *Associate Professor, Department of Physiology, Faculty of Medicine, Khon Kaen University*

*** *Associate Professor, Department of Pharmacology, Faculty of Medicine, Khon Kaen University*

**** *Assistant Professor, Department of Surgery, Faculty of Medicine, Khon Kaen University*

***** *Associate Professor, Department of Parasitology, Faculty of Medicine, Khon Kaen University*

****** *Lecturer, Department of Physiology, Faculty of Medicine, Khon Kaen University*

Introduction

Brain tumors are the most common occurrence of solid tumors in children under 15 years of age and represent 20% of all children cancers (Makino et al., 2010). The most common childhood brain cancer is medulloblastomas including primitive neuroectodermal tumors (PNETs) followed by glioblastoma multiforme (GBM) (Baldwin & Preston, 2004). These cancers are classified as grade IV tumors by WHO 2007 and their presence usually leads mortality (Louis et al., 2007). Hence, further studies are essentially required to study all aspects of brain cancers to improve treatment outcome and decrease mortality. Important materials for studying brain tumors are brain tumor cell lines, which are acceptable worldwide as standard method to research on human brain tumors.

In Thailand, brain tumors are the second most common disease occurring in children after leukemia. Additionally, the pediatric brain tumors (PBTs) have poor survival rate compared to other solid tumors such as retinoblastomas, germ cell tumors, renal tumors, soft tissue tumors and liver tumors (Gurney et al., 2001). In Thailand, the overall 5-year survival rate of PBTs is 41.7%, which is lower than that of the Western countries (Wiangnon et al., 2003). Currently, multidrug resistance of PBTs leading to chemotherapeutic resistance and poor treatment outcome is problematic issue worldwide including Thailand. Therefore, PBTs are extensively studied in many aspects to understand the origin of brain tumors, development of cancers and distinct characteristics of brain tumors including multidrug resistance mechanisms (Dean et al., 2005; Dean, 2009; Gottesman et al., 2002; Hartz & Bauer, 2011). If all significant secret knowledge of brain tumors can be revealed, PBTs are possibly cured in the future.

To study human brain tumors, brain tumor cell lines derived from patients with brain tumors are generally used. Currently, all commercial human brain tumor cell lines are derived from Caucasian and there is no available brain tumor cell lines derived from Thai patients. In addition, this will be important materials for further studies and researches in the field of brain tumor in Thailand. Previously, there are 2 main techniques to establish primary brain tumor cell lines; mechanical dissociation without enzymes and enzyme dissociation (Ali-Osman, 1996; Rutka et al., 1986; Wroblewska et al., 1975; Xu et al., 2011). However, mechanical dissociation technique provided low success rate of primary cell line establishment (Xu et al., 2011). Thus, our study is based on enzyme dissociation technique for primary cell line establishment (Ali-Osman, 1996; Rutka et al., 1986; Wroblewska et al., 1975). The previous study used enzyme cocktail including DNase type I, collagenase and hyaluronidase to dissociate brain tumor tissue for primary brain tumor cell line establishment (Al-Hajj & Clarke, 2004). Besides, some previous studies used less enzyme components to successfully establish cell lines (Aoki, 1974). There is no study to reveal the appropriate technique for primary brain tumor cell line establishment whether fewer enzymes or several enzymes provide high quality of primary cell lines and not affect the characteristics of parental brain tumors.

This study investigates the appropriate conditions of using enzyme cocktail for primary cell line establishment. Pilot study was performed to obtain information for setting enzyme cocktail conditions. Consequently, 4 enzyme cocktail conditions are used in this study: single enzyme (collagenase), two

enzymes (collagenase and DNase I or collagenase and hyaluronidase) and three enzymes (collagenase, DNase I and hyaluronidase) to dissociate brain tumor tissues obtained from patients. The quality of primary cell line establishment is determined in terms of efficiency of cell dissociation, clump cell size, percentage of live cells, time of cell attachment and time of cell growth to 80% confluency.

Objectives of the study

The objective of this study is to investigate the effects of different enzyme cocktail conditions on quality of primary brain tumor cell line establishment.

Methodology

Brian tumor tissues for primary cell line establishment

This study was approved by human ethics committees, Khon Kaen University (project code: HE 551016). Brain tumor tissues were derived from 9 months of age Thai girl patient with brain tumor. This patient was scheduledly admitted at Srinagarind hospital to surgically remove brain tumor during study period of this project. Brain tumor tissues removed from this patient were pathologically diagnosed as Pilomixoid astrocytomas.

Cell dissociation and clump cell size

Brain tumor tissues obtained from patient were cleaned and removed blood clots, blood vessels, gross brain tissue necrosis and other visible contaminations then washed in Hank's balance salt solution (HBSS). Tumor tissues were finely minced and incubated in 4 different enzyme cocktail conditions [1. collagenase, 2. collagenase and DNase I, 3. collagenase and hyaluronidase and 4. collagenase, DNase I and hyaluronidase] to dissociate cell into single cells. Dissociated brain tumor cells from 4 different conditions were plated in T25 flasks containing Dulbecco's Modified Eagle Medium (DMEM) with 15% Fetal Bovine Serum (FBS) then 6 different fields were captured under the light microscope to present the quality of cell dissociation. The averages of clump cell size were also investigated using image J program. Finally, established cell lines were incubated at 37ºC in 5% CO_2 and 95% humidity incubator.

Percentage of live cell

After dissociation procedure, tumor cells from 4 different conditions were stained with trypan blue to evaluate the number of live cells. Dissociated cells were counted duplicately using hemocytometer to determine the percentage of live cells.

Time of cell attachment

$5x10^4$ cells from 4 different conditions of cell dissociation were plated in five wells of 6-well plates then they were incubated at 37 ºC in 5% CO_2 and 95% humidity incubator for 5 days. To evaluate time of cell attachment, cells were detached at every 24-hour and counted the number of detached cells. Time of cell attachment was concerned when the number of cells reaches the plateau before rising again for cell proliferation.

Time of cell growth to 80% confluency

$5x10^4$cells from 4 different conditions of cell dissociation were plated in 6-well plate then they were incubated at 37ºC in 5% CO_2 and 95% humidity incubator. Cells were observed every 24-hour until they reached 80% confluency.

Results

Cell dissociation

Four different enzyme conditions as mentioned earlier were used for cell dissociation. The results present that all 4 conditions provided similar good cell dissociation with only few clump cells. All conditions efficiently disaggregated brain tumor tissues into single cells shown Figure 1.

Clump cell size

The averages of clump cell size were determined by using image J program in randomed 6 different fields of each enzyme cocktail condition. The average sizes of clump cells are 282.42 μm^2 for collagenase condition, 187.27 μm^2 for collagenase and DNase I condition, 138.10 μm^2 for collagenase and hyaluronidase condition and 158.29μm^2 for collagenase, DNase I and hyaluronidase condition (Figure 2). The more enzymes were used; the small clump cell size is obtained. However, 2-enzyme condition containing collagenase and hyaluronidase showed the lowest average size of clump cells compared to other conditions.

Percentage of live cells

The percentages of live cells from 4 different enzyme cocktail conditions are 60% for collagenase condition, 64.25% for collagenase and DNase I condition, 59.66% for collagenase and hyalronidase condition and 66.62% for collagenase, DNase I and hyaluronidase condition. The results revealed that the percentages of live cells from 4 conditions were insignificantly different (Figure 3). Several enzymes used for cell dissociation equally provided the number of dead cells similar to using fewer enzymes.

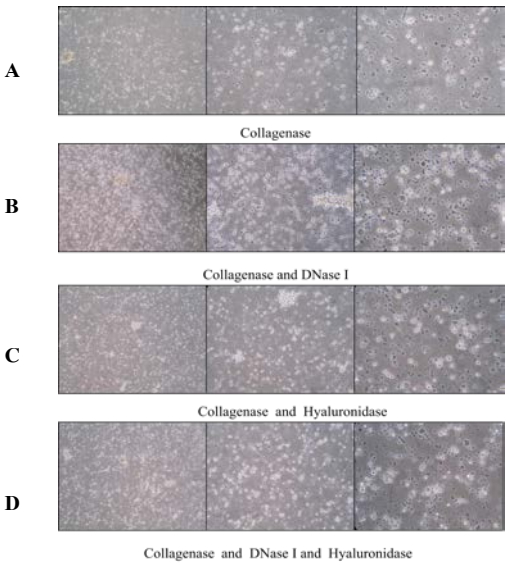

Figure 1 Cell dissociation from 4 different enzyme cocktail conditions (each panel: magnification x10, x20 and x40, respectively). All conditions properly dissociated cells into single cells with only few clump cells

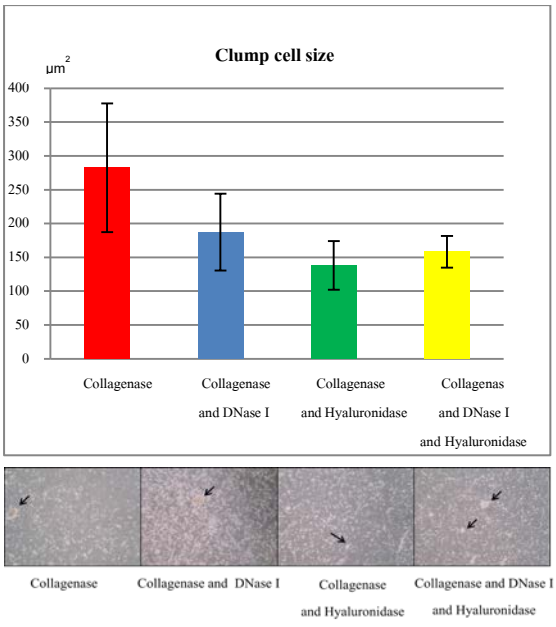

Figure 2 The average sizes of clump cells from 4 different enzyme conditions. The lower panel shows clump cells from each condition indicated by arrows

Time of cell attachment

Established cells from 4 different conditions were detached and counted every 24-hour for 5 days then numbers of cells from every 24-hour of each condition were plotted against time showed in Figure 4. The results obviously present that the condition containing 3 enzymes (collagenase, hyaluronidase and DNase I) provided the lowest number of cell attachment. Less than half of dissociated cells from 3 enzymes condition were able to attach the surface of cell culture flask whilst approximately 80% of seeded cells from the other 3 conditions using single or 2- enzyme for cell dissociation properly attached. Within 96 hours after seeding cells, seeded cells maximally attached to the surface of cell culture flasks before starting cell proliferation presented in Figure 4.

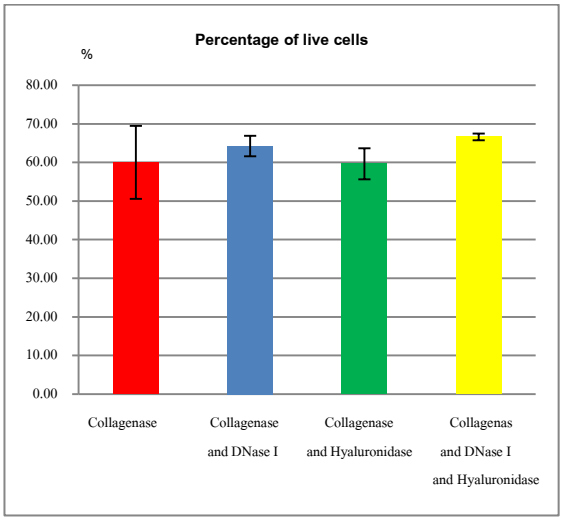

Figure 3 The percentage of live cells from 4 different enzyme cocktail conditions. All conditions showed similar percentages of live cells

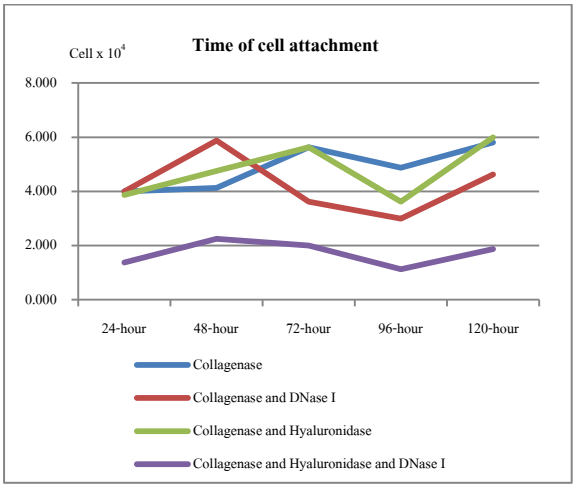

Figure 4 Time of cell attachment invested in 4 different enzyme cocktail conditions. The blue, red, green and purple line represents collagenase condition, collagenase and DNase I condition, collagenase and hyaluronidase condition and collagenase, hyaluronidase and DNase I condition, respectively

Time of cell growth to 80% confluency

Dissociated cells were plated in 6-well plate to evaluate cell growth to 80% confluency. Cells from only 2 conditions (collagenase condition and collagenase and hyaluronidase condition) were able to reach 80% confluency of cell growth (Figure 5, A and C) whereas the other 2 conditions required longer time to reach 80% confluency (Figure 5, B and D).

Statistical analysis

All continuous data from each experiment were presented with Mean±SEM and were analyzed by one-way ANOVA followed by post hoc Tukey's test, after normality assumption. Thep value less than 0.05 were considered statistically significant.

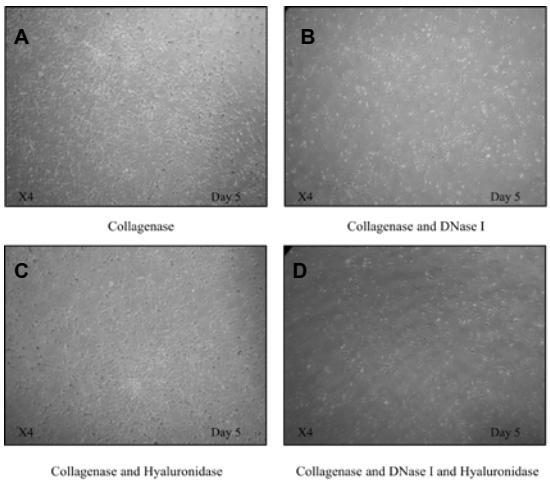

Figure 5 Time of cell growth to 80 % confluency from 4 different enzyme cocktail conditions. Cells from collagenase condition (A) and collagenase and hyaluronidase condition (C) stably grew and reached 80% confluency on 5th day. Collagenase and DNase I condition and 3-enzyme condition took longer time to reach 80% confluency

Discussion and Conclusions

Pilomyxoid astrocytomas obtained from 9-month Thai girl patient were established and investigated the quality of cell line establishment using 4 different enzyme cocktail conditions. There are 2 main techniques to dissociate cells for primary cell line establishment; mechanical dissociation (without enzyme) and enzyme dissociation. The first technique disaggregates cells into single cells using mechanical pipetting (Ali-Osman, 1996; Rutka et al., 1986; Wroblewska et al., 1975; Xu et al., 2011). Previous study using mechanical dissociation established primary cell lines from brain tumor tissues. They revealed that the success rate of primary cell line establishment is low (8.05 %) (Xu et al., 2011). Thus, the mechanical dissociation technique might be too harsh to brain tumor cells resulting in unsuccessful recovery of cells to grown in *in vitro* condition. Conversely, enzyme dissociation technique provide high successful rate (approximately 75 %) of primary cell line establishment (Ali-Osman, 1996). Each research center uses different enzyme cocktail components. Ali-Osman and colleagues successfully established primary cell lines from normal brain and malignant brain tumors using several enzyme components containing DNase type IA, neutral proteinase and collagenase (Ali-Osman, 1996). Additionally, Rutka and colleagues dissociated tumor tissues obtained from human gliosacroma using different enzyme components comprising proteinase, collagenase and DNase (Rutka et al., 1986). Both studies used different enzyme components to disaggregate tumor cells into single cells, however, both of them successfully established primary cell lines with high success rate. However, some studies used only 1 enzyme to dissociate cells for cell line establishment. Aoki, A. used pronase-P solution for cell dissociation (Aoki, 1974). The quality of cell lines established from human brain tissues is very important because these cell lines will be the representatives for further studies of human brain tumors. As mentioned earlier, different research centers used different techniques for cell dissociation. Nevertheless, there is no study, which investigates the quality of cell line based on different cell dissociation techniques. No previous study reveals whether fewer enzymes or more enzymes or no enzyme for cell dissociation provides better quality of primary cell lines. Hence, this study is interested in examining the quality of primary cell lines using different enzyme cocktail conditions.

The quality of cell lines is determined after cells are dissociated with different enzyme cocktail

conditions. This project attempted to investigate how well primary cell lines start growing in *in vitro* cell culture condition. The results of cell dissociation revealed that all 4 enzyme cocktail conditions provided similarly good cell dissociation (Figure 1). Brain tumor tissues were generally disaggregated into single cells with few clump cells (Figure 1). The clump cell sizes consistently depend on the number of enzymes. Bigger sizes of clump cells were found in fewer enzymes used whereas several enzymes used provide smaller sized of clump cells. However, the lowest averages of clump cell size were found in collagenase + hyaluronidase condition (Figure 2). This indicated that no matter what how many enzymes used to dissociate cells, only single enzyme is efficient enough to disaggregate cells into single cells for primary cell line establishment. Other aspects were also investigated to find the appropriate condition that has less harmful effects on the quality of cell lines.

The percentages of live cells were also determined to evaluate the toxic effects of enzymes on cells. Surprisingly, all 4 conditions provided similar proportion of live cells (approximately 60%) (Figure 3). Even if 3 enzymes were used to dissociate cells, the percentages of live cells were as high as using single enzyme. Thus, brain tumor cells were not physically affected by the quantity of enzymes. This might be because the concentration of each enzyme is low. Consequently, these enzymes did not harmfully destroy plasma membrane of brain tumor cells. Although, the condition containing 3 enzymes similarly provided the proportion of live cells as high as single enzyme condition, the other investigations showed the different results of cell growth quality among these 4 enzyme cocktail conditions.

The results of time for cell attachment presented that 3 enzymes cocktail condition obviously affected the attachment ability of brain tumor cells after seeding in cell culture flasks. Less than half of original seeding cell number was able to attach the cell culture surface (Figure 4). On the other hand, other conditions could maximally attach to the surface within 96 hours after seeding. The attachment of seeding cells is very important because cell lines are cultured in media containing fetal bovine serum (FBS), which enhances cells to grow as monolayers. Therefore, plating cells must attach to the culture surface before starting proliferation. This indicated that even though all enzyme cocktail conditions did not affect the physical structures of brain tumor cells determined by the percentages of live cells, several enzymes used for cell dissociation apparently disturbed the internal factors of cell growth capability resulting in less cell number of attachment. Additionally, the results of 80% confluency of cell growth showed that only these 2 conditions (collagenase and collagenase + hyaluronidase conditions) were able to grow and reach 80% confluency within 5 days after cell seeding (Figure 5) whilst collagenase + DNase I and collagenase + DNase I + hyaluronidase conditions required longer period of time. These results consistently confirmed that 3 enzymes condition were too harsh to brain tumor cells, consequently, they affected the ability of cell growth and cell proliferation of brain tumor cells.

Each enzyme used for cell dissociation provides different specific effects on cells. Collagenase cuts the peptide bonds in native, triple-helical collagen by hydrolyzing native collagen (Williams et al., 1995). DNase I is an endoneuclease, which has ability of lysing cells to reduce the viscosity

resulting in releasing DNA from damaged cells during cell dissociation process. Additionally, DNase I can also digest double-stranded DNA and high concentration of DNase I can also digest single-stranded DNA (Weir, 1993). Hyaluronidase is a polysaccharidase, which can cleavage endo-N-acetylhexosaminic bonds between 2-acetoamido-2-deoxy-beta-D-glucose and D-glucuronate. Hyaluronidase is often used in combination with a crude protease such as collagenase for dissociation of connective tissues. All enzymes have the effects on degradation of the extracellular matrix and disintegration of cell membranes. These capabilities of enzymes might lead to cellular damages when several enzymes are simultaneously used in cell dissociation for primary cell line establishment. Since DNase I is able to digest both double- and single-stranded DNA, damaged DNA might occur and can lead to genetic aberrations resulting in fatality. This might explain that both collagenase + DNase I and collagenase + DNase + hyaluronidase conditions could not properly proliferate and reach 80% confluency within 5 days.

This study revealed that fewer or more enzymes used for cell dissociation similarly provided good cell disaggregation with few clump cells. All conditions also provided similar proportion of live cells after cell dissociation. These indicated that several enzymes used for cell dissociation did not physically affect cell structures compared to single enzyme used. The physical quality of cells was indifferent among those 4 conditions; however, internal quality of cells in terms of cell growth and cell proliferation was obviously affected by using several enzymes for cell dissociation. Therefore, this study suggests that single enzyme used for cell dissociation in primary cell line establishment might be efficiently enough to establish cell lines from brain tumor tissues. It is able to preserve quality of the parental cells in growing and proliferating in *in vitro* culture condition and also provide a more economic solution resulting from using fewer enzymes.

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