CHAPTER 5.0 DISCUSSION

5.1 Formulation of low-cost materials for optimum growth of *G. neojaponicum*

The *G. neojaponicum* can be potentially cultivated as an alternative source for *G. lucidum* commercial production in Malaysia. The application of waste or low cost materials in cultivation medium can be highly effective to reduce cost in mushroom cultivation in large scale production. According to Miller & Churchill, (1986), the fermentation medium represented almost 30% of the cost for microbial fermentation.

Four types of low cost materials (molasses, spent yeast, corn steep liquor and brown sugar) were investigated in this study for the cultivation of *G. neojaponicum*. These low cost materials were selected due to their easy availability from sugar cane and brewery industry by-product in Malaysia. The amount of carbon and nitrogen in the raw materials were preliminary screened using AOAC (1980). The spent yeast and corn steep liquor are suitable as nitrogen sources while; brown sugar and molasses are suitable as carbon sources for *G. neojaponicum* cultivation.

The combination of spent yeast-brown sugar medium was the most successful cultivation medium for growth of *G. neojaponicum* in plate agar medium containing 0.06% (v/v) carbon and 6% (v/v) nitrogen, respectively, promoted the most effective mycelial growth of 20.86±0.02 mm/day. According to Hseih & Yang (2004), starch was found as the best carbon source for mycelial growth of *G. neojaponicum*. The Malt extract agar (MEA) and Potato dextrose agar (PDA) were used by Hseih et al., (2005) as cultivation medium of *G. neojaponicum* in petri dish. The optimum glucose for *G. neojaponicum* growth was at 40-80 g/L while, amount of nitrogen was optimum at 0.02%
(v/v) in ammonium nitrate (NH$_4$NO$_3$) for the *G. neojaponicum* mycelial growth (Hseih & Yang, 2004).

### 5.2 Further optimisation of spent yeast and brown sugar concentrations in the medium carried out in shake flask by RSM

Submerged culture is an alternative cultivation technique for consistency and reproducible harvest of bioactive compound and mycelial growth production of medicinal mushroom. The mycelial growth and bioactive compounds of mushroom production can reduce harvesting time 1-2 weeks compared to fruiting body which takes 3-4 months. The growth of *G. neojaponicum* was successfully cultivated using brown sugar-spent yeast medium in submerged culture in 6 days of cultivation time.

In this study, the spent yeast and brown sugar concentrations in the medium were selected based on the production of mycelial dry weight and polysaccharides of *G. neojaponicum*. The optimal medium composition for submerged fermentation of *G. neojaponicum* was predicted by RSM at 37.25 g/L of spent yeast, 91.3 g/L brown sugar with 0.5 g/L of KH$_2$PO$_4$, 0.5 g/L of K$_2$HPO$_4$ and 0.5 g/L MgSO$_4$.7H20 as basal medium. The combination of 37.25 g/L of spent yeast and 91.3 g/L brown sugar in cultivation medium for *G. neojaponicum* was produced 21.18±0.04 g/L mycelial dry weight.

*Ganoderma neojaponicum* was successfully grown in both medium containing commercial medium (glucose-yeast extract) in Experiment 3.2 and spent yeast-brown sugar in Experiment 3.6 as shown in Table 5.1. The optimum formulation for *G. neojaponicum* using glucose-yeast extract medium was at 10% (v/v) carbon and 0.1% (v/v) nitrogen to produce 30.12±0.02 g/L of mycelial dry weight (Experiment 3.2), while, low cost medium consisting of spent yeast-brown sugar medium was optimized at only
5.74% (v/v) carbon and 0.06% (v/v) nitrogen (Experiment 3.7) to produce 25.32 g/L of *G. neojaponicum*. The combination of minimum amount of brown sugar and spent yeast were proven suitable for replacement of expensive synthetic mediums such as glucose, yeast extract, malt extract and potato dextrose for cultivation of *G. neojaponicum*. The highest values of mycelial dry weight, total carbohydrate, and β-glucan production are 25.32 g/L, 115.89% (v/v), and 23.56 (g/L), respectively.

Our study recorded higher production of mycelial dry weight and polysaccharides in *G. neojaponicum* compared to Hseih & Yang (2004) that used 60% (v/v) brewery spent yeast (thin stillage) to produce 7.8 g/L mycelial dry weight and 7.5 g/L polysaccharides of *G. lucidum* in shake flask fermentation. Besides this, the use of 71.4 g/L brown sugar and 2.28 g/L yeast extract with other medium (malt extract, skim milk, sunflower oil and olive oil) produced 18.70 g/L mycelial dry weight and 0.420 g/L of polysaccharide by *G. lucidum* (Yang et al., 2000). Mizuno (1999) also reported the medium formulation consist of 50 g/L yeast extract and 20 g/L glucose with other mineral salt/ trace element produced 5.5 g/L mycelial dry weight and 1.71 g/L polysaccharides by *G. lucidum* in a shake flask culture.
Table 5.1: Summary of mycelial dry weight, total carbohydrate and β-glucan in non-optimized and optimum condition of shake flask and 2-L STR reactor fermentation of *G. neojaponicum*

<table>
<thead>
<tr>
<th>Fermentation Mode</th>
<th>Parameter studied</th>
<th>Parameter optimized</th>
<th>Mycelial dry weight (g/L)</th>
<th>Total carbohydrate (%)</th>
<th>β-glucan (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shake Flask (Experiment 3.2)</td>
<td>1. Glucose concentration: 0-20% (w/v) 2. Yeast extract concentration: 0-1.0% (w/v)</td>
<td>Glucose and yeast extract concentrations</td>
<td>30.12±0.02</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Shake Flask (Experiment 3.3)</td>
<td>1. Brown sugar concentration: 2-10% (w/v) 2. Spent yeast concentration: 0.02-1.0% (w/v)</td>
<td>Brown sugar and spent yeast concentrations</td>
<td>21.18±0.04</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Shake flask (Experiment 3.4)</td>
<td>1. Tween 80: 0-1% (v/v) 2. Vegetable oil: 0-1.2% (v/v)</td>
<td>Effect of surfactant types</td>
<td>12.14±0.8</td>
<td>47.7</td>
<td>12.55</td>
</tr>
<tr>
<td>Shake Flask (Experiment 3.5)</td>
<td>1. Tween 80: 0.1-0.5% (v/v) 2. Temperature: 22-30 °C</td>
<td>Tween 80 and temperature</td>
<td>22.21</td>
<td>ND</td>
<td>20.56</td>
</tr>
<tr>
<td>Bioreactor (Experiment 3.6)</td>
<td>1. Temperature: 22-29 °C 2. Aeration: 0.5-2 vvm</td>
<td>Optimization of physical condition</td>
<td>25.31</td>
<td>73.56</td>
<td>21.34</td>
</tr>
<tr>
<td>Bioreactor (Experiment 3.7)</td>
<td>Profiles for reducing sugar, pH, β-glucan and mycelial dry weight</td>
<td>Production at optimum condition</td>
<td>25.32</td>
<td>115.89</td>
<td>23.56</td>
</tr>
</tbody>
</table>

Note: All medium prepared in the same amount of basal medium (0.5 g/L MgSO₄·7H₂O, 0.5 g/L KH₂PO₄ and 0.5 g/L K₂HPO₄) at constant pH 6 and agitation speed of 160 rpm
In this study, the ratio of carbon to nitrogen (C/N ratio) was studied at the range of 0.1 to 9.84 for combination of spent yeast and brown sugar as growth medium for *G. neojaponicum*. The optimal C/N ratio of 2.9 was predicted in spent yeast-brown sugar medium to produce 20.23±0.02 g/L mycelial dry weight for *G. neojaponicum*. According to Baojing *et al.*, (2012), the medium cultivation of *G. lucidum* at C/N ratio of 5 produced mycelial dry weight and extracellular polysaccharide (EPS) at 7.24 g/L and 1.72 g/L, respectively. Besides that, Babitskatya *et al.*, (2005) suggested C/N ratio of 25 was suitable for *G. lucidum* cultivation medium. Thus, it can be concluded that the different growth medium sources and *Ganoderma* species were influenced by the suitable ratio of C/N in submerged cultivation medium (Miller, 2000).

5.3 Effect of surfactant for the growth of *G. neojaponicum* in shake flask

In this study, a selection of surfactant was made between Tween 80 and vegetable oil for the growth of *G. neojaponicum* in shake flask. The study of surfactant was conducted to disperse mycelial growth in cultivation medium; consequently this increases homogeneity and facilitates sampling of mycelium or broth of *G. neojaponicum*. The addition of Tween 80 in the cultivation medium increased the total carbohydrate and β-glucan content as opposed to the addition of vegetable oil in the medium for *G. neojaponicum*. The addition of vegetable oil showed significant amounts of mycelial dry weight compared to the medium containing Tween 80. Medium containing vegetable oil produced high viscosity due to the formation of emulsion.

The polysaccharides extracted from the medium containing vegetable oil cannot be considered as pure carbohydrates due to the presence of proteins and fats in the medium precipitate. Steve & Yolanda (2006) proposed the use of hexane to remove the fat
content for extraction of polysaccharides. Therefore, the yield of mycelial dry weight was higher for medium containing vegetable oil compared with that the medium with Tween 80. 0.5% (v/v) of Tween 80 was applied for the production of polysaccharides in *Trametes sp* (Bolla *et al.*, 2011).

The comparison between the addition of vegetable oil and Tween 80 to the medium of *G. neojaponicum* showed higher amounts of β-glucan and polysaccharides content in the medium containing Tween 80 compared with that containing vegetable oil. According to Cassiano *et al.*, (2007), the addition of vegetable oil and fatty acid aided mycelial dispersion, mycelial dry weight and polysaccharides production in *Botryosphaeria rhodina* growth. Furthermore, the medium containing vegetable oil was cloudy. This might be due to the formation of emulsion at higher concentrations of vegetable oil. This characteristic has resulted in vegetable oil not being suitable as an additive for mycelial production from *G. neojaponicum*. Meanwhile, the addition of Tween 80, interacted hyphae with each other resulting in reduction of pellet formations. The hyphae were either joined to one another by adhesive forces determined by their surface properties, or repulsed and formed free mycelia. This effect may be due to depression, induction or stimulation of secretion of the mycelial cell (Yang *et al.*, 2000).

It can be concluded that the suitable surfactant to be added to growth medium of *G. neojaponicum* was Tween 80 at 0.46% (v/v). The optimisation of Tween 80 and temperature was predicted by RSM at 0.46% (v/v) of Tween 80 at 26 °C and these conditions yielded mycelial dry weight of 22.21 g/L and β-glucan content of 20.56% (w/v).

It has been suggested that the effect of surfactants is on the permeability of cell membrane (Casssiano *et al.*, 2007). According to Cassiano *et al.*, (2007), several features
of the cell surface, which include surface antigens, hormone binding, cell recognition, adhesiveness and adsorption, play a role in the production of polysaccharides. The yield of intracellular and extracellular polysaccharides increased the mycelial growth, which correlated with the decrease of pellet size in the cultivation medium.

5.4 Optimum physical parameters for IPS and EPS production in shake flask and 2-L stirred tank reactor (STR) in spent yeast-brown sugar medium

The physical parameters such as pH, temperature, aeration and agitation were important factors to obtain optimum mycelial growth and bioactive substance in submerged cultivation. In this study, the optimum temperature for *G. neojaponicum* was successfully predicted by RSM at 26 °C in shake flask and 26.71 °C in 2-L STR reactor to produce mycelial dry weight of 22.21 g/L and 26.57 g/L, respectively. This finding is in agreement with Hseih (2004) who reported that the optimum temperature for the mycelial growth of *Ganoderma* spp. were at 24–28 °C. According to Wagner (2003), mycelial dry weight of *G. lucidum* produced at amount of 25 kg at temperature of 26 °C in 2-L of STR bioreactor. It is the simplest mode of operation in bioreactor systems. The use of bioreactor would be able to control the cultivation parameters easily and simultaneously. These parameters include medium composition, inoculation density, pH, temperature, aeration and agitation (Tang & Zhong, 2002b and Fang & Zhong, 2002b).

Temperature and aeration rates are the most crucial parameters of STR reactor employed for scale-up process (Felse & Panda, 2000). In this study, the use of batch
fermentation was carried out using a 2-L STR system (Biostat® A plus, B. Braun International, Germany Biostat A plus) to study the optimum conditions of temperature and aeration rate for G. neojaponicum polysaccharide production. The optimum conditions for polysaccharide production by G. neojaponicum was found to be an aeration rate of 1.339 vvm and temperature of 26.71 °C with a mycelial growth (26.57 g/L), total carbohydrate (75.56% w/v) and β-glucan content (21.57% w/v) in 2-L STR reactor.

Yang & Liau (1998b) noted that 22 g/L mycelial dry weight and 1.25 g/L extracellular polysaccharide of G. lucidum was produced at a temperature of 30 °C in bioreactor. Tang & Zhong (2002b) reported that 21.9 g/L mycelial dry weight and 0.87 g/L extracellular polysaccharides of G. lucidum at an aeration rate of 0.25-0.5 vvm in an agitated 2-L bioreactor. It is proven that different species of Ganoderma and different medium composition as well as different physical conditions producing different amounts of mycelial dry weight and polysaccharides in submerged fermentation (Fang & Zhong, 2002b).

5.5 Growth profiles of G. neojaponicum at optimum medium composition and physical conditions in a 2-L bioreactor

A trend of maximum yield of mycelial dry weight, β-glucan content and total polysaccharide were observed on day 4 of G. neojaponicum cultivation in submerged fermentation in 2-L STR reactor. The highest value was observed on day 4 based on mycelial dry weight, β-glucan and total polysaccharides, 25.32 g/L, 115.89% (w/v) and 23.56% (w/v) respectively. It can be concluded that the production of polysaccharides and β-glucan was associated with cell growth of G. neojaponicum. As comparison, the
mycelial growth of *Hericium erinaceus* was obtained at 14.021 g/L on 9th day of culture in a 5 L bioreactor, while, the polysaccharide produced by *H. erinaceus* was found to be 0.994 g/L at the 7th day of culture (Malinowska *et al.*, 2009). This showed that higher amount of mycelial dry weight and polycscharide content was produced in *G. neojaponicum* in shorter cultivation time compared to *H. erinaceus*.

The profiling of *G. neojaponicum* showed a declining pattern in the pH 6 to 4.2, and this might be caused by the production of organic acid from carbon sources during consumption and cell growth (Shih, 2006). Profiling of *G. neojaponicum* was revealed that higher amount of total carbohydrate content was obtained for IPS and EPS as compared to dried mycelium and broth. Meanwhile, the amount of IPS and EPS had low mycelia weight as compared to dried mycelium and dried broth of *G. neojaponicum*. This might be caused by the losses of nutrients or components in dried mycelium and broth during partial purification and extraction procedures.

The usage of ethanol is to eliminate the smaller molecules such as oligosaccharides or monosaccharides by interfering with the quantification of the polysaccharides. In this study, precipitation using ethanol had successfully developed a method of using concentration of 1:1 (v/v) of broth: 95 – 96% (v/v). This method was can reduce cost and time in polysaccharides production as compared to method suggested by Wagner (2003) at concentrations ratio of 1:4 (v/v) of broth: 95 – 96% (v/v). Table 5.1 shows the summary of mycelial dry weight, total carbohydrates and β-glucan in non-optimized and optimum condition of shake flask and 2-L STR reactor fermentation of *G. neojaponicum*. 
As a conclusion, the mycelial dry weight and β-glucan content of *G. neojaponicum* in submerged cultivation had increased up to 13.42% (w/v) and 12% (w/v), respectively by using bioreactor system as compared to shake flask fermentation system. The increment of mycelial dry weight might be due to the efficiency in controlling simultaneously among operational factors in bioreactor rather than in shake flasks. The use of bioreactor had increased mycelial growth up to 20% compared to shake flask culture.

### 5.6 Immunomodulatory properties of polysaccharides from *G. neojaponicum*

In the present study, dried mycelium, dried broth, intracellular polysaccharides and polysaccharides extracellular of *G. neojaponicum* extracts were tested further for *in-vitro* immune response activity. This was done by studying the activities of proliferation, phagocytosis and inhibiting NF-κB on macrophages (RAW264.7) and human colon cancer cell. The defense mechanism of macrophages against pathogens includes secreting cytokines, such as the tumor necrosis factor-α (TNF-α), and inflammation mediators, (NF-kB). The highest proliferation ability of macrophages exhibited at a concentration of 1000 μg/mL by IPS of *G. neojaponicum* with an increment of 226% compared with negative control samples.

It can be concluded that no significant cytotoxic effects were observed in macrophages treated with *G. neojaponicum* extract as compared with the negative control (P<0.05). All *G. neojaponicum* extracts were positively stimulated to RAW264.7 of phagocytosis assay. The mycelium of *G. lucidum* at 50 and 100 mg/kg inhibited the growth of RAW264.7 of S-180 in Balb/c mice with inhibitory rates of 37.8% - 78.1% (Hu & Lin, 1999). It was interesting to note that the highest level of phagocytosis activity in this study exhibited at 460% using 1000 μg/mL of IPS from *G. neojaponicum*. To
support the data, the range of 2.5-25 g/mL of *G. lucidum* stimulated RAW264.7 cells proliferation in a dose-dependent manner and with low associated cytotoxic effect (Guan, 2011). At the range of concentrations tested (0-100 g/mL), the 25 g/mL concentration produced a maximum 205.8% of the stimulation effect on macrophages (Guan, 2011).

Similar stimulating effect on macrophages was also observed in a study by Lee (2008) using polysaccharides from *Lentines edodes*. This result implies that *G. lucidum* triggers macrophage secretion of inflammatory cytokines, which is also promoted by the existence of lipopolysaccharide. This is possible by binding to dectin-1 and toll-like TLR-2/6 receptors, which activate NF-kappa B and trigger secretion of cytokines (Batbayar *et al.*, 2011). However, nitric oxide production is not enhanced by *G. lucidum* mycelium in RAW264.7 cells (Kuo *et al.*, 2006). Ryu (2010) had reported the inhibition of human colon cancer cell line (HT29) using polysaccharides extract from *Ostaracys japonicas* at a concentration of 2 mg/mL decreased dramatically to 28.43% as compared to the control.

In comparison, the inhibition of Nf-kB was reported on human breast cancer cell by Muller (2006) and prostate cancer cell (Sliva, 2003) of the extract of *G. lucidum*. Taken together, the antitumor activity of *Ganoderma* spp. are caused by the inhibition or activation of specific mechanisms and pathways. The reduction of proliferation of cancer cells might be due to the down-regulation of estrogen receptor and NF-κB signalling. Some of the effects on cancer cells are indirect and are caused by stimulation of the immune system by polysaccharides and the release of cytokines from activated macrophages and T lymphocytes. Other effects of *Ganoderma* spp. are targeted directly to the cancer cells by modulating their intracellular signaling and can affect the behavior of cancer cells.
Based on the findings on RAW264.7 cells, it could be deduced that *G. neojaponicum* has positive immune response. This is an indication that this wild Malaysian strain can be potentially used as immunomodulating agent. Further investigations on cytokines need to be conducted to confirm that this wild Malaysian strain of *G. neojaponicum* has immunomodulating properties. The inhibition trend of human colon cancer cell lines was observed of IPS and EPS of *G. neojaponicum*. Lowest inhibition of proliferation of IPS of *G. neojaponicum* was observed to be at a reduction of 28.8% as compared with negative control.

These findings suggest that the most active *G. neojaponicum* extract with the possession of anti-cancer activity of human colon cancer cell (HT29) is IPS. For comparison, the anti-cancer effect of *G. neojaponicum* was in agreement with the findings of *G. lucidum* reported by Yuen & Gohel, 2005. According to Wasser & Weis (1999), the polysaccharides do not respond directly to cytotoxic effects on tumour cells. Instead, they strengthen the host mediated immunomodulatory response by stimulating the immune system in curing cancer. The results related to inhibition of cancer cell proliferation were also supported by increment level of NF-κB activity on HT29. The highest inhibition rate (32.88%) of NF-κB activity of HT29 was determined of IPS as compared with the negative control.

Similar results were reported in a study by Anne et al., (2011), who showed that lipopolysaccharides inhibited the NF-κB activity. Inhibitory effects on proliferation on human breast cancer cell were also displayed through the use of *G. lucidum* extract. The reduction of proliferation of cancer cells might be due to the down-regulation of NF-κB signalling and estrogen receptor (Muller, 2006). The trend of dose dependent results can be seen clearly in proliferation on RAW264.7 as well as phagocytotic ability.
Meanwhile, in the present study, the concentrations of the cell line of HT29 were not dose dependent as seen in the NF-κB assay results. The Nf-kB assay had significant inhibitory activity of HT29 of mycelium and broth of *G. neojaponicum*. All *G. neojaponicum* extracts were further tested on human immune response *in-vitro* and showed immunostimulating effects by enhancement of proliferation and phagocytotic ability of RAW264.7 macrophages as well as decreasing proliferation of human colon cancer cell (HT29) in a dose dependent manner (*P*<0.05). Meanwhile, the IPS of *G. neojaponicum* showed the highest potential to stimulate the immune function and to act as anti-cancer agent against human colon cancer cells. It showed promising results on immune response of IPS and EPS compared with dried mycelium and broth of *G. neojaponicum*. Besides that, dried mycelium was the lowest cytotoxic effect on human colon cancer cell and also had positive immunomodulatory effect on macrophages cell *G. neojaponicum*.

5.7 Evaluation of *in-vivo* toxicity on dried mycelium of *G. neojaponicum*

The dried mycelium of *G. neojaponicum* was evaluated for its safety by analysis of *in-vivo* toxicology study. This is a method to ascertain the safety for consumption of polysaccharides extract of *G. neojaponicum* either as a drug or functional food. In this study, the dried mycelium of *G. neojaponicum* was evaluated for its safety and efficacy using a single dose testing for acute oral toxicity. This method is one of the most widely used in toxicology tests as according to the Organization of Economic Cooperation and Development (OECD) Guidelines (OECD, 1987). The adverse effects occurring within a short period of time after oral administration of a single dose of the substance was determined according to Chan & Hayes (1994). Acute toxicity study conducted revealed
that the administration of polysaccharide extract (up to a dose of 2000 mg/kg) of *G. neojaponicum* did not cause any significant changes in behaviour of the animals. No death was observed with doses up to 2000 mg/kg body weight. The rats were physically active. These effects were observed during the experimental period (14 days).

The results showed that in single dose, the polysaccharide extract had no adverse effect. This indicates that the medium lethal dose (LD50) could be greater than 2000 mg/kg body weight in rats. No toxic symptoms were observed for doses up to 2 g/kg body weight. All animals behaved normally. No neurological or behavioural effects were noted. No mortality was found throughout the 14-day study. Similar findings were reported in the aqueous extract of *G. lucidum* that was administered to mice (5 g/kg during 30 days); no differences in physical weight, organ weight or hematological parameters were observed (Wasser, 2005). In comparison with literature, the experiments of oral administration of hot water extract of *G. lucidum* (5,000 mg/kg) to mice for 30 days showed that there were no changes in body weight, haematological features and organ weight (Kim, 1986). Furthermore, an alcoholic extract of *G. lucidum* was given to young rats (1.2 and 12 g/kg daily during 30 days) by Wasser (2005) also showed no signs of toxicity in major organs as well as growth or development of the rats.

In this study, haematology test carried out on dried mycelium of *G. neojaponicum* including full blood count, renal function test, liver function test and glucose metabolism test did not show any significant abnormalities as compared to the control (*P*<0.05). In this study, no significant effect on total protein and albumin level were observed (*P*<0.05). This indicated that protein catabolism was not affected. Similar observation was noted with no changes in the blood test conducted after administration of *G. lucidum* (McKenna, 2002).
Therefore, in this study, no adverse effect level for polysaccharide extract was observed in the results. It can be concluded that doses of 2000 mg/kg body weight/day of dried mycelium of *G. neojaponicum* is safe to be used as functional food or medicinal application.
6.1 Conclusion

This study has demonstrated that mycelium of *G. neojaponicum* can be cultivated in submerged fermentation on low cost medium. A mixture of spent brewery yeast and brown sugar was the best combination among four sources (molasses, corn steep liquor, spent yeast and brown sugar) usable as cultivation medium. Optimal growth conditions as predicted by the Design Expert Software via RSM (Minitab Ver 16) were achieved (28.7 g/L of spent yeast, 50.6 g/L brown sugar, 0.5 g/L of KH₂PO₄, 0.5 g/L of K₂HPO₄ and 0.5 g/L MgSO₄.7H₂O). The C/N ratio of 2.9 was predicted as optimal in the use of spent yeast and brown sugar to produce 20.23±0.02 g/L mycelial dry weight for *G. neojaponicum*. The physical parameters were also predicted by RSM and were found optimal at temperature of 26.72 °C, aeration of 1.33 vvm, constant pH 6 and agitation of 160 rpm. The addition of selected surfactant (0.465 (v/v) Tween 80) was optimised the β-glucan production at 47.7% (w/v) in shake flask, and 26.39% (w/v) in 2-L-stirred tank reactor.

By fermenting *G. neojaponicum* according to the predicted growth medium requirements and physical parameters, mycelial dry weight obtained was at 25.32 g/L. The cultivation period for *G. neojaponicum* was discovered to be at optimal conditions on day 4 using 2-L STR. The amount of β-glucan and total carbohydrate content dried mycelium of *G. neojaponicum* were obtained on day 4 of cultivation time at 23.56±0.01% (w/v) and 115.89±2.78 g/L, respectively. *Ganoderma neojaponicum* showed positive immunostimulating effects by human immune response *in-vitro* test.
They enhanced the macrophages (RAW264.7) proliferation, increased phagocytosis activity and decreased human colon cancer cell (HT29) proliferation in dose dependent manner ($P<0.05$).

The findings of this study also indicate that dried mycelium, dried broth and IPS and EPS of *G. neojaponicum* have the potential to be used as immunomodulating agents to stimulate the innate immune system for fighting infectious diseases. Dried mycelium of *G. neojaponicum* had lowest cytotoxic effect on human colon cancer cell and also had positive immunomodulatory effect on macrophages *G. neojaponicum*. Thus, the dried mycelium at doses up to 2000 mg/kg body weight/day for 14 days did not cause either mortality or have any toxic effects on the rats. No adverse effects were observed for dried mycelium of *G. neojaponicum* based on the experimental results. Hence, dosage of 2000 mg/kg body weight/day has established its safety in regard to potential immunomodulatory properties.

Further research may focus on additional purification and identification of the specific polysaccharide(s) involved in the immunomodulatory action of these fungi to better understand its immunomodulatory properties for the benefits pharmaceutical industries and medicinal products for the public.

**6.2 Recommendation**

In this study, the production of polysaccharides and β-glucan have been successfully optimised using shake flask and 2-L stirred tank reactor. This application will also benefit the nutraceutical and pharmaceutical industry. This production on a large scale has to be validated further with regard to the application of polysaccharides of *G. neojaponicum* as immunomodulating agent. It is vital to purify the polysaccharides
further, focusing on water soluble polysaccharides with high molecular weight that would exert higher effect of immunomodulatory property.

However, limited studies have reported on the effects of polysaccharides from *G. neojaponicum* on immune system using *in-vivo* and *in-vitro* trials. Furthermore, lack of translational approach of β-glucan to animal studies or clinical trials need to be identified to apply knowledge of receptor and signal pathways. Therefore, it is of great interest in regard to the discovery of polysaccharides by *G. neojaponicum* which can either modulate positively or negatively the biologic response of immune system. The exact immunological actions and signalling pathway induced by β-glucan are still unclear and need to be defined further.