

Production of Fish Serum Products as Substitute for Fetal Bovine Serum in Hybridoma Cell Cultures from Surimi Industrial Waste

Fransiska Zakaria-Runkat¹, Wanchai Worawattanamatekul^{2*} and Ong-ard Lawhavit³

ABSTRACT

The hybridoma cell culture procedures mostly utilize fetal bovine serum (FBS) to supplement the culture media, which contributes to the high cost of production of monoclonal antibody. Previous research has shown interesting results on the utilization of fish serum (FS) prepared from wash water of surimi processing line. In this study, FS was used as a substitute for FBS in two types of hybridoma cell cultures. Cell viability was measured using MTT colorant while IgG monoclonal antibody secretion was measured using ELISA method. SDS electrophoresis was run to observe FS protein profiles. Nitrogenous compounds of FS were also determined. Based on the results, it was found that FS prepared from surimi factory wash water contained proteins with molecular weights ranging from 66 to 12 kD. The serum contained TVN and TMA, signalling the need to improve the washing process in the factory, particularly the waste water treatment in formulating the fish serum. The two hybridomas secreting IgG anti-chikungunya (105) and anti-dengue virus (H2) grew differently in the FS-substituted medium, where the 105 hybridoma showed less cell growth. The cells in all substituted cultures produced antibody until the third passage; however, substitution of 2% FBS with FS resulted in the higher antibody secretion, especially in cultures maintained for 96 hours after the third passage.

Key words: fish serum, goat fish, hybridoma cell culture, lizardfish, surimi waste utilization

INTRODUCTION

Hybridoma cells are cultivated to produce monoclonal antibody (Mab), which is used as reagent for diagnostic analysis and specific compound determination. Industrial production of commercial Mab has become a major biotechnological development since the 1980s. In clinical analysis, monoclonal antibodies are used in cell surface identification, cell separation, immuno-histochemistry, assays for regulating hormones or toxins, diagnostic imaging,

therapeutic uses in cancer, transplantation and specific protein or marker identification (Nakanishi *et al.* 1990; Jackson *et al.* 1998). Ten percent fetal bovine serum (FBS) is still used as supplement in media in many laboratories because it is applicable to almost all hybridoma cell lines. However, some technical disadvantages of using serum include its undefined nature, batch-to-batch variability in composition and the risk of contamination. There are also increasing concerns about animal suffering inflicted during serum collection that adds an ethical imperative to move

¹ Department of Food Technol Hmn Nutr, Bogor Agric University, Darmaga Bogor 16002, Indonesia.

² Department of Fishery Products, Kasetsart University, Bangkok 10900, Thailand.

³ Department of Veterinary Microbiology and Immunology, Kasetsart University, Bangkok 10900, Thailand.

* Corresponding author, e-mail: ffishw@ku.ac.th

away from the serum use wherever possible (Boschetti *et al.* 1991). FBS also interferes with downstream processing which, in turn, affects product recovery and quality.

Over the last 15 years, several serum-free media (SFM) and protein-free media (PFM) have been developed to enrich hybridoma cultures (Glassy *et al.* 1988; Schneider 1989). Hybridomas normally grow well in SFM composing of a basal medium, serum-derived proteins and hormones. However, most of these media require long cell-adaptation periods during which the cells could become fragile. Various natural components have been successfully used as a cell culture supplement for growth and monoclonal IgG antibody production. These include ultrafiltrate fraction (UF) of bovine colostrum (Pakkanen and Neutra, 1994), extract of bovine milk (Belford *et al.* 1995), serum and bovine serum albumin (Ozturk and Palsson, 1991), meat protein hydrolysate (Schlaeger, 1996) and lysine-containing peptides (Franek *et al.* 2003). Zakaria-Rungkat *et al.* (2003) reported the use of fish serum (FS) from wash water obtained after washing minced by-catch fish during surimi processing. In spite of the progress in expressing Mabs in yeast and bacteria, production of Mabs still depends largely on the use of animal cells. More cost-effective and defined methods of producing Mabs must be developed because of the increased demand for Mabs, especially for *in vivo* therapy.

Surimi, a popular food ingredient made of minced fish fillet, is used to formulate various fish-based food products including fish balls, kamaboko, artificial crab meat and fish sausages (Suzuki, 1981). In surimi processing, minced meat is washed with water, resulting in waste water containing fat, blood, proteins and water-soluble substances, which create major environmental pollution problem (Windsor and Barlow, 1981). A means to use this wash water is therefore desired. Zakaria-Rungkat *et al.* (2004) and Zakaria-Rungkat *et al.* (2003) reported the use of FS made

of lizardfish and pelagic fish meat surimi wash water as substitutes for FBS in lymphocyte and hybridoma cell culture, respectively. Since the reported FS from by-catch fish was made from laboratory-prepared surimi, it might not be the same as that produced industrially. Thus, it is interesting to verify the use of fish wash water obtained from a commercial surimi processing plant in different local hybridoma cells.

In this study, fish serum obtained from waste water of the surimi processing process was used in substitution of FBS in hybridoma cell cultures. The culture media were supplemented with various concentrations of FS. Cell growth and antibody secretion were also observed.

MATERIALS AND METHODS

Materials

Wash water was collected after decapitating, descaling, and mincing and washing minced fillets of shortfin lizardfish (*Saurida micropectoralis*) and black stripe goat fish (*Upeneus tragula*) from a surimi factory in Samutsakorn, Thailand. The water was brought immediately to the laboratory in an ice-packed cooling box. Two types of hybridoma cells, the first producing IgG anti-chikungunya (105) and the second producing antibody anti-dengue virus (H2), were sourced from the US-NAMRU-2 laboratory in Jakarta, Indonesia. RPMI-1640 medium, antibiotic-antimycotic, fetal bovine serum were obtained from Gibco Industries (Carlsbad, Ca) while other common chemicals were from Sigma (St. Louis, MO). All components of the medium were sterilized with 0.22 µm disposable sterile membranes.

Preparation of fish serum

Upon arrival at the laboratory, the wash water was filtered ice cold with cheese cloth, followed by Whatman No. 1 and 7 filter papers. The filtrate was evaporated at 47°C in a vacuum

wash water on SDS-PAGE is presented in Figure 1. All fish sera prepared contained proteins with molecular weights between 66 and 12 kD, with the major proteins having molecular weights slightly below 45 kD. Zakaria-Rungkat *et al.* (2003) reported that wash water obtained from pelagic fish contained sarcoplasmic proteins having small-molecule polypeptides comparable to myofibrillar proteins. Large proteins comparable to albumin were also present in the fish sera.

The amounts of total volatile nitrogen in all fish serum preparations were comparable. However, as for trimethylamine content, the serum from wash water of black stripe goat fish was twice

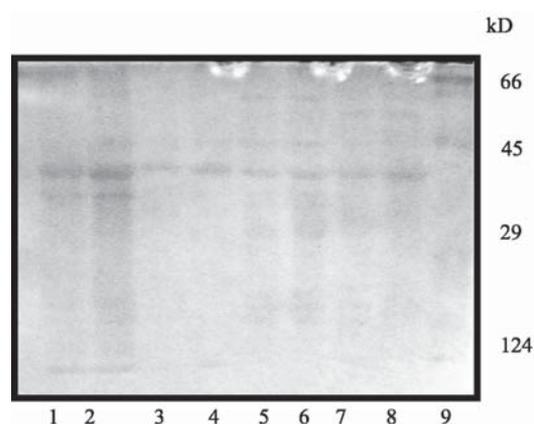


Figure 1 Molecular weight of proteins in fish serum prepared from waste water of lizardfish after mincing (1, 2); lizardfish after scaling (3, 4); black stripe goat fish after mincing (5, 6); black stripe goat fish after scaling (7, 8). Line 9 is the molecular weight standard marker.

the levels of the other sera (Table 1). TVN and TMA are volatile compounds resulting from the breakdown of trimethylamine oxide (TMAO), which is found in most marine species. The breakdown of TMAO has been implicated in the spoilage of fish. TMA has a very low odor threshold and is associated with fishy odor during spoilage. The suggested threshold value of TMA in fish products is 0.6 ppm. Skin and muscle represent the major source of TMAO, causing high TMA in the wash water after scaling and mincing (Morrissey *et al.* 2000).

On the other hand, FS contains other nitrogenous compounds such as urea and carnitine aside from protein. Urea might result from decomposition of fish body proteins that diffuse into the fish flesh and then into the serum, and is recovered during protein separation (Zakaria-Rungkat *et al.* 2003). In this study, only fish serum obtained from wash water after mincing lizardfish was used to substitute FBS in hybridoma cell culture. This is because FS from descaling black stripe goat fish contained high amount of undesirable non-protein nitrogenous compounds. It was also noted from the previous research (Zakaria-Rungkat *et al.* 2004) that FS from minced lizardfish had the highest protein content.

Hybridoma cultures and IgG antibody secretion

The first experiment was done to test cell survival in fish serum (FS)-substituted medium. As shown in Figure 2, both types of cells grew less in FS substituted medium after 24 hours of incubation. There were drops in living cells in

Table 1 Total volatile nitrogen (TVN) and trimethylamine (TMA) contents in fish serum obtained from two species and two of surimi processing steps.

Fish serum source	TVN (mg/100g)	TMA (mg/100g)
Lizardfish after descaling	109.15	17.43
Lizardfish after mincing	107.94	17.32
Black stripe goat fish after descaling	103.86	34.65
Black stripe goat fish after mincing	109.01	16.71

both H2 and 105 hybridoma cultures after 72 hours of incubation as the percentage of substitution increased. However, extended incubation until 96 hours did not lead to any further decrease in living cells. This suggests the potential of FS serum as a substitute for FBS, particularly at 2% substitution.

To further test the IgG antibody secretion, 105 hybridoma was used as the experimental model and fish serum substitution was limited to a maximum of 5%. The 105 hybridoma cells were used since they seemed to grow less than the H2 cells, making them a better representative as an experimental model considering that if the lesser

growing cells could survive, the other cells would have a better chance to survive.

The cells in all cultures produced antibody until the third passage which was done twice a week (Figure 3). The culture substituted with 2% fish serum had a similar antibody secretion pattern. However, the culture substituted with 5% FS seemed to secrete less antibody. In all three cultures, antibody secretion appeared to drop after each passage except for the culture substituted with 5% FS, where it increased slightly, probably due to the lower secretion during the first passage. This phenomenon is important where constant

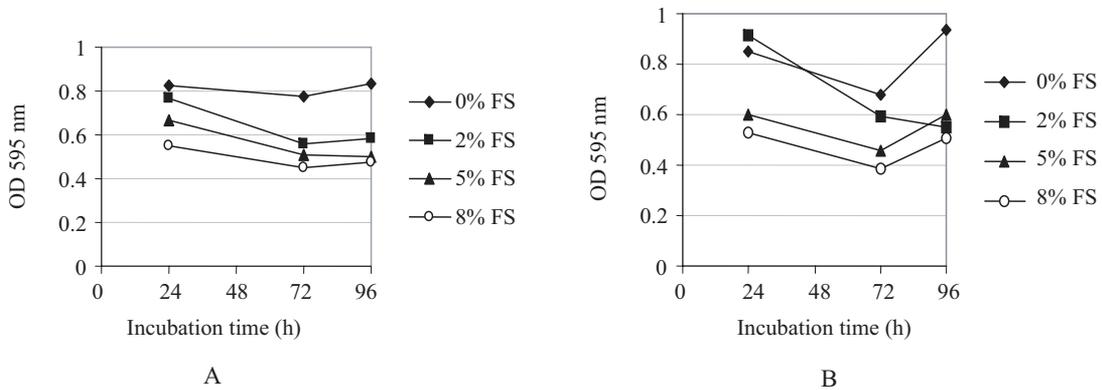


Figure 2 Growth of H2 hybridoma cells (A) and 105 hybridoma cells (B) in medium containing 10% FBS without (0% FS) or with substitution with 2, 5 or 8% fish serum (FS) obtained from lizardfish waste water processing. Cell growth was measured with MTT reagent.

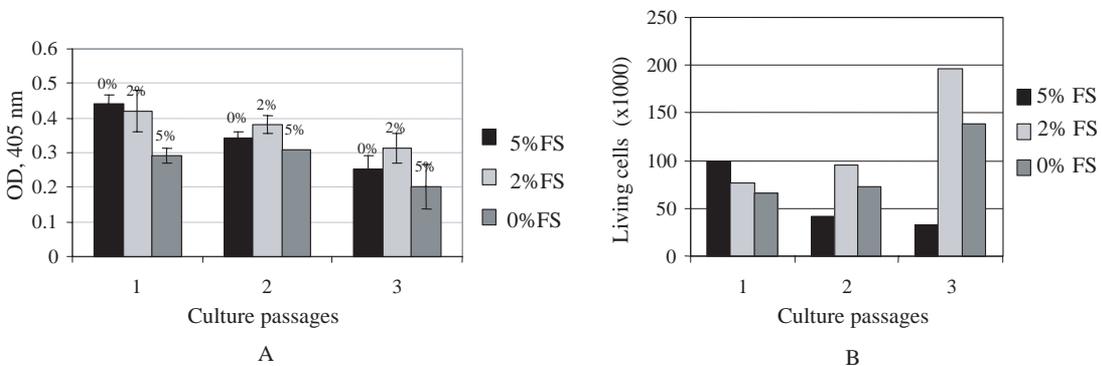
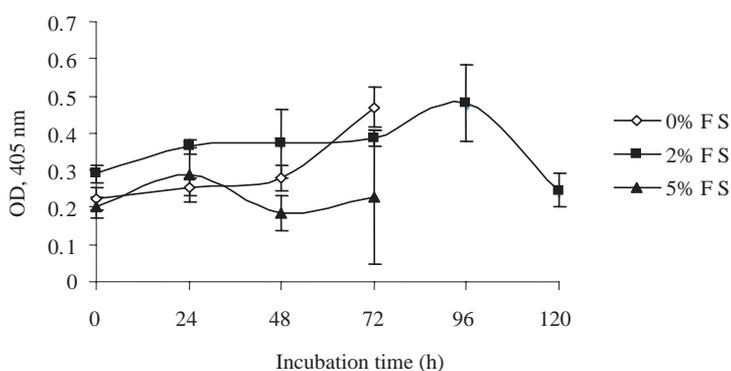


Figure 3 IgG antibody secretion (A) and cell growth (B) of 105 hybridoma cells cultured in medium substituted with 0, 2 or 5% FS prepared from lizardfish waste water processing. The observations were done at the first (1), second (2) and third (3) passages.

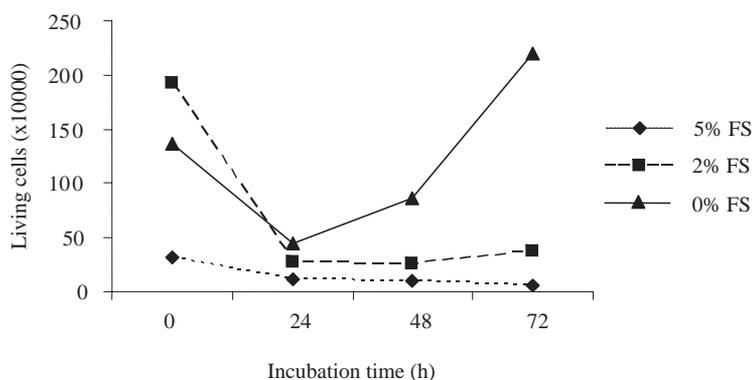
secretion up to the second passage can indicate the potential for at least short-term maintenance.

The performance of the 105 cells was observed after the third passage on a daily basis up to the third day for cultures without substitution and until the sixth day for those with 2% and 5% FS. Data in Figure 4A indicate that at the third passage, the secretion of IgG was slightly higher in cultures substituted with 2% fish serum starting from the first day of maintenance in incubation up to 96 hours after which the secretion dropped off. Both cultures without substitution and the one

substituted with 5% were maintained only up to 72 hours. However, the culture with 10% FBS without fish serum substitution appeared to steadily increase in antibody secretion. Living cells in cultures substituted with 5% fish serum dropped in accordance with the passage number (Figure 4B). However, those in cultures without or with 2% FS substitution appeared to grow better until the third passage. These growth patterns were not similar to the antibody secretion pattern, which was lowest at the third passage. After incubation up to 72 hours following the third passage, living



A



B

Figure 4 IgG antibody secretion (A) and growth (B) of 105 hybridoma cells in cultures substituted with 0, 2 or 5% fish serum (FS) obtained from lizardfish waste water processing. The cells were passaged for three times, then maintained in the same incubation for 4 to 5 days.

cells were much higher in the culture without FS substitution. Drops in living cell number were higher in cultures substituted with 5% FS, which also secreted the lowest antibody. The cultures substituted with 2% FS did not stimulate cell growth but antibody secretion was still high. These results are in accordance with the above data where FS substitution up to 2% might give promising result, particularly as a substitute for FBS in cell maintenance up to 96 hours after the third passage.

CONCLUSIONS

The results of this research showed that FS prepared from surimi wash water contained proteins with molecular weights ranging from 66 to 12 kD. The serum contained TVN and TMA. Improving washing procedures in the factory and handling of wash water in preparing the fish serum need particular attention to reduce the undesirable non-protein nitrogenous compounds. Using ultra filtration technique in serum preparation might also be of interest.

Hybridomas secreting IgG anti-chikungunya (105) and anti-dengue virus (H2) had different growth patterns in the medium substituted with FS. 105 hybridoma grew less and was subsequently used in the second experiments. The cells in all cultures produced antibody until the third passage where substitution up to 2 % had promising result, particularly as substitute for FBS in cell maintenance up to 96 hours after the third passage. This demonstrated that surimi wash water has potential in substituting FBS in hybridoma cell culture for the production of monoclonal antibody. This could mean less cost in antibody production and possibility for adding value to waste. Nevertheless, the specification of the constituents, particularly proteins and growth factors, and exploration of other fish species as sources of fish serum, other cell cultures and other microbe and tissue culture, is necessary to study the potential of FS as a substitute for FBS and as a supplement for other culture media.

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evaporator to concentrate the liquid to achieve 3-4% protein contents. The concentrated fish serum was kept at -20°C until its use. All components of the medium were sterilized with 0.22 µm disposable sterile membranes.

Hybridoma culture

Frozen hybridoma cells were immediately brought to 37°C in a water bath, washed three times by centrifugation at 600Xg for 10 minutes with RPMI-1640. The cells were then resuspended in the same medium, supplemented with 10% FBS, antibiotic-antimycotic and cultivated by 5 ml in 25-ml flask in 5% CO₂ incubator at 37°C and 95% humidity. Living and dead cells were recorded and cultures were regularly maintained every 3-4 days. Cell suspension at the lag phase was counted, using tryphan blue and hemacytometer. It was then adjusted to 10⁴ cell/ml in standard RPMI-1640 for subsequent experiments.

The survival of the two types of cells was observed in the FS-substituted medium. 4.5 ml cell suspension was placed in 25-ml flask. FS was added at 0, 1, 2.5 or 4 ml, and FBS supplemented at 5, 4, 2.5 or 1 ml for FS substitution at 0, 2, 5 or 8%, respectively. The experiments were performed in triplicate. Living cells were analyzed using MTT reagent and color formation was monitored by measuring the absorbance at 570 nm.

In the second experiment, only 105 hybridoma cells were grown in 5-ml culture medium containing 10% FBS as the control culture. The rest of the cultures had 2, 5 or 8 % FS. Cultures were incubated following the above procedures and recultured twice a week until the third passage. Passaging was done by allocating 1 ml of the cultures into new 25-ml flasks and adding 3.5 ml fresh RPMI medium. FBS supplementation and substitution with FS were done as above. Before passaging, 50 µl of cell suspensions were counted using tryphan blue and another 50 µl were used for the ELISA analysis.

IgG antibody secretion was analyzed by standard sandwich ELISA method using anti-mouse IgG as the coating antibody, before adding 100 µl of culture supernatants. Blocking was done using 3% casein. After incubation at 37°C for 2 hours, plates were washed with 0.5% Tween 80 solution; second anti-mouse IgG conjugated with horse radish peroxidase was then added. After incubation and washing, ABTS substrate was used to develop the color, which was measured by a spectrophotometer at 450 nm.

After the third passage, cell cultures were incubated up to 5 days without passaging. One flask was taken for cell counting and IgG analysis on a daily basis.

Analysis of protein and nitrogenous compounds

Total FS proteins were measured using the Lowry method (Lowry *et al.* 1951). FS proteins were separated by SDS polyacrylamide gel electrophoresis (PAGE) (Coligan *et al.* 2001) using low molecular weight (LMW) Standard markers (Pharmacia, Piscataway, NJ). Total volatile nitrogen (TVN) and trimethylamine (TMA) (Yamagata and Kim, 1992) were determined by NaOH titration and were calculated using the following formula:

$$\text{TVN (mg/100g)} = \frac{14 \times (30 + w) \times (15 - V1) \times 0.01}{5} \times \frac{100}{m}$$

$$\text{TMA (mg/100g)} = \frac{14 \times (30 + w) \times V2 \times 0.01}{5} \times \frac{100}{m}$$

V1 = volume of 0.01 M NaOH used in first titration

V2 = volume of 0.01 M NaOH used in second titration

m = sample mass (g)

w = moisture content (g/g wet mass)

14 = nitrogen atomic mass

RESULTS AND DISCUSSION

Electrophoresis profiles of fish sera prepared from lizardfish and black stripe goat fish

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