CONSTRUCTION OF BINARY VECTOR WITH WOUND INDUCIBLE PROMOTER FOR *HBsAG* EXPRESSION: DEVELOPMENT OF PLANT-BASED EDIBLE HEPATITIS B VACCINE FROM INDONESIAN ISOLATE

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ABSTRACT

Hepatitis B is a serious infectious disease in the third world countries including Indonesia. Vaccination is the most effective way to prevent the spread of the disease; therefore the demand for HBV vaccine is high. In order to produce more vaccine at lower cost, transgenic plant can be chosen to express the vaccine with the above criteria. Several researches were successfully producing transgenic plants expressing HBsAg that formed virus-like particles and induced immune response in human. However, HBsAg expression in transgenic plant needs to be improved especially on gene expression control system. Here, we describe the construction of *HBsAg* structural gene under the control of wound inducible promoter, *MeEF1* promoter from *Manihot esculenta* Crantz. The *HBsAg* gene was amplified using PCR from HBV genome isolated from an Indonesian patient. The gene was subsequently fused with VSPáS signal peptide, which targeted the reticulum endoplasm of plant cell. The construct was cloned into binary expression vector for *Agrobacterium* plant transformation in near future.

Keywords: HBsAg, VSPáS signal peptide and *MeEF1* promoter

INTRODUCTION

Hepatitis B that is caused by Hepatitis B virus (HBV), is one of the world's most common and serious infectious diseases. It is estimated that more than one third of the world's population has been infected with the hepatitis B virus. About 5% of the populations are chronic carriers of HBV, and nearly

25% of all carriers develop serious liver disease such as chronic hepatitis, cirrhosis, and primary hepatocellular carcinoma. HBV infection causes more than one million deaths every year (http://www.who.int/csr/disease/hepatitis). Data from Departemen Kesehatan Republik Indonesia (Ministry of Health the Republic of Indonesia) demonstrated that 14-16 million of Indonesian people were infected and 200.000 die each year (Achmadi, 2002).

Vaccination is the best way to prevent HBV transmission. Hepatitis B vaccine has an outstanding record of safety and effectiveness. For example, intramuscular vaccination has shown 95% effective in preventing children and adults from developing chronic infection if they have not yet been infected. In many countries where 8% to 15% of children used to chronically infected, the rate of infection has been reduced to less than 1%. (http://www.who.int/mediacentre).

So far, effective immunization and protection from HBV infection have been achieved by intramuscular vaccination of yeast-derived recombinant hepatitis B surface antigen (HBsAg) (Sojikul *et al.*, 2003). Yeast-derived HBsAg can produce virus-like particles (VLP) that assemble highly immunogenic virion-like structures devoid of viral genetic material (Huang & Mason, 2004). Chimeric HBsAg-S proteins carrying foreign epitopes allow particle formation and have the ability to induce anti-foreign humoral

and cellular immune responses (Vietheer *et al.* 2007)

However, despite of yeast-derived HBsAg vaccine's effectiveness, administration of this vaccine causes pain, which is uncomfortable, especially for children. Furthermore, less expensive vaccine is still a need, especially in most developing countries (Thanavala *et al.*, 2006). In Indonesia, injection of yeast derived-HBsAg vaccination will cost approximately IDR 300.000,00, which values almost half of minimum payment for a full time job. To circumvent these problems, plant-derived oral vaccines was developed as a prospective alternative. The recombinant antigen is expressed in transgenic plants. As in yeast, plant-derived HBsAg antigen is also assembled into virus-like particles (VLPs) and was proven to be orally immunogenic in mice and human (Kong *et al.*, 2001; Richter *et al.*, 2000; Thanavala, 2005). In addition, the compact and highly ordered structures of VLP very likely provide resistance to digestive enzymes in the gut (Huang *et al.*, 2004). The cost of vaccine production using this technology will be reduced to more than ten times of the cost of the vaccine production using microbes or yeast (Mason *et al.*, 1992). The cost of delivery and storage can also be reduced since plant based vaccine does not need to be stored and transported under cold condition (Sala *et al.*, 2002; Thanavala *et al.*, 2006).

So far, the HBsAg gene has been successfully transformed into tobacco and potato (Mason *et al.*, 1992; Kong *et al.*, 2001). Unfortunately, the HBsAg expression level in these plants is still low. Attempts to increase the HBsAg expression level in the plant system have been made by using various regulatory elements and subcellular targeting signals (Richter *et al.*, 2000; Sojikul *et al.*, 2003). They found that the new system gave expression of 20-100 times higher than previous construct. However, the promoter they used is a constitutive promoter which drives expression at anytime and on every single tissue in the plant.

The aim of our research was to develop a construct which uses strong and wound-inducible promoter derived from cassava elongation factor 1 alpha gene (*MeEF1*) to drive HBsAg gene. *HBsAg* gene is constructed in plant expression vector pCAMBIA1390 with VSPαS signal peptide under the control of *MeEF1* promoter. The promoter is more efficient than CaMV35S promoter and will be active as soon as the tissue wounded or broken mechanically (Suhandono *et al.*, 2001). Using this promoter, antigen production can be controlled whenever and wherever we need the most. Since the recombinant HBsAg will be expressed the most in wounded tissue, the HBsAg protein will not be produced in normal tissue (Suhandono *et al.*, 2001). This would exclude the possibility that the protein may interfere with the physiological process of the plant. This report will only include the construction of the gene into the expression vectors.

Materials and Methods

Isolation and cloning of HBsAg

HBV genome was isolated from 200 µL patient blood serum using *High Pure Viral Nucleic Acid Kit* (ROCHE™). The S gene of HBsAg was amplified using Taq Polymerase (Fermentas) and cloned into pGEM-T® easy (PROMEGA). Primer pair of HBsAg (HBsF: 5'-GGATCCATGGAGAACATCGCATCAG-'3 and HBsR: 5'-GAATTCGGTAACCATGAAGT TGAGAGGGAGTAG-3') were used to amplify a full length of S gene. The result was sequenced at Macrogen, Korea and Agency for the Assessment and Application Technology (BPPT), Indonesia.

Determination of HBV genotype

Determination of HBV genotype was based on the sequencing data analysis using viral genotyping tool from NCBI. HBV subtype was determined using amino acid analysis, which was made based on Purdy method (2007).

Construction of HBsAg- VSPαS signal peptide gene fussion.

PCR amplification of the S gene orf (open reading frame) from pGHB using primers contained VspαS sequence (*vegetative storage protein* from soybean, targeted to reticulum endoplasma) (Sojikul, 2003) and end-on *Nco*I and *Bst*EII restriction sites (Forward:5'-CCATGGATG AAAAT GAAGGTCCTTG TTTTCTTCGTTGCTA CAATTTTGG TAGC AT GGCA ATGCCATGCGATGGAGAACAT CGCATCAGGACTCC-'3; Reverse: GGT AACCTTAAATGT ATACC CAAA GACA

AAAGAAAA TTGGTA ACA GCGGCA TAAAGGGACT CAAGATG) and subsequent cloning into pGEM-T easy vector would result in pGHBS. Subsequently, digestion of pGHBS with *Nco*I and *Bst*EII endonuclease, would allow for ligation of VspαS-HBsAg into pCAMBIA-MeEF cut with the same endonuclease, resulting pAFHBS.

Results and Discussion

Clone of S gene that can express HBsAg VLP

Genomic DNA was isolated from blood serum of a patient from Hasan Sadikin Hospital, Bandung as described in material and methods. In an attempt to obtain the orf of *S* gene, primer HBsF (5'-GGATCCATGGA GAACATCG CATCAG-'3 and HBsR (5'-GAATTC GGTA ACCATGA AGTTGAGAGGGAGTAG-3') were used to amplify a full length of *S* gene. The PCR product was purified from agarose gels and ligated into pGEM-T easy vector to generate pGHB. Restriction fragment analysis of plasmid pGHB using *Eco*RI revealed two fragments, representing the 3 kb linearized plasmid and the 0.7 kb *S* gene insert (Fig. 1). Sequencing of the plasmid were aligned using BLAST (Basic Local Alignment Search Tool) search in the NCBI database and identified as the S genes with 99% identity.

Characterization of the "a" determinant, immunodominant region of HBsAg

The HBsAg amino acid sequence contains a highly conformational, hydrophilic domain from positions 100 to 160 namely "a" determinant (Coleman, 2006), which represents the immunodominant region of HBsAg. The "a" determinant consists of two peptide loops. The first loop is a large laminar loop stabilized by disulfide bonds between cysteine residues (Cys) 108–138 and 121–124. The second loop projects from the viral membrane and is stabilized by disulfide bonds between Cys 136–149 and Cys 139–147. The human immune response to HBsAg is primarily directed against these disulfide-bonded conformation. Alteration of these conformational epitopes may result in failure to neutralize viral infection (Coleman, 2006). As can be seen in Figure 2, the deduced amino acid sequence analysis confirmed that the "a" determinant was also present in cloned HBsAg (pGHB). This region was completely identical to amino acid sequence of HBsAg (M54923). All

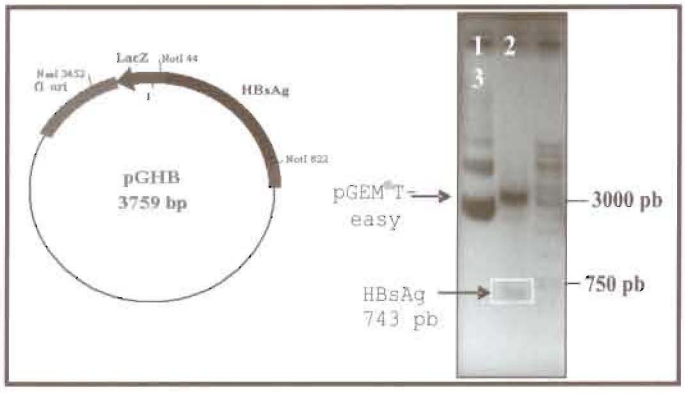

Figure 1. Cloning of the open reading frame (orf) of the *S* gene encoding HBsAg VLPs. Lane 1: pGHB uncut; lane 2: restriction enzyme digested of pGHB by *Eco*RI, Lane 3: molecular weight marker (1 kb DNA ladder-FERMENTASI).

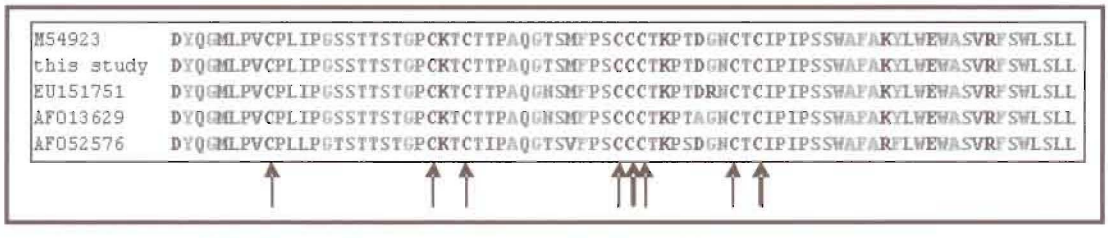

Figure 2. Amino acid sequence alignment of the "a" determinant

of the cystein residues (Cys 108-138, Cys 121-124, Cys 136-149, Cys 139-147) were also found. Therefore, they provide a structural architecture facilitating disulfide-bonded conformational epitopes that are important for neutralizing viral infection.

Another concern related to the immunogenecity of the "a" determinant is HBV mutants (Coleman, 2006; Purdy, 2007). In the past 10 years, a wide range of HBV mutants have been found including many amino acid substitution, insertions and deletion. One of the mutation, Gly/Arg145 substitution, alters the projecting loop (aa 139–147) of the "a" determinant, hence it was termed as vaccine-escape mutant (Coleman, 2006; Purdy, 2007). Other substitution mutant such as Met/Val133 & Thr/Ser143 ; Asp/Ala144 (Fang *et al.*, 1998; Ni *et al.*, 1995) have also been identified. If these mutants are used as a template for producing antigen in vaccine generation, the resultant antibody will not neutralize wild type HBV, which is still dominant as the causative agent. As can also be seen in Figure 2, since the allignment of the DNA sequences of the "a" determinant from pGHSB and corresponding region in the NCBI database was shown to be identical, all of those mutants in the "a" determinant were not present. Therefore, this HBsAg is a good candidate for generating wild type HBV vaccine.

Deduced amino acid alignment of "a" determinant from cloned HBsAg pGHBS and corresponding region of other HBsAg sequence from in the NCBI database was shown to be identical. All of the cystein (shown by black arrows) residues were found to be present while mutants that can influence epitope confirmation e.q Gly/Arg145, Met/Val133 & Thr/Ser143 , Asp/Ala144 were absent.

Characterization of HBV genotype and subtype HBV can be classified into nine immunological subtypes or eight genotypes (Purdy, 2007). Based on the sequencing data analysis using viral genotyping tool from NCBI, it was revealed that HBV that was used in this research was belong to genotype B. Subseqent amino acid analysis based on Purdy method (2007) was shown that the isolate was subtype adw2. This was due to the presence of amino acid residues Lys, Lys and Pro at position 122, 160 and 127, respectivelly, which were responsible to determine the HBsAg to be subtype adw2.

Some published data related to Indonesian genotype and subtype have been reported. Norder *et al.*, (2004) found that the subgenotypes of B and C differed in their geographical distribution. Subgenotype B1 is dominating in Japan, while B2 is dominating in China and Vietnam. B3 is confined only to Indonesia. All strains specify subtype ayw1. Another study by Lusida *et al.* (2003) revealed that all HBV isolates obtained from HBsAg-positive HBV carriers, including healthy blood donors; patients with acute hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma; and patients on hemodialysis all located in Surabaya belonged to genotype B, with more than 90% of them being classified into subtype adw (Lusida, 2003). Another survey in Yogyakarta, found that subtype adw was found in 34 (74%) of 46 HBsAg samples and adr in five (11%); compound subtypes, such as adyw and adyr were detected in the remaining seven (15%) (Hadiwandowo, 1994).

Therefore, cloned HBsAg indicate that isolated HBV belongs to genotype B and subtype adw in Indonesia were dominant, HBsAg which were identified with the same genotype and subtype in this research, were very likely to be used for vaccine generation that can neutralize dominant HBV infection in Indonesia.

Construction of fusion gene for expression fusion HBsAg VLP and VspάS under the control of MeEF1 promoter

Previously, Sojikul (2003) has created a gene encoding a recombinant HBsAg that was fused with a plant signal peptide in its amino terminus under the control of CaMV35S promoter with dual enhancers. The signal peptide which derived from soybean vegetative storage protein vspA (VSPάS) could direct the HBsAg into endoplasmic reticulum in plant cells and could enhance HBsAg accumulation. The fusion protein (VSPάS-HBsAg) expressed in plant cells and contained more disulfide bonds. Furthermore, this fusion protein was able to form virus like particles (VLP). Unlike conventional signal peptide such as KDEL, the VSPάS signal peptide gives the HBsAg protein more stablility, thus did not degrade in the vacuole. The VSPάS-HBsAg also stimulated higher levels of serum IgG than native HBsAg when injected into mice (Richter, 2000).

The same strategy was subsequently used in our construction. In order to increase the expression of HBsAg, we tried to construct fusion protein of VSPαS-HBsAg under the control of *MeEF1* promoter, a wound inducible promoter. The promoter is more efficient than CaMV35S promoter and will be active as soon as tissue is wounded or broken mechanically (Suhandono, 2002). Using this promoter, antigen production can be controlled whenever and wherever we need the most. Using the *MeEF1* promoter, the recombinant HBsAg protein will be expressed the most in wounded tissue and should not be produced in unwounded tissue. This will give the plants a chance to grow and develop normally without additional metabolic load such as expressing the recombinant protein.

As can be seen in Figure 3, the first step in construction strategy is making gene fusion between *VSPαS* and *HBsAg*. This was generated by PCR amplification from the *S* gene orf (open reading frame) from pGHB using forward primers tailed with *Nco*I-*VspαS* sequence and reverse primer tailed with *Bst*EII sequence (Supraba, 2007). This amplicon was subsequently ligated into pGEM-T easy vector to gain pGHBS.

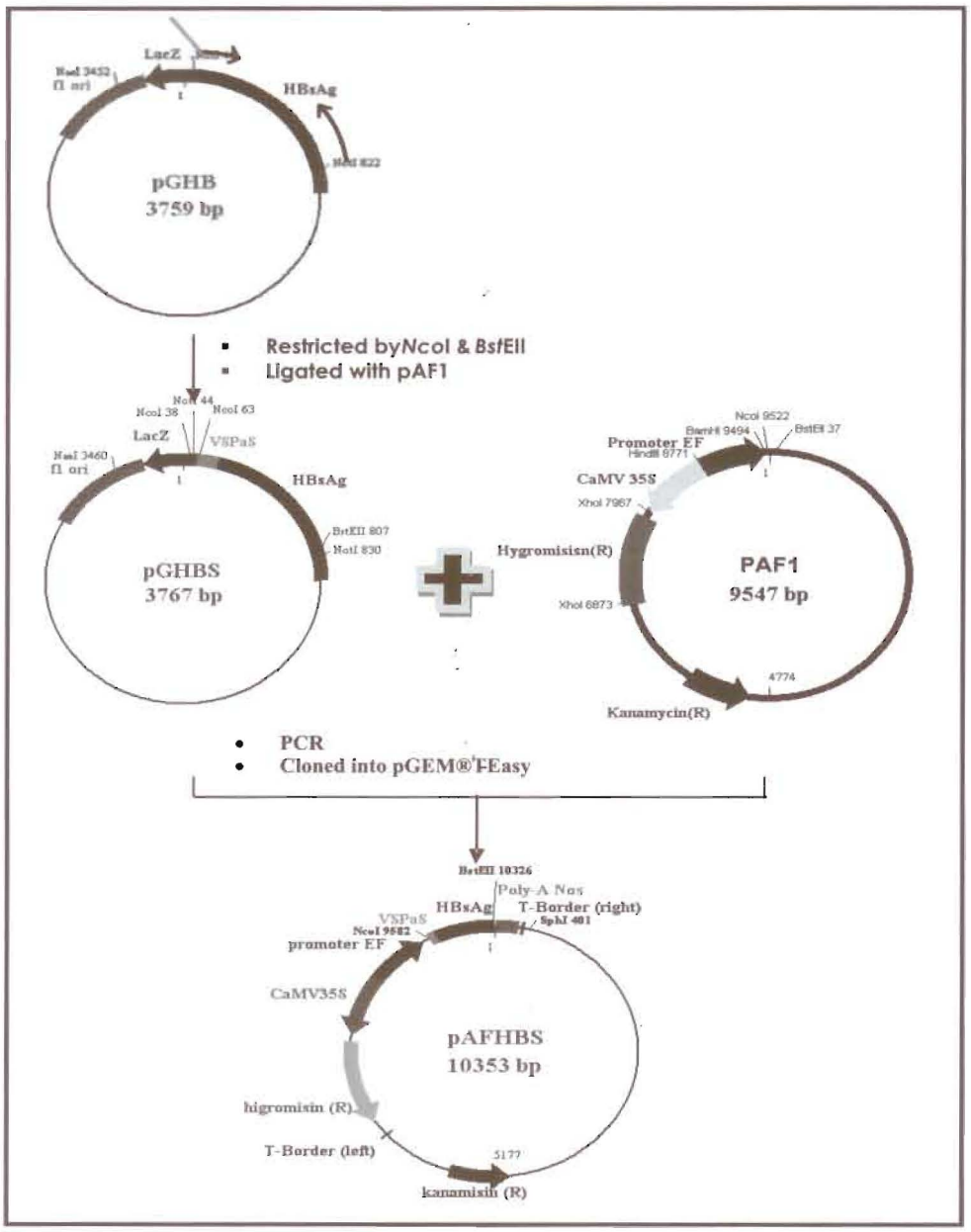

Figure 3. Construction strategy

It was proven that restriction with the same enzyme would result approximately 0.7 Kb (Fig. 4). Digestion of pGHBS and pAFI with both *Nco*I and *Bst*EII would allow the ligation of VspαS-HBsAg into pAF1 (a binary vector derived from pCAMBIA 1390 containing *MeEF1* promoter) (Fig. 5).

As can be seen in Figure 5, pAFHBS that contains VspάS-HBsAg fusion under the control MeEF1 promoter has been generated. Since pAF was originated from pCAMBIA 1390, all components from this plasmid were also included (Suhandono, 2005). Therefore, pAFHBS also consist of poly-A nopaline synthase (NOS polyadenilation signal for stabilizing expressed mRNA), right and left T borders, which are important for plasmid recombination into plant genome (Fig. 5). By expressing this construct, we can specifically choose the plant tissue to produce the HBsAg protein and control the time of antigen production.

Conclusions

Plasmid pAFHBS, that contains promoter *MeEF1*, fusion gene of HBsAg – VSPάS, and poly-A Nos has been generated. The cloned HBsAg gene sequence is originated from a common strain in Indonesia, genotype B and subtype adw2. We suggested that the HBsAg gene can be expressed in wounded plant tissue, accumulated in the ER of the plant cell and can be detected using commercial detection system.

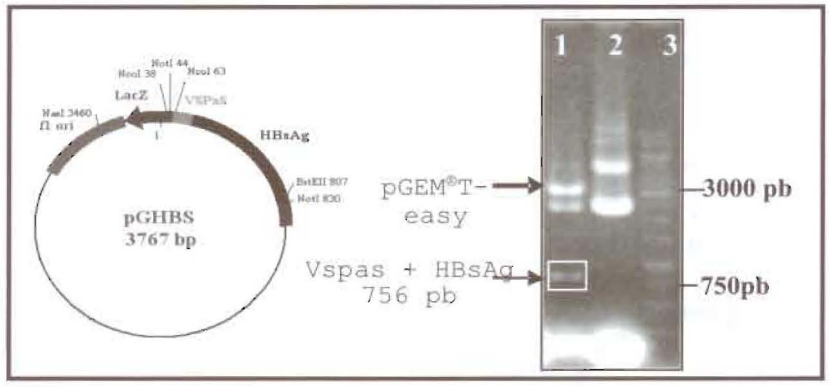

Figure 4. Cloning of VspαS-HBsAg. Lane 1: restriction enzyme digest of pGHB by *Nco*I and *Bst*EII; lane2: pGHB uncut; lane 3: molecular weight marker (1 kb DNA ladder-FERMENTAS)

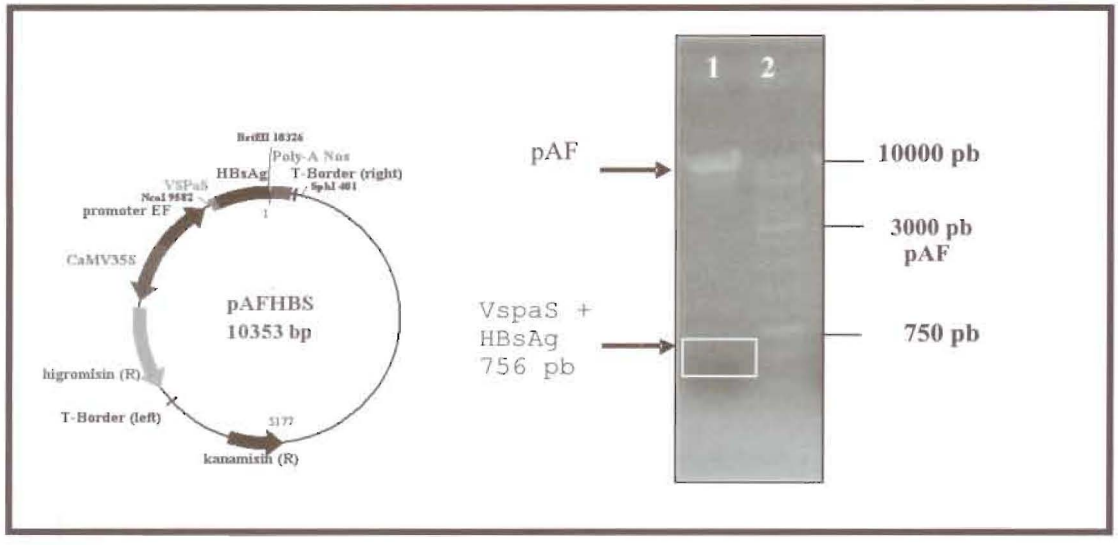

Figure 5. Cloning of VspαS-HBsAg under the control of MeEF1 Lane 1: restriction enzyme digest of pAFHBS by *NcoI* and *BstEII* Lane 2: (1 kb DNA ladder- FERMENTAS)

Further experiments need to be performed in order to conclude our work.

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