Proceedings of the 2nd Biotechnology Colloquium

14th-15th April 2009

Published by:
Department of Molecular Biology
Faculty of Resource Science and Technology

ISBN 978-983-9257-99-1

TABLE OF CONTENTS

Preface

The event of the 2nd Biotechnology Colloquium and production of an associated proceeding represent another success (2 years in a row) of the Department of Molecular Biology in research and publication endeavors. More importantly, this culture of scientific conference among postgraduate students has truly become an annual 'ritual'. Most importantly, the contributions in the proceeding will be a stepping stone (or starting point) for many postgraduate students to realize publications in refereed journals. Researchers and students at the Department of Molecular Biology have indeed carved a lasting research ideology where intellectualism embodies a culture of regular scientific discourse via seminars and publications.

The event of the 2nd Biotechnology Colloquium and production of an associated proceeding represent another success (2 years in a row) of the Department of Molecular Biology in research and publication endeavors. More importantly, this culture of scientific conference among postgraduate students has truly become an annual 'ritual'. Most importantly, the contributions in the proceeding will be a stepping stone (or starting point) for many postgraduate students to realize publications in refereed journals. Researchers and students at the Department of Molecular Biology have indeed carved a lasting research ideology where intellectualism embodies a culture of regular scientific discourse via seminars and publications.

This year's colloquium highlighted the application of Biotechnology in biofuel science and technology. A keynote address on this was presented by Prof. Dr. Kopli Bujang, a foremost authority and expert in fermentation and bioprocess technology in this region. An article by Prof. Kopli on biofuel in this proceeding explains the current trend and knowledge on this subject and is information worth reading. Comprehensively, contributions in this year's proceeding are evidence of active research in the four main niche areas of the department, namely Sago Biotechnology, Environmental Microbiology, Cancer Genetics and Bioassay, and Forest Genomics. Of emerging prominence are the Zoonotic Disease (Molecular Division) Group whose contributions represent new inclusion to the current repertoire of research activities at the department. It is my hope that new groups such as this and the Transgenic Animal team will be recognized formally as niche areas in the department. Their contributions in next year's colloquium will be among the important deciding factors.

My sincere compliment and gratitude to Dr. Ho Wei Seng and Ms. Hashimatul Fatma Hashim for coordinating the 2nd Biotechnology Colloquium and the proceeding thereof. Many thanks to the team of postgraduate students who have assisted in this event, and to all academic staff and students at the department who have contributed to the success of the colloquium and compilation of another informative proceeding. Together, let us make the Department of Molecular Biology a beacon of research culture in UNIMAS.

Dr. Edmund Sim Ui Hang
Associate Professor and
Head of Molecular Biology Department

Plenary

ARE WE BIOFUELED FOR THE FUTURE?

The abundance of our agricultural wastes which require only simple processing or the appropriate cultivating methodology of microalgae from our local environment can provide huge opportunities to minimize future dependence on the depleting fossil fuel

Prof. Dr. Kopli Bujang

Department of Molecular Biology, Faculty of Resource Science & Technology,
Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak

Introduction

Rapid depletion from extensive use of the natural energy resources such as petroleum and coal enhance the use of alternative energy sources as in synthetic fuels and biofuels. Synthetic fuels or synfuel are derived from catalytic processes such as Fischer-Tropsch and Mobil Process. The former was developed in Germany and has been applied on fossil feedstock including natural gas and coal. Synfuel from biomass or BtL has the potential to be applied on almost any types of biomass such as tree barks, grasses, energy crops, waste paper or even reclaimed wood. A National Non-Food Crop Centre (NNFCC) study in the UK estimated that a total of 1.3 billion tones of biomass are needed to generate 66 tones of biofuel, which may make the process more expensive than starch-based biofuels.

The word Biofuel normally connotes two types of biologically produced fuel, bioethanol and biodiesel. Bioethanol is commonly produced from corn, sugarcane and wheat, and the feedstock for biodiesel includes palm oil and its derivatives, alga, soybean, jatropha and rapeseed oil (McIvor and Evans, 2007). Production of bioethanol involves the use of microbe and hence is a fully microbiological process through fermentation. Biodiesel, on the other hand, although started as a biological process ends with a chemical synthesis through etherification to convert the plant lipid to biodiesel.

Depletion of fossil fuel may positively enhance future development of biofuels

In 2006, the combined industry of biodiesel and bioethanol was valued at US$20.5 billion globally, with the production of biofuel for 2005 at about 20 million tones (McIvor, 2007). The mainstream of the biofuel industries is ethanol at 17 million tones produced by Brazil (8.2 million tones) and the US (7.2 million tones). Within the same period, production of biodiesel was about 2.9 million tones, of which 2.5 million tones was produced by the European countries. The prominent division between the producers of biofuel as in Europe which is more towards biodiesel and the US and Brazil towards bioethanol is due to the demand by the automobile industries in these continents.

FEEDSTOCK

Sugarcane efficiently bridged the need for food and energy from biomass

Other than the use of sugarcane in Brazil which has received world recognition for production of bioethanol from sugarcane, starch can be a potential substrate once its usage can be carved away from the food chain. Among the starch plants of the world, sago starch productivity is the highest at 4 times greater than rice, 5 times than corn and wheat and almost 17 times of tapioca (Ishizaki, 1997).

Technically, any plant materials can be harnessed to produce biofuel as long as the starch and cellulose is available and convertible to fermentable sugars. Plant biomass will have to undergo pre-treatments to release cellulose from the tough ligno-cellulosic structure.

The main feedstock for bioethanol is sugar. More than 50% of the global ethanol production comes from sugarcane which can be used directly for fermentation, followed by corn (35%), and the rest is from other sources such as wheat and cassava. However, continuous pressure from the public will certainly shift the focus to cellulosic ethanol in the future. Cellulosic ethanol can be fueled by plant biomass and hence will not interfere with the food chain.

In most cases, the types of feedstock for biodiesel production depend on their local availability to minimize transportation charges, and in most cases these are plants normally grown in the tropical regions. As such, the extensive use of local plants for biofuel will not change drastically the normal flora of the immediate surrounding environment – a major plus point if future increases in fossil fuel price influence the decision of governments to boost production of biofuel. However, the main implication is that, tropical regions of South East Asia and a huge part of Africa will be developed as the primary producers of such plants in order to meet the demand as feeder for biofuel industries of temperate or colder regions of the world.

Although each sago log generates between 15-20kg of starch, the soft fibre is being tuned as the main substrate for production of bioethanol from biomass in Sarawak.

Hydrolysis of sago fibres can generate between 4-5 tons of fermentable sugars per day

UTILISATION OF SAGO BIOMASS FOR BIOETHANOL

The fermentation works for the pilot-plant bioethanol project in UNIMAS is based on the Ishizaki Process, a high speed process that is proven to generate 40ml/Lhr of ethanol from each kg of starch (Bujang *et al*, 2008).

The process utilizes high cell density fermentation with on-demand substrate feeding and coupled with cell recycling to maintain maximum biomass in the system. The pre-commercialisation project was fully funded by a Techno-Fund grant from the Ministry of Science, Technology and Innovation (MOSTI).

Large-scale fermentation is always plagued by the high cost of nutrients and media. The use of mieki (a liquid by-product from a local food additive industry) was studied as an alternative to yeast extract. Mieki, a soybean meal acid hydrolysate is a mixture of amino acids containing 24g N/liter (Nomura *et al*, 1998). With hydrolyzed sago starch (HSS) as the substrate for bio-ethanol production, mieki was able to generate comparable amount of ethanol (61.2 g/L) to yeast extract (65.9g/L), at the same nitrogen concentration of 5g/L within the same period and similar fermentation parameters (Niyong and Bujang, 2008).

As in all processes for production of biofuel from a food source, the cost of the raw material or use of the food source itself as the raw material will inadvertently draw criticisms from various sides. Although the use of sago starch can be seen as a method of increasing the income of the sago farmers, we have shifted our focus to sago waste solids for sustainability of our biofuel process.

Cultivation of micro-algae with high lipid concentration is the best prospect for the future of biodiesel

UTILISATION OF SAGO EFFLUENT FOR BIODIESEL

At the Faculty of Resource Science and Technology, we are improving parameters for efficient filtration of sago effluent and harvest of sago fibres for production of fermentable sugars. Previous results showed that about 20-25% of fermentable sugars can be extracted using our modified methods in enzymatic pre-treatment of sago fibres.

Per acre, production of biodiesel from alga is about 14,000 gallons, compared to palm (579 gal) and jatropha (300 gal)

A typical sago mill consumes about 1,000 logs per day, producing about 20 tons of starch. Previous research revealed that a minimum of 400 tons of wastewater, containing 5% solids (20 tons) is generated everyday from this mill (Bujang et al, 1996). At a conservative conversion of 20% for enzymatic hydrolysis of cellulose, it is possible to produce a minimum of 4 tons/day of fermentable sugars from these fibres alone (Janggu and Bujang, 2008). This indeed is a considerable feat since adopting this method will definitely reduce the reliance on the supply and cost of sago starch, while concomitantly minimizing the effects of environmental pollution from the sago factories.

The current interest in the utilization of alga as an efficient producer of lipid for biodiesel is the best choice since it does not interfere with food supply. Yields of oil-crops per acre are also low, leading to massive clearance of agricultural and marginal land for fuel. Microalgae are normally selected due to their extremely efficient biomass producing ability and also the energy-rich storage lipid which is a useful product for conversion to biofuel (Hall and House, 1995). Most plants require large area of fertile farm lands and need time to mature, about 10-12 yrs in the case of sago palm. An algal cultivating facility can be constructed within weeks so the return obtained will be much faster. Although an algal farm requires substantial initial capital, but on a per ton of fuel produced basis, this is lower compared to both palm and jatropha. Production of biodiesel from an algal farm is about 14,000 gal/acre, compared to palm at 579 gal/acre, and jatropha at 300 gal/acre (Corden, 2006).

Current research in our Biochemistry Lab at FRST has established standard parameters for efficient culture of the alga Spirulina on modified sago effluent (MSE) which can be harvested after 20 days. Spirulina biomass has a very high protein content with a well-balanced amino acid composition, rich mineral content (Fe, Se, Zn, Ca, Mg, etc), vitamins (especially B complex and B12), antioxidants (carotenoids), phyco-biliproteins, essential fatty acids (γ-linolenic) and polysaccharides. The use of Spirulina cultivated in effluent is compelling because they yield an algal biomass that is easy to harvest at appropriate scales and not environmentally dangerous because it rests on the principle of natural ecosystems (Converti et al, 2006).

The cultivation of Spirulina in MSE was able to yield higher biomass when supplemented with nutrient sources like NaHCO3 (Manggi and Bujang, 2008). The final objective will be marketing of this product as a halal source of protein and organic health supplements. The use of sago effluents not only will reduce the price of substrate inputs, thus making the process more economical on a large-scale, but will concomitantly minimise the detrimental impact from the sago industries to the environment and thus adding commercial value to a potential pollutant.

REFERENCES

Bujang, K.B.; Apun, K. and Salleh, M.A. (1996). A study in the Production and Bioconversion of Sago Waste. Proc. 6th International Sago Symposium. Sago: The Future Source of Food and Feed. Universitas Riau, Pekanbaru, Sumatra. Rep. of Indonesia. 9-12th December, 1996. pp: 193-200.

Bujang, K.B.; C. Nolasco-Hipolito, L. Niyong and A. Ishizaki (2008). Maximizing Pilot-Scale Production of Bioethanol for Fuel from Sago Starch (in press).

Converti, A., Scapazzoni, S., Lodi, A., Carvalho, J.C.M. (2006). Ammonium and urea removal by Spirulina platensis. J. Ind. Microbiol. Biotechnol. 33: 8-16.

Corden, R. (2006). Biodiesel Feedstock and PetroAlgae. Cleantech. ISSN 1756-2244. Issue 11, (1): 43.

Hall, D.O. and House, J.J. (1995). Biomass: A Modern and Environmentally Acceptable Fuel. Solar Energy Materials and Solar Cells. (38):521-542

Ishizaki, A. (1997). Concluding Remarks for the 6th International Sago Symposium. Riau. Indonesia. Sago Comm. July, 8(2): 22-24.

Janggu, U. and Bujang, K.B. (2008). Development of Cellulose Treatments Procedures for Production of Fermentable Sugars from Sago Hampas for Ethanol Fermentation. 10th Symposium of the Malaysian Society of Applied Biology. 6-8th November 2008. Kuching, Sarawak, Malaysia.

Manggi, M. and Bujang, K.B. (2008). Maximising Growth of Spirulina platensis as Organic Health Supplement in Modified Sago Effluent. 10th Symposium of the Malaysian Society of Applied Biology. 6-8th November 2008. Kuching, Sarawak, Malaysia.

Niyong, L. and Bujang, K.B. (2008). Use of Mieki in Large-scale Production of Ethanol for Fuel from Sago Starch. 10th Symposium of the Malaysian Society of Applied Biology. 6-8th November 2008. Kuching, Sarawak, Malaysia.

McIvor, A. (2007). From Field to Wheel. Cleantech. ISSN 1756-2244. Issue 11, (1): 22-26.

McIvor, A. and Evans, L. (2007). A Sustainable Biofuelled Future? Cleantech. ISSN 1756-2244. Issue 11, (1): 5-10.

Nomura, Y., Hallsworth, J.E., Iwahara, M., Tanaka, K., and Ishizaki, A. (1998). Rapid and Efficient production of L-lactate from Xylose Using Electrodialysis Culture-associated Product Separation. World J. Microbiol. Biotechnol. 14:911-916.

Oral Presentation

DOWNREGULATION OF *RPS26* IN NASOPHARYNGEAL CARCINOMA CASES

Ma Xiang Ru[1], Edmund Ui Hang Sim[1], Tiong Thung Sing[2] and Alan Khoo[3]

[1]*Department of Molecular Biology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, 94300 Kota Samarahan, Sarawak, Malaysia.*
[2]*Faculty of Medicine and Health Science, Universiti Malaysia Sarawak, Lot 77, Jalan Tun Ahmad Zaidi Adruce, 93150 Kuching, Sarawak, Malaysia.*
[3]*Molecular Pathology Unit, Cancer Research Centre, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia.*

Abstract

There is growing evidence that ribosomal proteins may have extraribosomal functions despite their conventional role in protein biosynthesis. This group of genes has also been associated with various human disorders and malignancies. In this study, we examined the expression of ribosomal protein S26 (*RPS26*) in local nasopharyngeal carcinoma samples via reverse transcription-PCR approach. We revealed significant downregulation of *RPS26* in four nasopharyngeal carcinoma samples relative to their normal controls at $p<0.05$. Our data confirmed our previous findings with an increased sample size.

Introduction

Nasopharyngeal carcinoma (NPC) is a distinct type of head and neck cancer. It refers to the malignancy of the nasopharynx tissue, and about 80% of these tumours arise from the squamous epithelial cells (Carlos *et al*., 1990). Based on the degree of cell differentiation, the World Health Organization (WHO) classification distinguishes 3 histopathological types of NPC, namely Type I, II and III. NPC has its highest incidence in South East Asia, and is more prevalent in population of Chinese heritage. According to Malaysian National Cancer Registry Year 2003 Report, NPC ranked second and twelfth in males and females respectively, and Chinese was identified as the highest risk group (Lim & Halimah, 2004). In the East Malaysian state of Sarawak, by comparing population-based registry, the native Bidayuh population was found to exhibit highest age-standardized rates of NPC occurrence in the world (Devi *et al*., 2004).

Traditionally, ribosomal proteins (RP) are thought to play important role mainly in catalysing protein translation. However, recent progress provides growing evidence that RPs could have other extraribosomal functions such as replication, transcription, RNA processing and DNA repair that are independent of their own involvement in the protein biosynthesis (Wool, 1996). Ribosomal proteins have also been implicated in human diseases and disorders. Gazda *et al*. (2006) reported association of *RPS19* with Diamond-Blackfan Anemia, in which mutations of *RPS19*, together with downregulation of other RP genes, alter transcription, translation, apoptosis and oncogenic pathways in the disease. In colorectal carcinoma, the overexpression of *S3, S6, S8, S12, L5* has been identified by Pogue-Geile *et al*. (1991) whereas Kasai *et al*. (2003) reported differential expression of RP genes in normal and neoplastic colorectum. Study by Amesterdam's group (2004) using Zebrafish as model suggested RP genes to be

candidate cancer causing genes. In 2006, Uechi *et al*. reported developmental defects in RP knockdown Zebrafish. A recent study by Maclnnes *et al*. (2008) reported loss of p53 synthesis in Zebrafish carrying heterozygous mutations for 17 different RP genes hence possibly predisposing Zebrafish to malignant peripheral nerve sheath tumours.

In a recent study by our group, 2 RP genes were identified to be downregulated in nasopharyngeal carcinoma (Sim *et al*., 2008). Our previous study has also reported consistent differential expression of 33 RP genes on Malaysian colorectal carcinoma cases (Sim *et al*., 2006). With this evidence, we postulated that RPs are strongly associated with cancer progression and a more detailed investigation is warranted. This study aimed to verify the data from our previous preliminary findings (Sim *et al*., 2008) with increased sample size, as well as to identify any nucleotide aberrancy that might alter the expression of *RPS26*.

Materials and Methods

Biopsies of nasopharyngeal (NP) and nasopharyngeal carcinoma (NPC) tissues were obtained from patients admitted to The Sarawak General Hospital (SGH) and Serian Hospital via the Forcep-Biopsy method. Half of each biopsy was given to SGH pathology unit, while the other half was kept in cryovials containing RNAlater™ and brought to the research laboratory (UNIMAS). A number was assigned to each specimen, which corresponds to details of the patient concerned. Consent forms (in 3 languages) were given to each patient, explaining that biopsies can be subjected to research analysis. Total RNA was extracted from biopsy specimens using the Trizol method (Invitrogen). Assessment of RNA quality and quantity was performed via spectrophotometric analysis and agarose gels.

Reverse transcription polymerase chain reaction (RT-PCR) was then carried out. First strand cDNA was synthesized from 2 µg of total RNA using oligo-dT primers, catalysed by MML-V reverse transcriptase in a reaction volume of 25 µl. Then, 0.5 µl of the first strand cDNA was used as template for subsequent PCR amplification. PCR was carried out in a mixture volume of 25 µl with final reaction concentrations of 1X GoTaq™ Reaction Buffer, 0.2 mM dNTPs, 1 mM $MgCl_2$, 1 pmole/µl of each *RPS26* specific primers (Malygin *et al.,* 2003) or 0.4 pmole/µl of each *β-actin* specific primers (forward: 5'-GGACTTCGAGCAAGAGATGG- 3'; reverse: 5'-AGCACTGTGTTGGCGTACAG- 3') and 1.25 U GoTaq® Flexi DNA Polymerase. 0.6 pmole/µl PCR amplifications were performed using PTC-2000 Peltier Thermal Cycler (MJ Research). The mixture was first incubated at 96°C for 1 min, followed by denaturation at 94°C for 40 s; annealing at 54°C for 40 s; and extension at 72°C for 40 s. The complete amplification procedure was carried out for 25 cycles followed by further incubation at 72°C for 5 mins. Housekeeping gene *β-actin* served as an internal control. Aliquots of PCR products were later size-fractionated by agarose gel electrophoresis.

Images of the RT-PCR ethidium bromide-incorporated agarose gels were acquired with a gel documentation system (Alpha Digi Doc system). The gel images were then exported as TIFF images. The expression of transcripts was determined quantitatively using AlphaEaseFC™ Software version 4.0.0 (Alpha Innotech Corporation, USA). Band intensity was expressed as relative concentration units by comparing the density of desired PCR product bands to density of reference bands (with known

concentrations). The ratio of *RPS26/ β-actin* expression in each reaction was calculated and subjected to statistical analysis using SPSS® software version 15.0 (SPSS Inc., USA). For sequence analysis, the PCR product was purified using Gel Purification Kit (VIOGENE) prior to sequencing service (First Base Sdn Bhd, Malaysia). The sequences obtained were later compared with the sequences in the NCBI database using the BLAST tool.

Results

Two primers sets were used in the amplification of *RPS26*. The first primer set, RPS26F and RPS26R, was designed using Primer3 software. However, these primers could not produce consistent product band although many PCR tests were carried out under various parameters. Amplification using established primers (Malygin *et al,* 2003), RS26F and RS26R, resulted in a 373 bp amplicon (**Figure 1**).

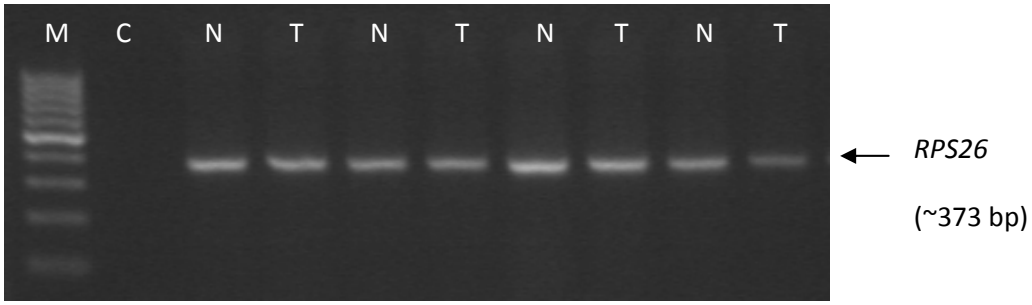


Figure 1: Representative gel image of *RPS26*. M: 100bp marker; C: negative control; N: normal sample; T: tumour

Expression study of *RPS26* revealed expression pattern as observed in **Figure 2**. The expression level of *RPS26* was calculated as the ratio of *RPS26/ β- actin*, in which expression of *RPS26* was normalised against *β- actin* expression. Out of 4 cases studied, *RPS26* was found to be under-expressed in all NPC samples relative to their normal controls with a mean difference of 0.04. Statistical analysis using Student's Paired T-test revealed that the difference is significant ($p<0.05$, $n=4$) with a t-value of 3.301.

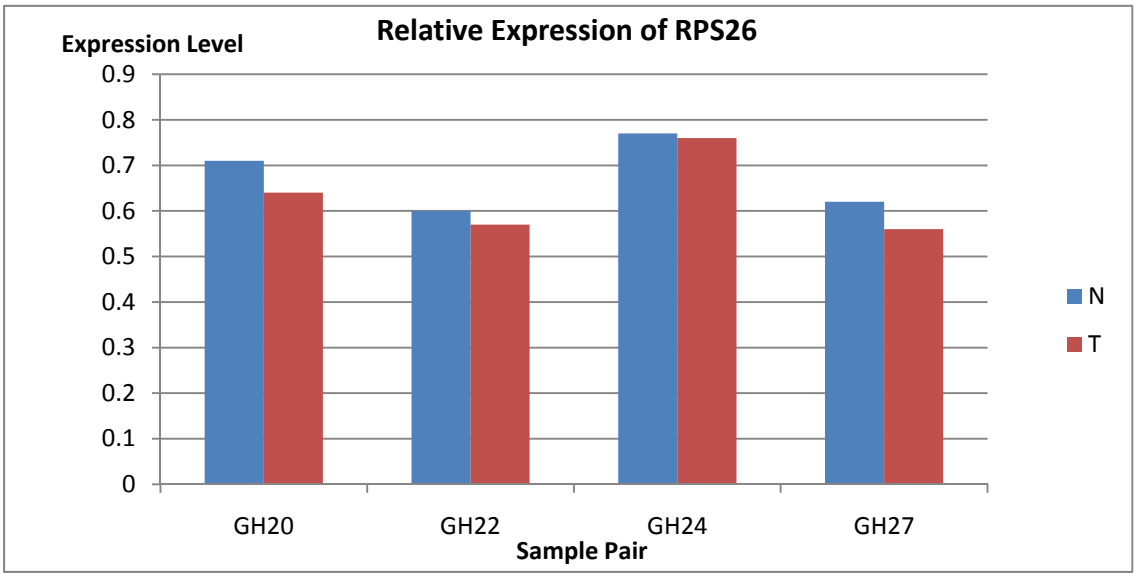


Figure 2: Relative expression of *RPS26* in NPC samples relative to their normal controls on two replicates. N: normal; T: tumour; GH20, GH22, GH24 and GH27: sample name.

Discussion

Our result obtained via reverse transcription polymerase chain reaction approach confirmed previous findings by our group (Sim *et al*., 2008) that *RPS26* was under-expressed in NPC cases. Furthermore, the study was performed on paired samples (normal-tumour) and with increased sample size (n=4) and was proven to be significant using Paired T-test. As far as we are concerned, there is no published report on *RPS26* association with NPC prior to our group. Ivanov *et al*. (2004, 2005) reported that rpS26 protein might suppress the splicing of its pre-mRNA and demonstrated the ability of rpS26 to interact with its pre-mRNA intron I and mRNA fragment using HeLa cells. They also suggested that the mechanism regulating the splicing of the rpS26 pre-mRNA could be a feedback mechanism in which excessive free *RPS26* proteins bind to the first intron of its own pre-mRNA and thereby suppress its splicing. This could probably result in lower copy number of mRNA transcripts of this gene. Our result which revealed under-expression of *RPS26* at transcript level correlates their inferences. However, little is known as to why in a diseased state the transcript level is lower compared to normal situation and immediate next step in our study may be to assess for aberrancy in the splicing region of *RPS26* in NPC cases.

References

Amsterdam A, Sadler KC, Lai K, Farrington S, Bronson RT, Lees JA, and Hopkins N. (2004). Many ribosomal protein genes are cancer genes in zebrafish. PLOS Biology 2(5):690-698.

Carlos R, Harley JP, and Noback CR. (1990). Human Anatomy and Physiology (2nd Ed) McGraw-Hill, Inc., NY USA: 721-722.

Devi BCR, Pisani P, Tang TS, and Parkin DM. (2004). High incidence of nasopharyngeal carcinoma in native people of Sarawak, Borneo. Cancer Epidemiology, Biomarkers & Prevention 13(3):482-486.

Gazda HT, Kho AT, Sanoudou D, Zaucha JM, Kohane IS, Sieff CA, and Beggs AH. (2006). Defective ribosomal protein gene expression alters transcription, translation, apoptosis, and oncogenic pathways in Diamond-Blackfan Anemia. Stem Cells 24:2034-2044.

Ivanov AV, Malygin AA, and Karpova GG. (2004). Human ribosomal protein S26 inhibits splicing of its own pre-mRNA. Molecular Biology 38(4): 568-574.

Ivanov AV, Malygin AA, and Karpova GG. (2005). Human ribosomal protein S26 suppress the splicing of its pre-mRNA. Biochimica et Biophysica Acta 1727: 134-140.

Kasai H, Nadano D, Hidaka E, Higuchi K, Kawakubo M, Sato TK, and Nakayama J. (2003). Differential expression of ribosomal proteins in human normal and neoplastic colorectum. The Journal of Histochemistry and Cytochemistry 51(5):567-573.

Lim GCC, and Halimah Y(Eds). Second Report of the National Cancer Registry. Cancer Incidence in Malaysia 2003. National Cancer Registry. Kuala Lumpur 2004.

Maclnnes AW, Amsterdam A, Whittaker CA, Hopkins N, and Lees JA. (2008). Loss of p53 synthesis in zebrafish tumors with ribosomal protein gene mutations. Proceedings of the National Academy of Sciences of the USA 105(30): 10408-10413.

Malygin A, Baranovskaya O, Ivanov A, and Karpova G. (2003). Expression and purification of human ribosomal proteins S3, S5, S10, S19 and S26. Protein Expression & Purification 28:57-62.

Pogue-Geile K, Geiser JR, Shu M, Miller C, Wool IG, Meisler AI, and Pipas JM. (1991).Ribosomal protein genes are overexpressed in colorectal cancer: isolation of a cDNA clone encoding the human S3 ribosomal protein. Molecular and Cellular Biology 11(8):3842-3849.

Sim EUH, Bong IPN, Balraj P, Tan SK, Jamal r, Sagap I, Nadeson S, Rose IM, and Lim PKM. (2006). A preliminary study of differentially expressed genes in Malaysian colorectal carcinoma cases. Journal of Bioscience 17(1):19-37.

Sim EUH, Toh AKL, and Tiong TS. (2008). Preliminary findings of down-regulated genes in nasopharyngeal carcinoma. Asia Pacific Journal of Molecular Biology and Biotechnology 16(3):79-84.

Uechi T, Nakajima Y, Nakao A, Torihara H, Chakraborty, Inoue K, and Kenmochi N. (2006). Ribosomal protein gene knockdown causes developmental defects in zebrafish. Plos ONE 1(1):e37. doi: 10.1371/journal.pone.0000037.

MICROSATELLITE MARKERS DEVELOPMENT FOR *D. moluccana* – PRELIMINARY RESULTS

Liew Kit Siong[1], Ho Wei Seng[1] and Julaihi Abdullah[2]

[1]Department of Molecular Biology, Faculty of Resource Science & Technology
Universiti Malaysia Sarawak (UNIMAS), 94300, Kota Samarahan, Sarawak
Email: liewks46@yahoo.com / wsho@frst.unimas.my
[2]Applied Forest Science and Industry Development (AFSID), Sarawak Forestry Corporation
93250, Kuching, Sarawak

Abstract

We have isolated sixteen microsatellite markers from *D. moluccana* using dual-ISSR suppression method. Fragments flanked by two microsatellite sequences were amplified using SSR primers namely; $(GTG)_6$, $(AC)_{10}$ and $(AG)_{10}$. ISSR-PCR amplicons ranged from 500bp to 3kb were cloned. As a result, 9 recombinant plasmids DNA were isolated and sequenced. Subsequently, a primer IP1 designed from the known region flanking the SSR and, for nested PCR, another primer IP2 based on the sequence between IP1 and the SSR were prepared. These two primers were used to determine the other sequence flanking the microsatellite by a "walking method". Five of the adaptor-ligated, restricted DNA libraries were then constructed. The amplified nested-PCR product were cloned and sequenced as described above. Six of the SSR markers were identified until now. Subsequently, IP3 was designed on the newly-sequenced region flanking each of the microsatellite. Thus, IP1/IP3 or IP2/IP3 primer pair was used as the SSR marker in order to amplify microsatellite region. Finally, the characteristics of each developed microsatellite marker will be investigated.

Introduction

The discovery of simple sequence repeats (SSRs) are being increasingly utilized in selection of plus trees, quality control in seed production and measurement of genetic diversity for conservation management. This is because SSR marker has a number of advantages: 1) PCR-based marker; 2) high discriminatory power; 3) high information content arising from their multiallelic nature; 4) co-dominant marker; 5) randomly distributed throughout the genome; 6) transferability across closely related taxa (Saha *et al*., 2003; Masi *et al*., 2003; Ujino *et al*., 1998). In addition, the attractive attribute of this marker is especially in the case of species which show a low level of genetic variation, inbred populations and geographically close populations (Rakoczy-Trojanowska and Bolibo, 2004).

However, the isolation and characterization of SSR marker for forest trees is still limited until now because involves time-consuming and highly development cost (Lian *et al*., 2001). Several improved protocols have been reported e.g. enrichment technique (Brondani *et al*., 2000) and vectorette PCR strategy (Ujino *et al*., 1998). However, Lian *et al*. (2001) reported that enrichment technique is inefficient due to only a few of microsatellite markers were obtained from each restricted DNA fragments libraries. Besides, they also found that many of the microsatellite regions were not amplified by the designed SSR primer pairs.

In this study, ISSR-Suppression PCR without enrichment and screening procedures based on Lian *et al*. (2001) was used for SSR markers development. *D*. *moluccana* (Sawih) was chosen for study due to it's economically and fast growing ability. To date, baseline genetic information of *D*. *moluccana* is very limited and none of the DNA-based markers has been developed until now. Thus, the objectives of this study are 1) to establish an efficient protocol for isolating pure and high-molecular weight genomic DNA from *D*. *moluccana*; 2) to develop a set of simple sequence repeats markers specific for genotyping *D. moluccana* trees, and; 3) to determine the characteristics of each newly developed SSR marker.

Materials and Methods

The leaf sample of Sawih seeding (Figure 1a) was collected from the Seed Bank, Sarawak Forestry Corporation (SFC), Kuching. Genomic DNA of Sawih was isolated from leaves using a re-optimized CTAB-based method. Three SSR primers namely $(GTG)_6$, $(AC)_{10}$ and $(AG)_{10}$ were used to amplify the fragments flanked by two microsatellite sequences oriented in opposite direction. Re-optimization of ISSR-PCR amplification conditions were carried out in order to obtain better results. ISSR-PCR amplicons ranged from 500bp to 3kb were cloned into pGEM®-T Easy vector and then transformed into JM109 competent cells. The recombinant plasmids DNA were isolated using Wizard® *Plus* SV Minipreps DNA Purification System (Promega, USA) and sequenced. Subsequently, initiating primers (IP1 & IP2) were designed from the known region flanking the SSR sequence. The sequence of the other flanking region of each microsatellite was determined by the "walking method" (Siebert *et al*., 1995). Subsequently, IP3 was designed in order to amplify microsatellite. The characteristics of each newly developed microsatellite markers will be investigated.

Results and Discussion

DNA Isolation via a Re-optimized DNA Isolation Protocol

Total genomic DNA of Sawih was successfully isolated using a re-optimized DNA isolation protocol. As a result, a sharper and distinct DNA band was observed (**Figure 1c).**

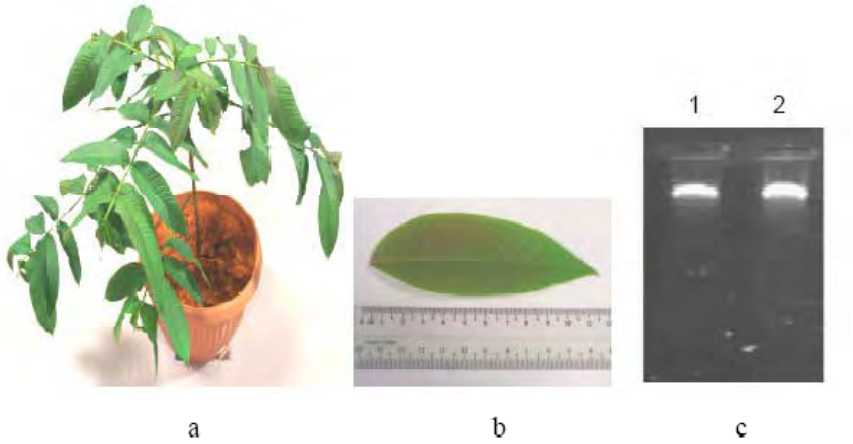


Figure 1: (a) *D*. *moluccana* seedling, and (b) Leaf and (c) Electrophoresis genomic DNA on 0.8% agarose gel. Lanes 1 and 2: Genomic DNA isolated from *D*. *moluccana* leaves.

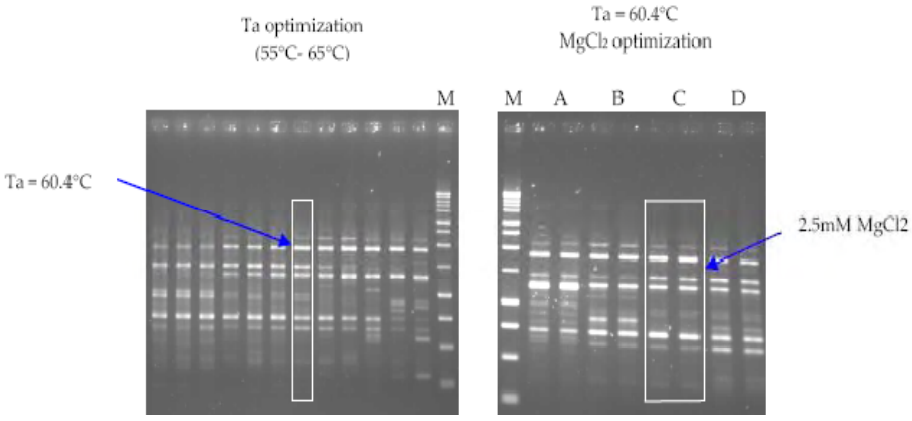
Cloning and Sequencing of Amplified Fragments of Inter-simple Sequence Repeats and Primer Design

Optimization of ISSR-PCR Conditions

In order to obtain reproducible, scorable and informative bands, PCR conditions (annealing temperatures and Magnesium concentrations) were optimized. The optimal PCR profiles for each of the ISSR primer were determined (**Table 1**). For example, the optimized PCR profiles for $(GTG)_6$ primer as shown in **Figure 2**.

Table 1 Optimum ISSR-PCR conditions for $(GTG)_6$, $(AC)_{10}$ and $(AG)_{10}$ primers

Primer	Optimal Parameters	
	Ta (°C)	$[MgCl_2]$
$(GTG)_6$	60.4	2.5mM
$(AC)_{10}$	58.8	2.5mM
$(AG)_{10}$	59.6	2.5mM



(a) (b)

Figure 2 Electrophoresis of PCR amplified ISSR products on 1.5% agarose gel using $(GTG)_6$ primer. (a) Ta optimization, (b) $MgCl_2$ optimization. A: 1.5mM $MgCl_2$, B: 2.0mM $MgCl_2$; C: 2.5mM $MgCl_2$ and D: 3.0mM $MgCl_2$. Lanes M: 1Kb DNA ladder. Lanes A-D: Genomic DNA isolated from *D. moluccana* leaves.

Cloning and Sequencing of ISSR fragments

The excised ISSR-PCR amplicons ranged from 500bp to 3kb (**Figure 3**) were cloned into pGEM®-T Easy vector and then transformed into *E. coli* JM109 (**Figure 4a**). The picked white colonies were further verified using M13 reverse and forward sequence primers (**Figure 4b**). Overall, 9 recombinant plasmids DNA were isolated and sent for sequencing (**Figure 5**). Two primers, IP1 and IP2 were designed from the

known region flanking the SSR sequence in order to determine the sequence of the other flanking region of each microsatellite by using a "walking method".

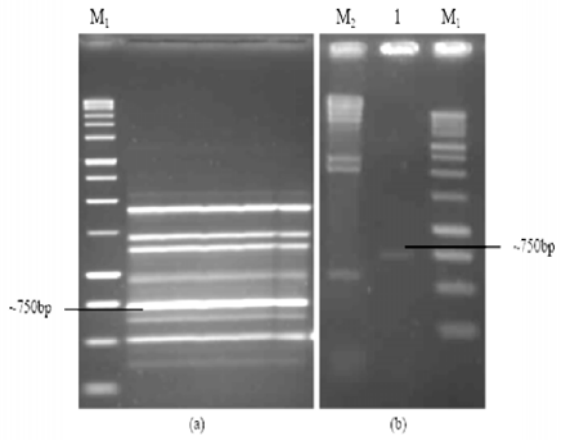
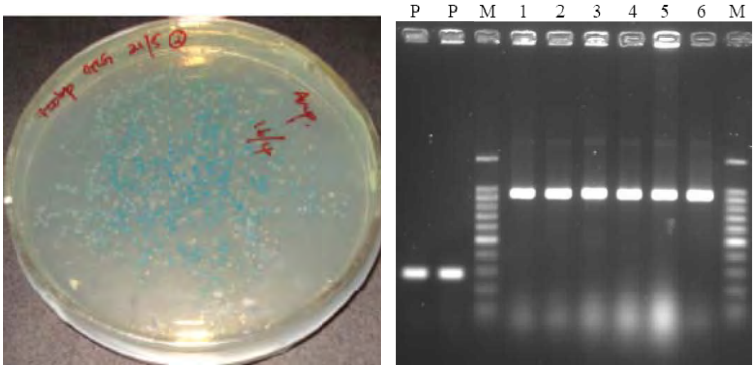


Figure 3 Gel electrophoresis of ISSR- PCR products using $(GTG)_6$ primer on 1.5% agarose gel. (a) Unpurified ISSR-PCR product. (b) Purified ISSR-PCR product. Lane 1: ~750bp. Lanes M_1: 1kb DNA ladder. Lane M_2: λ *Hind*III DNA marker.



(a) (b)

Figure 4 (a) Growth observed on a LB culture plate containing ampicillin, IPTG and X-gal. (b): Electrophoresis of PCR products on 1.5% agarose gel using M13 reverse and forward sequence primers. Lanes 1-6: White colony with insert. Lanes P: Blue colony without insert (Negative control). Lanes M: 100bp ladder marker (Promega, USA).

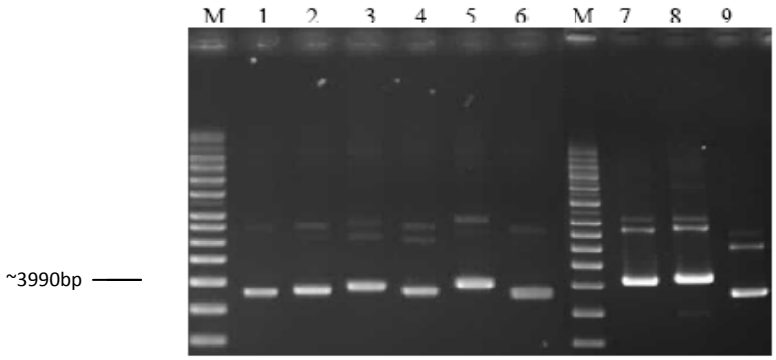


Figure 5 Gel electrophoresis of recombinant plasmids DNA on 0.8% agarose gel. Lane 1: DMGTG485. Lane 2: DMGTG602. Lane 3: DMGTG602. Lane 4: DMAC584. Lane 5: DMAC859, Lane 6: DMAG418. Lane 7: DMGTG1248. Lane 8: DMGTG1407. Lane 9: DMAG550. Lanes M: Supercoiled DNA ladder (Invitrogen, Brazil).

Construction of DNA libraries

The genomic DNA was digested separately with a restriction enzyme namely: *Alu* I, *EcoR* V, *Hae* III, *Rsa* I and *Ssp* I (**Figure 6**). The digested DNA was then ligated with 48-mer adaptor.

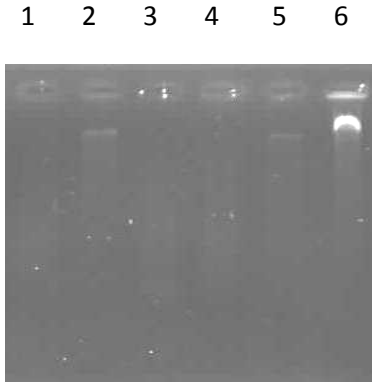


Figure 6 Rrestriction enzyme analysis of genomic DNA isolated from *D. moluccana* on 0.8% agarose gel. Lane 1: *Alu* I, Lane 2: *EcoR* V, Lane 3; *Hae* III, Lane 4 *Rsa* I and Lane 5: *Ssp* I. Lane 6: Undigested genomic DNA (Negative control).

Determination of the Sequence beyond the Determined ISSR Sequence

Walking method (Siebert *et al*., 1995) was used to determine the sequence of the other flanking region of each microsatellite. Two steps of PCR amplification from adaptor-ligated, restricted DNA libraries were performed using the primers prepared based on sequences of each ISSR fragment (IPl and IP2) and adaptor primer (APl and AP2). The primary PCR produced smeared banding patterns (**Figure 7a**), but multiple bands were obtained in the nested PCR (**Figure 7b**). In order to obtain a single major band, nested PCR conditions were re-optimized. Since PCR products yielded identical band patterns with different Ta (55°C-65°C) (**Figure 8**), this indicates that the effects of T_a on PCR products was not

significant. Serial dilution (10^{-1} to 10^{-10}) of 1st PCR products was performed (**Figure 9**). A single major band was obtained with the dilution 10^{-5} (**Figure 9, Lane 5**). A single-banded PCR product was cloned and sequenced as described above. Sixteen of the SSR regions were identified until now and IP3 was designed in order to amplify SSR region.

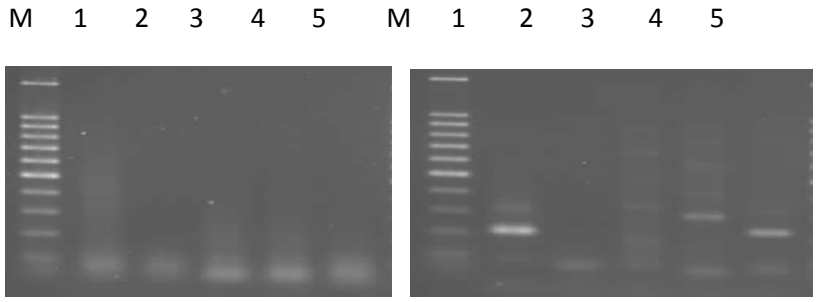


Figure 7 Example of amplification PCR products obtained using a walking method (Siebert *et al*., 1995) to determine the other flanking region of a microsatellite. (a) Primary PCR products amplified with the AP1 and DMAC584 IP1. (b) Nested PCR products amplified with the AP2 and DMAC584 IP2. Lanes 1-6: PCR products obtained from the lane 1: *Alu* I, lane 2: *Eco*RV, lane 3: *Hae*III, lane 4: *Rsa*I and, lane 5: *Ssp*I restricted-DNA libraries, respectively

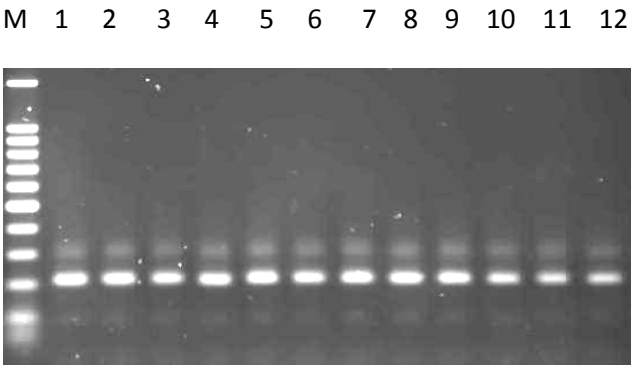


Figure 8 Nested PCR products amplified with the AP2 and DMAC584 IP2 for restricted DNA library *Alu* I. Lanes 1-12: Ta optimization (55°C-65°C). Lane M: 100bp DNA ladder (Promega, USA).

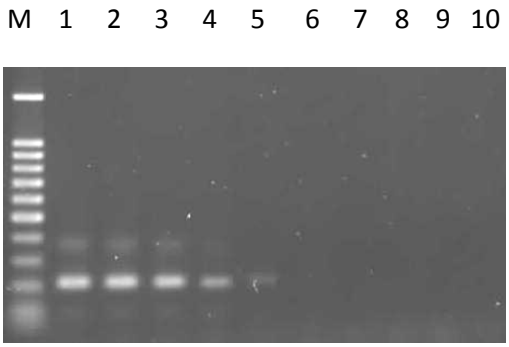


Figure 9 Nested PCR products amplified with AP2 and DMAC584 IP2 for restricted-DNA library *Alu* I. Lanes 1-10: Serial dilution (10^{-1} to 10^{-10}). Lane M: 100bp DNA ladder (Promega, USA).

Conclusions

The total genomic DNA of *D. moluccana* was successfully isolated using a re-optimized DNA isolation protocol. In addition, an optimum ISSR-PCR conditions were established for $(AG)_{10}$, $(AC)_{10}$ and $(GTG)_6$ primers. A total of 9 ISSR amplicons had been cloned and sequenced. Nine primers were synthesized and successfully used in the determination of the sequence beyond the determined ISSR sequence by a walking method. Sixteen of the SSR regions had been identified and subsequently, IP3 was designed in order to amplify each of the identified microsatellite. The characteristics of each newly developed SSR marker will be determined.

Acknowledgements

This study was supported by the Sarawak Forestry Corporation (SFC) and Universiti Malaysia Sarawak (UNIMAS) research grant.

References

Brondani, C., Brondani,. R.P.V., Garrido., L.D..R., & Ferreira, M.E. 2000. Development of microsatellite markers for the genetic analysis of Magnaporthe grisea. Genetics and Molecular Biology 23(4) 753-762.

Chakraborti, D., Sarkar,A., Gupta, S. & Das, S. 2006. Small and large genomic DNA isolation protocol for chickpea (Cicer arietinum L.), suitable for molecular marker and transgenic analyses. African Journal of Biotechnology 5(8): 585-589.

Doyle, J.J. & Doyle, J.L. 1990. Isolation of plant DNA from fresh tissue. Focus 12:13- 15.

Masi, P., Spagnoletti Zeuli, P.L. & Donini, P. 2003. Development and analysis of multiplex microsatellite markers sets in common bean (Phaseolus vulgaris L.). Molecular Breeding 11: 303-313.

Lian, C., Zhou, Z. & Hogetsu, T. 2001. A simple method for developing microsatellite markers using amplified fragments of inter-simple sequence repeat (ISSR). Journal of Plant Research 114:381-385.

Rakoczy-Trojanowska, M. & Bolibok, H. 2004. Characteristics and a comparison of three classes of microsatellite-based markers and their application in plants. Cellular and Molecular Biology Letters 9: 221-238.

Saha, S., Karaca, M., Jenkins, J.N., Zipf, A.E., Reddy, O.U.K. & Kantety, R.V. 2003. Simple sequence repeats as useful resources to study transcribed genes of cotton. Euphytica 130: 355-364.

Siebert, P. D., Chenchik, A., Kellogg, D. E., Lukyanov, K. A. & Lukyanov, S. A. 1995. An improved PCR method for walking in uncloned genomic DNA. Nucleic Acids Research 23: 1087-1088.

Ujino, T., Kawahara, T., Tsumura, Y., Nagamitsu, T., Yoshimaru, H. & Ratnam, W. 1998. Development and polymorphism of simple sequence repeat DNA markers for Shorea curtisii and other Dipterocarpaceae species. Heredity 81:422-428.

FUNGAL AMYLASE PRODUCTION UNDER SUBMERGED AND SOLID STATE FERMENTATION

Noorhidayah Mut and Awang Ahmad Sallehin Awang Husaini

Department of Molecular Biology, Faculty of Resource Science & Technology
Universiti Malaysia Sarawak (UNIMAS), 94300, Kota Samarahan, Sarawak

Introduction

Starch is among the most abundant polysaccharide on earth and a very important source of energy for most organisms. However, for starch to be transform into usable energy it needs to be hydrolyzed to its monomer, i.e. glucose and the enzymes responsible for this action are the starch-degrading enzymes (amylase). Most of the starch-degrading enzymes belong to the α-amylase family (van der Maarel et al., 2002). The α-amylase cleaves α-1,4-glycosidic linkages within the starch molecule (Gupta et al., 2003) while β-amylase cleaves α-1,4-glycosidic bonds of external glucose residue (van der Maarel et al., 2002). Industrial production of α-amylase involves the use of *Aspergillus niger* and *A. oryzae* (Nigam and Singh, 1995). Another significant class of amylase is glucoamylase, an enzyme that hydrolyzed the cleavage of α-1,4- and α-1,6-glycosidic bonds of external glucose residue (van der Maarel et al., 2002). This enzyme is produced industrially by Aspergillus, Rhizopus and Endomyces species (Nigam and Singh, 1995). Fungi can be utilized to produce amylase under submerged fermentation (SmF) or solid state fermentation (SSF). The SSF occur in the absence or near absence of free water, hence it often involve microorganisms that grows well under the low water condition (Pandey et al., 2000). The substrate for SSF provides the required nutrients and anchorage for the microbial cells, and preferably cheap and easily available (Pandey et al., 2000). The most suitable substrate is the agro-industrial residue as it satisfies those requirements. An example of such substrate is the sago hampas and rice husk.

Materials and Methods

Isolation of fungi isolates

Five isolates (Aspergillus sp. SW003, Aspergillus nomius SW004, Aspergillus sp. SW005, Ceratocyctis paradoxa OSP and Aspergillus niger PAN) were obtained from previous study. Six isolates (NSH1, NSH4, NSH5, NSH6, NSH9 and NSH11) were isolated from sago humus and three (NMYL1, NMYL2 and NMBS) were from rotten sago bark. Extracellular amylase production was confirmed by using starch agar plate method.

Screening of extracellular amylase production by fungi isolates

The fourteen isolates were grown in minimal salt medium (MSM) (% w/v: KH2PO4; 0.05, (NH4)2SO4; 0.05, MgSO4.6H2O; 0.05) amended with 1% starch and incubated at 28°C for 96h. Three types of starch were used as carbon source which were soluble starch, sago flour and raw sago starch. Crude enzyme was harvested by filtration through Advantec no. 1 filter paper and amylase activity was determined by using DNSA method (Miller, 1959).

Submerged Fermentation vs. Solid State Fermentation

Three isolates with highest amylase activity (from screening) were selected. For SSF, the isolates were grown at 28°C in 250ml Erlenmeyer flasks containing 5g of sago hampas and 1ml of mineral salts solution pH6 (% w/v: MgSO4.6H2O; 0.1, KH2PO4; 0.1, CaCl2; 0.1, FeSO4; 0.05 and (NH4)2SO4; 0.1) (Omemu et al., 2005). Distilled water was added to adjust the moisture content to 50%. For SmF, the isolates were cultured at 28°C in 250ml Erlenmeyer flasks containing 5g of sago hampas and 50ml of mineral medium pH6.18 (g/L: NaCl; 2, KH2PO4; 2.5, MgSO4.6H2O; 1, CaCO3; 5, NaNO3; 5 and (NH4)2SO4; 5) (Kekos and Macris, 1983). The flasks were incubated at 28°C for 72h and 96 h and with shaking (115rpm) for SmF flasks. The SSF flasks were harvested by adding 50ml of cold sterile distilled water, followed by shaking at 250rpm for 20 min. The homogenized medium was filtered through muslin cloth before it was centrifuged at 6000rpm for 20 min. The supernatant was then filtered through Advantec no.1 filter paper to obtain the crude enzyme. For the SmF flasks, the same method of harvesting was employed except the medium was not added with cold distilled water.

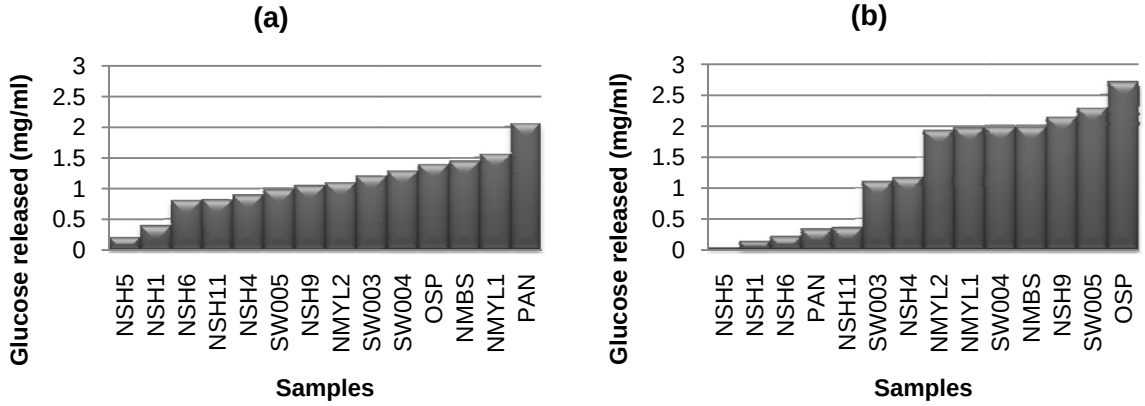
Substrates Comparison

Solid state fermentation was repeated by using the same protocol as above except the sago hampas was replaced with rice husk.

Results

Screening of extracellular amylase production by fungi isolates

Among the fourteen isolates only a few showed relatively high enzyme activity by the released of reducing sugar as glucose. By looking at glucose released from the raw sago starch culture, three isolates were chosen for the next experiments. Those isolates were OSP, PAN and NSH9.



.