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# PRODUCTION OF MONOCLONAL ANTIBODY AGAINST DOMOIC ACID (DA) BY MURINE HYBRIDOMA USING CONDITIONED CELL CULTURE MEDIUM *IN VITRO*

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## ABSTRACT

Domoic acid is a highly potent marine toxin which is produced by *Pseudo-nitzschia*. It causes amnesic shellfish poisoning in humans after consumption of contaminated shellfish. In the present study, hybridoma technology has been used to develop a murine hybridoma cells producing monoclonal antibody by the conditioning of cell culture media with different carbohydrates *in vitro*. Carbohydrates are important organic enhancements in cell culture media that considerably increase hybridoma cell growth and antibody production.Indirect enzyme-linked immunosorbent assay was established to determine the antibody titers of cell culture supernatants. A hybridoma C4H5 cell line was developed by the fusion of Sp2/0 myeloma cells with spleen cells that were harvested from Balb/c mice immunized with complete DA antigen.The influence of carbohydrates on cell growth and higher protein production of C4H5 cells has been investigated. Galactose, fructose and maltose significantly increased protein production by the factor of 1.7, 2.0 and 2.2, respectively.The study extends understanding of augmented use of superior carbohydrate in cell culture media to improve growth of hybridoma cells and subsequently higher yields of antibody.

**Keywords:** Domoic acid, Amnesic shellfish poisoning, Cell culture, Hybridoma, Monoclonal antibody, Fetal bovine medium, Carbohydrates.

**Abbreviations:** DA (Domoic acid); MAb (Monoclonal antibody), ELISA (Enzyme linked immunosorbent assay); CHO (Carbohydrates); ASP (Amnesic shellfish poisoning).

## INTRODUCTION

Domoic acid (DA) is primarily produced by marine diatomic algae of the genus *Pseudo-nitzschia* (Perl *et al.* 1990). The broad salinity survival range of this alga permits its growth in global marine watersand estuarine environment(Jackson *et al.* 1992). Furthermore, the algae are consumed by shellfish thatleads to the final consumers: sea mammals, birds and eventuallyto the humans. Consequently, the intoxication results in neural tissue damageand memory loss, therefore,the disease was later named asamnesic shellfish poisoning(ASP) which is an acute state of DA intoxication (Wright *et al.*1989, Todd, 1993, Jeffery *et al.*2004). In 1987, there were reports of three deaths and 100 people became ill after consuming shellfish heavily contaminated by DA from Prince Edward Island, Canada (Bates *et al.* 1998; Smith *et al.* 1990b; Horner *et al.* 1997). The symptoms of ASP include vomiting, abdominal convulsions, diarrhea, severe headache, unconsciousness, seizures and memory loss (Perl *et al.*1990; Pulido, 2008; Kumar *et al.*2009; Lefebvre and Robertson, 2010; Todd, 1993). To this day, a large number of mammals, marine birds and anchovies deaths have been associated with DA poisoning (Lelong *et al.* 2012; Trainer *et al.* 2012).

Hybridoma cell culture *in vitro* has become an essential molecular tool in basic cell and biotechnological research. The growth of hybridoma cells is dependent on internal and autocrine factors present in the cell culture media (McKeehan *et al.* 1990; Schaeffer, 1990; Hartung, 2007; Lindl and Gstraunthaler, 2008).

Cell culture media are complex mixtures of growth factors containing various nutrients. Carbohydrates (CHO) are supplied largely in the form of glucose as a carbon source for the growth ofhybridoma cells. In some cases, glucose is substituted with superior carbohydrates such as galactose and other simple sugars to improve cell growth, viability and protein production (Wagner *et al.* 1991; Altamirano*et al.* 2000; Altamirano*et al.* 2004; Wilkens *et al.* 2011).

The aim of this study was to prepare a monoclonal antibody (mAb) clone C4H5 with high specificity against DA utilizing carbohydrates conditioned cell culture media and to establish indirect enzyme-linked immunosorbent assay (iELISA)for DA detection. DA is a small molecular haptendevoid of immunogenicity. Therefore, DA–bovine serum albumin (DA–BSA) and DA–ovalbumin (DA–OVA) conjugates were used as immunogen and coating antigen,respectively. The current study describes positive

influence of carbohydrate conditioning in basic cell culture media on hybridoma growth and mAb production.

## MATERIALS AND METHODS

**Animal, reagents and cell culture apparatus:** All animal experiments were carried out according to the rules by the Animal Welfare Committee of Fujian Agriculture and Forestry University, Fuzhou, China. Female Balb/c mice were obtained from Shanghai SLAC Laboratory (Shanghai, China). The mouse myeloma cell line SP2/0 (ATCC) was from American Type Culture Collection. Roswell Park Memorial Institute medium (RPMI-1640), 96-well plates, Tween 20, Goat anti-mouse-peroxidase conjugate (IgG-HRP), substrate solution 3, 3′,5,5′-tetramethylbenzidine (TMB), polyethylene glycol (PEG- 1500), carbohydrates, bovine serum albumin (BSA), ovalbumin (OVA), phosphate buffer saline (PBS), hypoxanthine/thymidine/aminopterin (HAT) and fetal bovine serum (FBS) (Sigma-Aldrich and Thermo-Fischer Scientific, China) were used for cell cultures *in vitro*. All cultures were incubated (Heracell™ $CO_2$ Incubator, USA) in humidified environment at 5% $CO_2$ and 37ºC. 96-well ELISA plates were read by Thermo-Fischer Scientifi cMultiskan™ microplate reader, USA.

**Production of mAb:** Three female Balb/c mice (virus free and 6–8 weeks old) were subcutaneously injected with antigen DA-BSA. After 5 times injection, the mice serum titer of antibody (anti-DA) from the immunized mouse was determined by iELISA. The mouse with best titer was selected to sacrifice for the isolation of potent splenocytes and later used for the cell fusion with mouse myeloma cells.

**Carbohydrate conditioning:** Various carbohydrates as carbon source were used for the conditioning of cell culture media. The basic media RMPI-1640 + 10% FBS was used in C4H5 cell cultures on 96-wells plates *in vitro*. This media was conditioned with 0.08mM/mL glucose, 0.08mM/mL fructose, 0.08mM/mL galactose and 0.04mM/mL maltose respectively.

**Murine myeloma cell cultures:** The mouse myeloma cell line SP2/0 was cultured in RPMI-1640 (added 10% FBS), 2.2 g/l $NaHCO_3$, 100 U/ml penicillin, and 100 g/ml streptomycin. Cells were maintained in a humidified atmosphere with 5% $CO_2$ at 37°C until the logarithmic growth phase based on the cell growth curve. The adherent and suspension cells were harvested for the following experiment.

**Cell fusion:** Sp2/0 myeloma cells were grown in RPMI-1640 culture medium (added 10% FBS) for 4-5 d at 37ºC in a 5% $CO_2$ atmosphere.Before the fusion, the mouse with best anti-DA titer was given booster injection. On the day of fusion, the mouse was sacrificed by cervical dislocation and the spleen was removed aseptically. The splenocytes were harvested and fused with myeloma cells (SP2/0) at a 10:1 ratio in the presence of 1 mL 50% PEG 1500 as a fusing reagent. Later, 50 mL of HAT-1640 medium, feeder layer cells (added with 20% fetal bovine serum) were supplemented to the fused cells. This medium was transferred (100 µL/well) onto 96-well cell culture plates. After eight-ten days, supernatants from hybridoma were screened with iELISA to determine the positive wells containing single growing colonies, the positive well culture showing significant DA recognition activity were expanded from the cultures in the 96-well plate to a 24-well plate, and subcloned three times by the limiting dilution. Using 8-channel micropipettor added 100 µL culture medium to all the wells of 96 well cell culture plates except well A1. Added 200 µL of cell suspension to well A1 then transferred 100 µL from A1 to H1 in entire column by using single channel pipettor and mixed gently. After this, added 100 µL additional medium to this column and transferred 100 µL from first column to the entire plate. The plates were incubated at 37ºC in humidified $CO_2$ incubator; colonies were seen growing under microscope after 4 to 5 days and subsequently expanded after 7 to 10 days in 12 or 24 well cell culture plate. The technical route designed in this study for hybridoma technology is shown in **Fig. 1**.

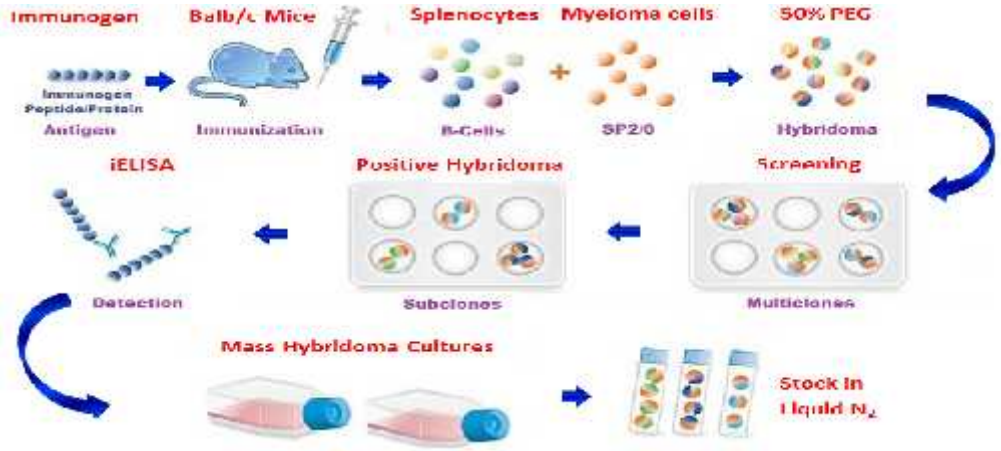


**Fig. 1.Technical route of hybridoma technology. The illustration shows different steps used in this study for the production of C4H5 anti-DA mAb by hybridoma technology.**

**Determination of cell growth:** Cell density of hybridoma cells suspension was determined by trypan blue exclusion method using a hemacytometer. Trypan blue stock solution 0.1 mL was added to 1 mL of the hybridoma cells (0.4% trypan blue solution in PBS (phosphate buffer saline), pH 7.2 to 7.3). Hemacytometer was loaded and cells were blue stained. Total numbers of cells were examined immediately under the microscope in low magnification.

**Indirect Enzyme-linked immunosorbent assay (iELISA):** The antibody titer was tested by iELISA in three separate assays in replicates, using the procedure described below. The microplates were coated with coating antigen DA-OVA at 1 µg/mL (100 µL/well) by overnight incubation at 4°C. Plates were washed with PBST three times and were blocked with 200 µL/well with PBSM, followed by incubation for 2 h at 37ºC. Blocking buffer was discarded and the plates were washed three times with PBST. Fifty µL/well of hybridoma supernatant were added, and the plates were incubated for 1 h at 37°C. After another washing procedure, goat anti-mouse IgG-HRP(1:5000, 100 µL/well) was added, followed by incubation for 1 h at 37°C. The plates were washed, and 100 µL/well freshly prepared TMB substrate solution A and B (1:1, v/v) was added. After incubating for 15 min at 37ºC, the reaction mixture was stopped using 2 mol/L $H_2SO_4$. The absorbance was measured at 450 nm by ELISA plate reader.

**Statistical analysis:**Statistical tests were performed using GraphPad Prism. Data are presented as mean±SD. All the experiments in the present study were performed in replicates and results were confirmed in at least three independent experiments.

## RESULTS

**Production and screening of monoclonal antibody:** Following immunization and iELISA analysis of serum titer showed highest anti-DA activity with titer 2.5 in mice 1 compared to the control (**Fig. 2**), From cell fusion, positive single growing colony of hybridoma clone C4H5 was obtained successfully (**Fig. 3**).

**Effect of carbohydrates on C4H5 anti-DA titer *in vitro*:** The hybridoma clone C4H5 supernatants were assayed by iELISA after 8d *in vitro*conditioned with different carbohydrates respectively. The anti-DA titer of C4H5 in medium conditioned with galactose showed antibody titer 0.78 which was 1.7 fold, fructose showed titer 0.99 with 2.0 fold, and maltose showed highest MAb titer reached up to 1.19 which was 2.2 fold higher compared with glucose as a control which showed very low titer of 0.49 (**Fig. 4**).

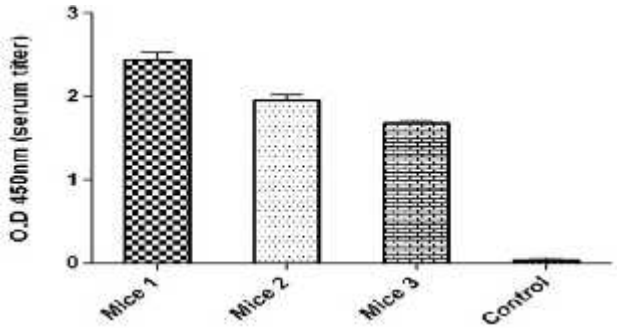


**Fig. 2. Mice 1 had the highest serum titer determined by iELISA and selected for the cell fusion.Control, non-immunized mice. The values are mean ± SDof three independent experiments. Vertical bars are SDs.**

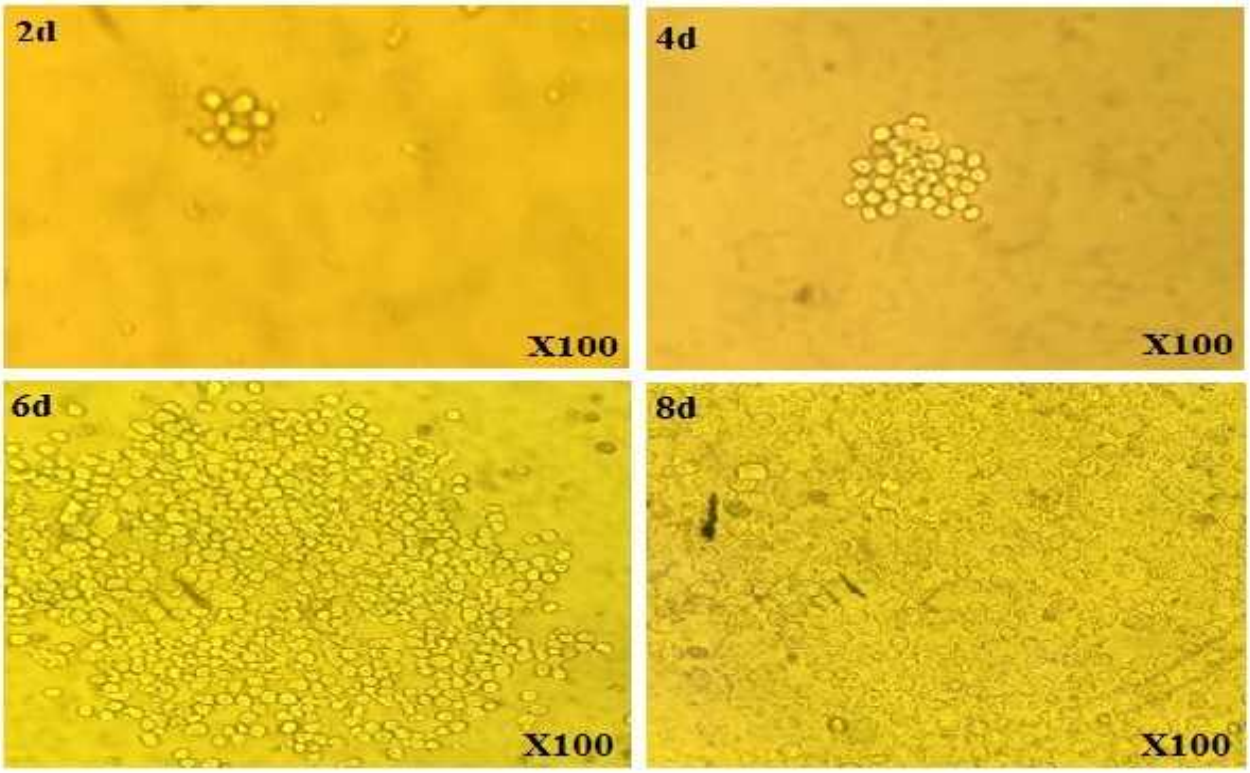


**Fig. 3.Hybridoma clone C4H5 grown in culture medium observed after 2d, 4d, 6d and 8d. The monoclonal cell colony is visualized as fewer cells in 2d to a fairly large colony as seen in 8d.**

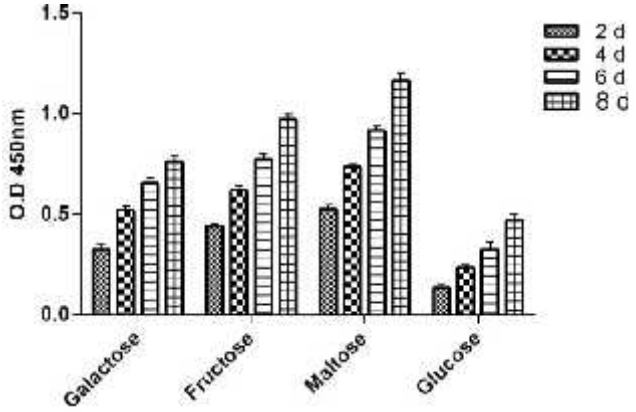


**Fig. 4.Immunoassay of C4H5 supernatant mAb by iELISA at days 2, 4, 6 and 8 respectively grown in conditioned cell culture media.The values are mean ± SD of three independent experiments. Vertical bars are SDs.**

**Cell density:** C4H5 grown in cell culture media conditioned with maltose showed considerable increase in hybridoma cell growth and the cell density was significantly increased up to 2.0 x $10^8$ cells after 8d (**Fig. 5**).

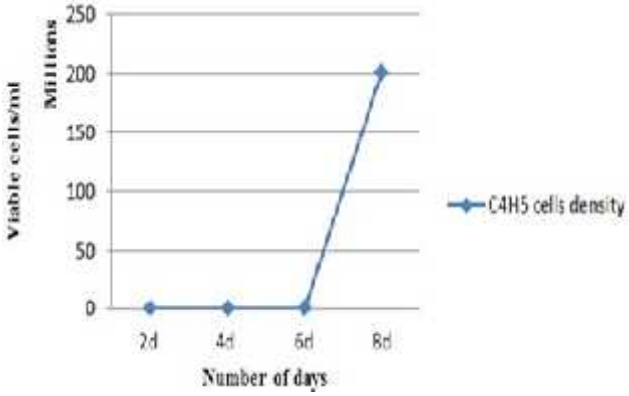


**Fig. 5. C4H5 viable cell density at 2d, 4d, 6d and 8d observed after 8d of cell culture. The density of the viable cells increased rapidly with the incorporation of the superior carbohydrates up to 2.0 x $10^8$ at 8d.**

## DISCUSSION

DA is a characteristic hapten with a low molar mass of 311 g/mol, and it cannot induce immune response in animal. Therefore, a hapten-protein conjugate was developed and DA-BSA was injected at multipoint subcutaneously into the mice to induce the immune response in Balb/c mouse. Subsequently, iELISA of

mouse serum showed high antibody titer following immunization (Yu *et al.* 2005; Gefen*et al.* 2015).

On the base of recent study experiences, the chance of getting stable hybridoma with high mAb titer was dependent on cell culture medium, appropriate screening and early subcloning methods in aseptic conditions. Feeder layer cells were essential for cells fusion and increased C4H5 hybridoma growth because they provided numerous growth factors and cleared dead myeloma cells, splenocytes, and cell debris by phagocytosis; they also conveyed antimicrobial activity (Smith, 2001; Li *et al.* 2010; Ahn*et al.* 2010).

C4H5 clone produced mAbthat recognized a single and distinct epitope of DA antigen. There is a little research available on the supplementation of superior carbohydrates such as fructose, maltose and galactose for hybridoma cell growth. In the present study, hybridoma cells grew healthy as determined by viable cell density and produced high mAb titer in cell culture media containing superior carbohydrates due to freely available carbon source for the growth of cells (Kuby, 2007). Maltose was the best carbohydrate substitute in cell culture medium which raised mAb titer to a significant level. Glucose in general metabolizes to form lactic acid which hinders the cell growth or slows growth and has a negative effect on cell proliferation. The substitution of glucose with fructose, galactose and maltose favored the growth of C4H5 clone and improved the growth conditions (Gambhir *et al.* 2003; Korke *et al.* 2004; Anand *et al*. 2009).

It was observed that highest MAb titer (1.19) in maltose supplemented growth medium was directly proportional to growth, cell viability, available attachment space and proliferation of the cells in still culture in accordance with previous reports (Kurano*et al.*, 1990; Sathya*et al.*, 2008). Superior carbohydrates and optimum incubation temperature (37°C) in the medium significantly enhanced the number of hybridoma cells and affected the viability and density of the cells (Sadettin and Bernhard, 1990; Evelyn *et al.*, 2006: Alireza *et al.* 2007).

**Conclusion:** It is concluded that cell culture conditions such as various nutrients, optimal incubation temperature and $CO_2$ could augment hybridoma growth, high anti-DA activity and cell viability. The present study demonstrated that cell culture media supplemented with superior carbohydrate could significantly increase mAb titer. The current study could be utilized for improved protein production in cell culture and may have widespread applications in various fields of biomedical, biotechnology and immunology research.

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**Author contribution:**A.F.S wrote the manuscript andS.A carried out statistical analysis.

**Conflicts of Interest:** The authors declare no conflict of research.

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