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Aspects of glucosamine production using microorganisms

[1]Sitanggang, A. B., [2]Sophia, L. and [3]*Wu, H. S.

[1]*Department of Food Science and Technology, Bogor Agricultural University, Bogor 16680, Indonesia; Southeast Asian Food and Agricultural Science and Technology (SEAFAST) Center, Bogor Agricultural University, Indonesia*
[2]*Department of Chemical and Biomolecular Engineering, University of California, Los Angeles, USA*
[3]*Department of Chemical Engineering and Materials Science, Yuan Ze University, Taoyuan, 32003, Taiwan*

Abstract: Glucosamine (GlcN) is an amino monosaccharide that has physiological importance to the body. The amino sugar has a potential to prevent joint structure change in patients with osteoarthritis. This bio-monomer is one of the building blocks of chitin and chitosan that are commonly present in crustacean shell waste and fungal cell walls. Because of the economical, environmental, and physiological disadvantages of using crustacean waste, the alternative source of GlcN production, namely microorganisms is being looked at. This paper presents the production of GlcN using microorganisms and focuses on the different fermentation systems for microorganism cultivation, the microorganisms commonly used and the characteristics of the produced chitinous material or GlcN, and the methods for GlcN isolation and quantification.

Keywords: Glucosamine, fermentation, cell wall, separation, quantification

Introduction

Chitin is a biopolymer composed of β-(1-4)-linked *N*-acetyl-D-glucosamine (*N*-GlcNAc) monomers. The polysaccharide is the second most abundant biopolymer following cellulose on the earth. Chitin is considered to be synthesized in nature by an enzyme which is capable of affecting a glycosyl transfer of the *N*-GlcNAc from uridinediphosphate-*N*-acetyl-D-glucosamine to a preformed chitodextrin acceptor, forming the polysaccharide. In addition to being found in the exoskeleton of crustaceans and insects, chitin is also found in the cell walls of fungi. The deacetylation of chitin results in chitosan, a linear polysaccharide of β-(1-4)-linked-D-glucosamine (GlcN) units. Chitosan is utilized in many different industrial sectors such as agriculture, industry, and medicine (Dodane and Vilivalam, 1998). The biopolymer has been cited to contribute to cosmetics, drug delivery, feed additives, semi-permeable membranes, and pharmaceutics (Li *et al*., 1992).

The hydrolysis of chitosan results in monomers of GlcN, an amino monosaccharide with physiological importance to the human body. GlcN has been reported to have a potential to prevent joint structure change in patients with osteoarthritis and has currently been incorporated into supplements (Noack *et al*., 1994; Reginster *et al*., 2001; Richy *et al*., 2003). The amino sugar is naturally synthesized in the body and because of its extensive applications there are emerging commercial interests in the commercialized production of GlcN.

As previously stated, natural sources of GlcN are found within the shell waste of crustaceans such as shrimp, crab, and lobster. The GlcN is extracted via enzymatic or acid hydrolysis of chitosan (Ferrer *et al*., 1996; Sashiwa *et al*., 2002). In addition to this, GlcN can be directly obtained through the hydrochlorination of chitin (Rupley 1964; Hsieh *et al*., 2007; Chang *et al*., 2011). This source of GlcN (crustacean derived GlcN) has disadvantages due to shellfish's seasonal dependence, its role as a potential allergen, as well as its unpredictable size and composition (Rane and Hoover, 1993).

In regards to these limitations, the production of GlcN from microbial systems has been gaining attention in recent years. The predominant sources of alternate GlcN production are bacteria and fungi. Bacterial strain *Escherichia coli* have been engineerined to directly synthesize GlcN and method of GlcN production using *E.coli* takes place under traditional submerged method (Deng *et al*., 2005). Common fungi used to produce GlcN include fungal strains of the *Aspergillus, Rhizopus,* and *Mucor*. The cultivation technique of filamentous fungi can vary. Up to two decades ago, fermentation processes had often taken place under submerged conditions, however there has been increasing attention directed

*Corresponding author.
Email:*cehswu@saturn.yzu.edu.tw*
Tel: (+886) 3 4638800 2564; Fax: (+886) 3 4559373

towards understanding solid-state fermentation (Crestini *et al*., 1996). GlcN production using both methods will be discussed.

Following microbial cultivation, isolation and purification of GlcN and *N*-GlcNAc are necessary. Because the mechanism of GlcN production differs, the extraction process is also different. GlcN is directly secreted by bacteria into the fermentation medium while the extraction of chitosan and chitin from the fungal mycelia and subsequent decomposition is required for the fungal sources. Common methods for GlcN quantification include colorimetric assays (Wagner, 1979), radioactivity (Setnikar *et al*., 1984), gas chromatography (Shinohara, 1981), capillary electrophoresis (Guttman, 1997), and high liquid performance liquid chromatography (HPLC) (Shao *et al*., 2004). The amount of GlcN derived from the microbial samples varies between cultivation conditions. As the production of GlcN using microbial systems have gained attention, hence, this review article will attempt to discuss the GlcN production from microbial sources and evaluate the potential of these methods, because research papers that have been reported only focus on the chitin or chitosan production and their characteristics.

Production of GlcN using microorganisms

GlcN can be obtained from the direct breakdown of chitin substrates from shellfish waste, but in addition to directly utilizing chitin, efforts have been made to generate GlcN through fermentation processes (Rattanakit *et al*., 2003; Kuk *et al*., 2005). The microorganisms discussed in this paper utilize different methods of GlcN production. Bacteria, specifically *E.coli* has been genetically engineered for enhanced production of GlcN through the over-production of certain enzymes and the inhibition of transport processes. Production of fungal GlcN mainly utilizes Zygomycotina and Ascomycotyna subdivision fungi. Production of GlcN from fungal fermentation can either be the main motivation or it can also be run in parallel with production of other substances such as organic acids and enzymes. Compared to bacteria, using fungi for the production of GlcN will require the further conversion of the biomass content obtained from the fungal culture into GlcN monomers. This process can be expensive and the chitosan or chitin content in the fungi can vary depending on the cultivation technique. This conversion will be discussed later in the section concerning extraction and isolation of GlcN.

Bacterial fermentation

Previous characterization regarding the synthesis and metabolism of GlcN via the bacterium *Escherichia coli* has been utilized to metabolically engineer a bacterium species that is able to express high levels of GlcN. A method devised by Deng *et al*. (2005) involved the over-expression of the GlcN synthase (GlmS) gene and the inactivation of genes involved in GlcN transport and catabolism in *E. coli* ATCC 25947. Further optimization of cultivation conditions such as the media's pH and cell confluence led to *N*-GlcNAc production of over 110 g l^{-1} (Deng *et al*., 2005). However, Deng *et al.* (2005) reported that GlcN was degraded under more acidic condition during fermentation process. The pH was needed to be shifted from 6.5-7.0 into 5.0 during fermentation. Hence, Deng *et al*. (2005) tried another alternative by producing *N*-GlcNAc during fermentation since it was stabile in the pH range of 6.5-7.0 instead of GlcN. Eventually, *N*-GlcNAc resulted from fermentation process would be hydrolyzed using mild acid to obtain GlcN.

In addition to modification of the GlmS gene in the bacterium *E. coli*, further work has been done to enhance GlcN or *N*-GlcNAc production through the over-expression of the GlcN-6-phosphate deaminase (*nag*B) gene (Deng *et al*., 2006). Though it was shown that over-expression of *nag*B alone only increased cell growth, the simultaneous coupling of over-expressed *nag*B in *E. coli* with GlcN-6-phosphate acetyltransferases (GNA1) from *Saccharomyces cerevisiae* resulted in *N*-GlcNAc production that is comparable to bacterial strains over-expressing GlmS and GNA1.

Fungal fermentation

GlcN production from microorganisms is not limited to bacteria, but also fungal sources. There are four subdivisions of fungi under the Amastogomycota division: (1) Zygomycotina, (2) Ascomycotina, (3) Basidiomycotina, and (4) Deuteromycotina, but only the subdivision of Zygomycota and Ascomycota will be discussed in this article because the absence or lack of chitin and chitosan in the cell wall structures of the Basidiomycotina and Deuteromycotina classes (Schaechter and Lederberg, 2004). For further comparison, tabulation of several general cell wall components in fungi is shown in Table 1.

Fungal cell walls are comprised of glycoproteins (mannoproteins) and polysaccharides, mainly glucan (β-(1,6)-glucan and β-(1,3)-glucan) and chitin. The glycoproteins present in the cell wall are extensively modified with both N- and O-linked carbohydrates (Bowman and Free, 2006). Additional minor cell wall

Table 1. Production of GlcN using different fungal fermentation systems with several cell wall components

Microbial	Cell-wall component	Medium	System	Time (h)	Biomass (g l⁻¹)	GlcN Conc. (g l⁻¹)	content (mg/gdw cells or *gds)	Reference
Rhizopus oligosporus NRRL 2710	Chitin-Chitosan	SDB[a]	SmF	72	-	-	0.11	Sparringa and Owens, 1999
Aspergillus sp.	Chitin-Glucan	WBS[b]	SSF	120	-	-	24.16	Carter *et al*., 2004
Rhizopus oligosporus NRRL 2710	Chitin-Chitosan	SDA[c]	SSF	24	-	-	0.11	Sparringa and Owens,1999
Monascus pilosus	-	RSA[d]	SmF	120	-	0.26	-	Yu *et al*., 2005
Aspergillus sp BCRC 31742	Chitin-Glucan	GP[e]	SmF	168	18.50	3.43	185	Hsieh *et al*., 2007
Monascus pilosus BCRC31527	-	RSA	SmF	168	17.70	0.72	40.40	Hsieh *et al*., 2007
Rhizopus oligosporus BCRC 31996	Chitin-Chitosan	SDB	SmF	168	2.09	0.40	188	Hsieh *et al*., 2007
Rhizopus oryzae ATCC 20344	Chitin-Chitosan	Dairy manure	SmF		-	-	160	Liao *et al*., 2008
Aspergillus sp BCRC 31742	Chitin-Glucan	WF[f]	SmF	168	21.56	5.48	250	Chang *et al*., 2011
Pleurotus ostreatus	-	GY-C[g]	SSF	288	-	-	40.21*	Mishra and Kumar, 2007
Aspergillus sp BCRC 31742	Chitin-Glucan	WFM[h]	SmF	120	28.68	7.48	260	Sitanggang *et al*., 2010

[a]medium: sabouraud dextrose broth, [b]medium: wheat bran and soy bean meal, [c]medium: sabouraud dextrose agar, [d]medium: rice bran + sucrose + ammonium chloride, [e]medium: glucose - peptone, [f]medium: white fine granulated sugar – peptone, [g]medium: groundnut shell and yeast with supplementation of copper (1.0 mM), [h]medium: white fine granulated sugar – peptone with addition of methanol (1.5% v/v)

components are varies amongst species of fungi and also depend on the cultivation systems of fungi. Since the cell wall of the fungi (especially subdivision of Zygomycotina and Ascomycotina) contains both of chitin and chitosan, so it can be converted into GlcN units through enzymatic or acid hydrolysis (Ferrer *et al*., 1996; Sashiwa *et al*., 2002). The bioconversion sometimes results either in GlcN and *N*-GlcNAc. It can be shown simply in Figure 1. The chitin and chitosan content within fungal species can vary and is also dependent on fungal cultivation as well as the fermentation system. Recently, there has also been research in the co-production of biochemicals such as fumaric acid, lactic acid, citric acid, oxalic acid and alcohol in parallel with chitin from fungal sources (Sumbali, 2005). Generally, the production of GlcN in fungal systems associates with the biomass concentration of the fungi as well as chitin and chitosan are found in the cell walls. Through the fungal fermentation, production of GlcN is usually represented by the production of either chitin and chitosan resulted from fermentation process.
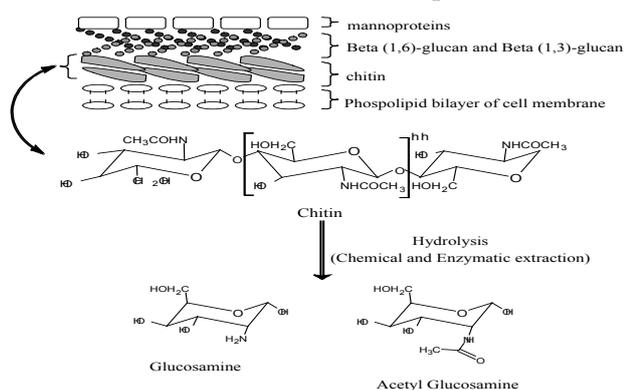
Figure 1. Bioconversion of chitin using chemical and enzymatic extraction

The two subdivisions of Zygomycotina and Ascomycotina can further be divided into the fungal classes more commonly studied for biomass production associates with GlcN production: Zygomycetes and Ascomycetes. The fungi found within these classes are used because of their high chitin and chitosan content. The fungal cell walls of the Zygomycete class have been generalized to be predominately composed of chitin and chitosan and subsequently have been extensively analysed for their chitosan production (Bartnicki-Garcia, 1968).

The fungal species *Mucor rouxii* of the Zygomycetes class has been heavily studied because of its high chitosan content as well as the ease at which it is cultivated (Bartnicki-Garcia and Nickerson, 1962). Many research groups have explored the variability at which the *M. rouxii* species is able to produce chitosan. For example, the effects of different media such as molasses salt medium (MSM), potato dextrose broth (PDB), and yeast extract peptone glucose (YPG) on the chitosan production under submerged conditions of *M. Rouxii*. It resulted in no significant difference in the concentration of chitosan obtained but differing average molecular weights (Chatterjee *et al*., 2005).

According to the results of Katz and Rosenberger (1971), the inhibition of protein synthesis by cycloheximide in *Aspergillus nidulans* could lead to significant changes in the deposition of GlcN in cell walls. Through this finding, White *et al*. (1979) tried to increase the GlcN content in the cell wall of *M. Rouxii* by adding the cycloheximide into the fermentation medium of *M. Rouxii*. Addition of cycloheximide (80 μg/mL), and incubation time of

48 h could only increase the GlcN content slightly, from 37 (μg GlcN/mg of cell wall) to 42 (μg GlcN/mg of cell wall). However, addition of cycloheximide resulted in low concentration of biomass from 3 gdw/L to 7 gdw/L.

Other fungal species of the Zygomycetes class commonly studied for the production of chitosan via solid fermentation, such as *Rhizopus oryzae, Mucor rouxii, Rhizopus pusillus, Gongronella butleri, Absidia coerulea*, etc. Sometimes, fermentation of chitosan-producing fungi characterizes chitosan in terms of GlcN. Wang *et al.* (2008) studied about physical properties of fungal chitosan and characterized three fungal chitosan in terms of GlcN, molecular weight, degree of deacetylation. Wang *et al*. (2008) reported that there was no significant difference in the GlcN yield from the three species of chitosan-producing fungi quantitatively, such as *A. coerulea*, *M. rouxii*, and *R. oryzae* incubated in the same time. The difference was found only in the physical property of molecular weight. Another comparison of chitosan production of fungi from the Zygomycetes class was done by Tan *et al*. (1996) which the measurements were performed at the end of the exponential growth phase of each fungus. The resulting biomass and chitosan content between species and genera varied due to the different extraction times. This further underscores the idea that different strains of fungi will result in chitosan with different GlcN yields and different physical properties.

The other class of fungi also examined for chitosan and or GlcN content is the Ascomycete class. The cell wall composition is characterized to be made up of chitin and glucan (Schaechter and Lederberg, 2004). However, it is necessary to take note that the make-up of each fungal species is not constant and is dependent on environmental conditions (Bartnicki-Garcia, 1968).

Though there are studies solely concerned with enhancing the fungal biomass concentration as source of GlcN, the focus of many experiments concerns the production of other biochemical materials with the fungal biomass cultivation being a side product. For example, Liao *et al.* (2008) explored the co-production of fumaric acid and chitin using fungal species *R. oryzae* ATC 20344 because of the fungi's capability for efficient fumaric acid production. *R. oryzae* NRRL 395 was also shown to be able to produce lactic acid alongside chitin. Jangbua *et al*. (2009) looked at the production of gamma linolenic acid (GLA) and biomass by *M. rouxii* grown on different substrates and inoculated at different spore concentrations.

The *Aspergillus* fungus is widely employed for enzyme and organic acid production and the chitosan content of the cell wall is also looked at for GlcN extraction (Vandenberghe *et al.*, 2000; Jacobs *et al.*, 2008; Sitanggang *et al.*, 2009; Sitanggang *et al*., 2010). For example, *Aspergillus niger* demonstrated the ability to maximize gluconic acid production at the same concentration of maximal cell growth rate. This process was carried out in both of submerged fermentation and solid-state fermentation (Singh *et al*., 2003). Optimization of enzyme naringinase production by *A. niger* MTCC 1344 is examined and it was found that an increase in carbon source results in a decreased naringinase level but an increase in cell mass. This inverse relationship highlights the necessity for an optimization process to enhance enzyme activity, yield, and biomass concentration (Puri *et al*., 2003).

Hsieh *et al*. (2007) reported about kinetics of GlcN production using several fungi, such as *Rhizopus oligosorus* BCRC 31996, *Monascus pilosus* BCRC31527, and *Aspergillus sp.* BCRC31742. They reported that the best fungus from those to produce GlcN was *Aspergillus sp.* BCRC31742 which the GlcN concentration was up to 3.43 g l^{-1}. This fungi was cultivated in submerged fermentation containing glucose-peptone (GP) medium. The generation culture of fungi and the pH were found to play an important role in enhancing the yield of glucosamine. The specific growth rate of the microorganism and the biomass, content, yield, and productivity of glucosamine were calculated as well by Hsieh *et al*. (2007). Using the same strain (*Aspergillus sp.* BCRC31742), Sitanggang *et al*., (2010) improved the production of GlcN to up to 7.48 g/L with addition of methanol (1.5% v/v) as the stimulating factor. Methanol markedly depressed the synthesis of cell protein in early stages of the cultivation. Therefore, deposition of glycoprotein in cell wall would decrease due to depression by alcohol, especially methanol (Moyer, 1953; Sitanggang *et al*., 2010).

Fermentation systems of fungal GlcN: SSF versus SmF

The fermentation systems of microorganisms can be generalized into two types: submerged liquid fermentation (SLF) or can also be called submerged fermentation (SmF) and solid-state fermentation (SSF). The predominant difference between the two systems is the amount of free liquid content consisted in the fermentation medium (Pandey, 1992). SmF involves the cultivation of microorganisms in a broth that can contain up to a water content of 95% (wt) since it calls for the immersion of the culture

in a fermentation broths, whereas SSF denotes fermentation on moist solid substrates in the absence or near absence of an aqueous phase (Mitchell *et al.*, 2000). SmF is currently a well-standardized commercial process as a result of Western antibiotic production in the 1940s. On the other hand, SSF is a more widespread practice in Eastern countries due to its roots in the food industry (Hölker and Lenz, 2005). There has been more focus in recent years to normalize SSF processes because of the potential for enzymes, antibiotics, organic acids and secondary metabolites productions.

Comparison between fungal biomass production via the SmF and SSF is the focus of many studies. A study of the growth kinetics of fungal species *Aspergillus niger* as a result of varied initial glucose concentration has reported that SSF results in an increased growth of filamentous fungi due to lack of catabolite repression (Favela-Torres *et al.*, 1998). This increase in biomass through SSF would subsequently result in a higher GlcN production. The same strain of fungi grown either in SmF and SSF can result in different biomass concentration. The differences in biomass concentration were thought to be derived from many factors such as the variable substrate as well as the initial glucose concentration or even the environmental condition. These ultimately influenced the metabolic activity of the microorganism through catabolic repression (Solís-Pereira *et al.*, 1993; de Azeredo *et al.*, 2007).

Focusing on biomass concentration difference regarding to the different cultivations, Crestini *et al.* showed that SSF of fungal species *Lentinus edodes* yielded a biomass concentration up to 50 times greater than that of SmF after twelve days of incubation, the time of maximum production for both conditions. The substrate used for SSF was humidified wheat straw and the medium composition was consistent between the two fermentation sources (Crestini *et al.*, 1996).

While there are many reports that SSF processes appear to be more efficient in biomass production, there have been cases when biomass levels produced from SmF either exceed that of SSF or are comparable. Mazumber *et al.* (2009) compared biomass levels of *Pleurotus ostreatus* production through SSF and SmF over time. The substrate used was synthetic polyurethane foam (PUF) and was inoculated with the same amount of cultured used for the SmF process. Analysis of dry mycelia demonstrated that there was no significant difference between biomass concentrations in the initial growth period. Following the first two days of growth SmF is able to produce a higher concentration of *P. ostreatus* biomass.

From these studies of fermentation systems, it becomes apparent that it is difficult to derive a solid comparison between SmF and SSF in terms of biomass concentration. The variations in biomass production suggest variable dependence on fermentation conditions. For example, in the previously discussed articles that evaluate microorganism activity during SmF and SSF, fungal biomass concentrations and subsequent GlcN amount differed. However, the potential benefits of SSF push for a deeper understanding about this fermentation process. The differences are thought to arise due to substrate choice, incubation temperature, medium composition, etc. (Bhargav *et al.*, 2008).

In SSF, the substrate can be homogenous or heterogeneous (Aidoo *et al.*, 1982, Zhu *et al.*, 1994). An example of a homogenous substrate is polyurethane foam (PUF). By using a synthetic substrate designed to mimic a natural state, one introduces the element of uniformity. Homogeneous substrates allow the control of the medium composition, feed rate, oxygen transfer, and facilitated quantification of biomass. Besides synthetic polymer devices, SSF can also take place on heterogeneous waste products such as rice bran, soy bean, wheat straw or other agricultural byproducts. Fungal growth of *M. rouxii* was demonstrated to be dependent on both natural substrate type and fermentation temperature (Jangbua *et al.*, 2009).

A disadvantage of using SSF and more specifically a heterogeneous substrate is the difficulty for measuring the fungal biomass (Zhu *et al.*, 1994; Sparringa and Owens, 1999). This results in poor characterization of fungal growth kinetics and transport and consequently optimization and standardization of SSF is difficult. The substrate's physical properties heavily influence the rate of nutrient and oxygen diffusion, water activity, and therefore metabolic activity. The detailed mechanisms by which these varying factors affect the fungal growth will not be discussed in this paper.

The difficulty in SSF for recovering fungal biomass from fermentation system will affect the extraction process of GlcN. This condition sometimes pushes the other scientists to produce GlcN in SmF instead of SSF. Simple step for calculating the biomass in SmF, which is the most basic parameter routinely followed during the course of fermentation makes this fermentation system is preferred to produce GlcN. Calculating the biomass in SSF will need another method rather than calculated the total solid mass particularly for GlcN measurement (Cui *et al.*, 1998). However, since the water activity of the medium in SmF is very high, makes it prone to contaminants if the aseptic treatment is not maintained and it also produces much water waste at the end

of fermentation process when recovering fungal biomass. Moreover, high agitation to provide adequate mixing and filtration process will lead to high energy expenditure. Comparability, energy expenditure is a good consideration for choosing SSF, but the ease of recovering fungal biomass is also the good reason for choosing SmF. Table 1 shows the production of GlcN from fungal fermentation including fermentation system and optimum harvesting time.

To enhance the productivity and the growth of fungal mycelium, many groups have looked into supplementing traditional fermentation medium with additional nutrient sources. Nwe and Stevens (2004) demonstrated that the addition of urea in SSF medium resulted in different amounts of total chitosan produced by fungal species *Gongronella butleri*. This additional N-source is thought to aid in the synthesis of more chitosan. The variation in amount of urea also resulted in differences in molecular weight of extracted fungal chitosan. Mishra and Kumar examined the effects of the alternate N-sources ammonium sulfate, urea, yeast extract and dry cyanobacterial biomass of *Anacystis nidulans* in the production of laccase and GlcN content by *Pleurotus ostreatus*. It was reported that the use of cyanobacterial biomass resulted in the greatest recovery of both enzyme and GlcN due to its high nutritional value (Mishra and Kumar, 2007).

Benjamin and Pandey (1997) demonstrated that the use of coconut oil cake (COC) as a substrate with various different minerals and different N and C sources resulted in varying lipase activities and GlcN concentrations from the fungus *Candida rugosa*. The study involved optimizing both N and C sources and combining the parameters to yield an increase in lipase activity but no difference in GlcN concentration. In addition to use additional N and C sources, the effects of treating a solid substrate with acid, phosphate, ethanol and conditioned at different moisture contents on citric acid production and biomass content by *A. niger* ATCC 9142 have been explored. The use of additional supplements overall did not enhance citric acid production but the treatment of substrate with acid demonstrated a significant ability increasing the biomass concentration (Xie and West, 2009).

The effect of different carbon sources on the production of the enzyme pectinase as well as the biomass content on *A. niger* is studied by Solís-Pereira *et al*. (1993) in SmF. The addition of glucose, sucrose or galacturonic acid with pectin was tested and it was found that the supplemental carbon sources resulted in increased biomass concentrations (Solís-Pereira *et al*., 1993). Other additional biochemical materials are added to submerged fermentation media in efforts to enhance biomass content and therefore increase

the yield of GlcN extract. Nwe *et al*. explored the effects of different nitrogen sources on chitosan yield in both SmF and SSF processes. From the nitrogen sources tested—urea, ammonium sulfate, peptone, and sodium nitrate—urea was found to best enhance the mycelia production but ammonium sulfate was found to yield the greatest amount of chitosan (Nwe and Stevens, 2004).

Plant growth hormones were also reported to improve the mycelium growth of fungi in SmF. Chatterjee *et al*. (2008) studied the effects of gibberellic acid, auxins and kinetin on the growth of chitosan by *R. oryzae* and *M. rouxii* in whey medium and molasses-salt medium, respectively. Low concentrations of plant hormones resulted in an increase in both mycelia growth and chitosan content in *R. oryzae* and *M. rouxii* while growth in both aspects were inhibited at higher plant hormone concentrations. Also, the molecular weight of extracted also was increased (Chatterjee *et al*., 2008; Chatterjee *et al*., 2009*)*.

From the explanation above, it is clearly stated about the differences between SmF and SSF in terms of GlcN production through fungal recovery from fermentation process. The differences in experimental design result in variations in isolation methods, surface area, oxygen and nutrient transport, and many other factors that will influence the microorganism growth and production. The media composition for each fermentation process can be supplemented with additional nutrient sources. For choosing suitable system for the production of fungal GlcN, several factors need to be considered for feasible system to be scaled up or developed and low production cost.

Isolation, purification and quantification of GlcN

With the outstanding developments in the area of separation process, spectroscopic techniques and other emerging techniques, natural product research is enjoying its attention for providing interesting chemical scaffolds. The various available hyphenated techniques i.e., GC-MS, LC-MS, LC-FTIR, LC-NMR, LC-NMR-MS have been utilizing for isolation and determination of crude extract of various natural products.

Generally, the quantitative analysis of GlcN from various sources uses colorimetric assay (Wagner, 1979), radioactivity (Setnikar *et al*., 1984), gas chromatography (Shinohara, 1981), capillary electrophoresis (Guttman, 1997) and high performance liquid chromatography (HPLC) techniques (Shao *et al*., 2004; Hsieh *et al*., 2007; Sitanggang *et al*., 2009; Sitanggang *et al*., 2010).

Traditional methods for the extraction and purification of GlcN and GlcNAc from shellfish waste involve demineralization, deproteinization, and bleaching of shells to yield chitin. Eventually chitin is hydrolyzed using hydrochloric acid for 4 h at 100°C (Mojarrad *et al.*, 2007) This process can be considered uneconomical due to the step numbers and different harsh chemicals employed (Hayes *et al.*, 2008). The movement towards of GlcN production via microbial organisms results in different methods for isolation and quantification.

In regards to bacteria, *E. coli* has been reported to directly synthesize and secrete *N*-GlcNAc into the fermentation broth. Further hydrolysis with mild acids such as acetic acid or hydrochloric acid to deacetylate the monomers of *N*-GlcNAc will yield the GlcN product. To quantify the GlcN derived from bacterial systems, the amino sugar is assayed using a modified version of the Elson-Morgan (Deng *et al.*, 2005; Deng *et al.*, 2006). The general method for GlcN quantifition begins with the high performance liquid chromatography (HPLC) analysis to isolate the samples of interest. The eluted samples are then confirmed through colorimetric assays. This involves acid hydrolysis of the solution, treatment with acetylacetone and Ehrlich's reagent (Johnston *et al.*, 1951). The resulting solution is a colored product (Róden *et al.*, 1997).

For the isolation and purification of GlcN from fungal species, it is begun with recovering fungal biomass from the medium fermentation. This process depends on the fermentation system being employed. For SmF, recovering fungal biomass undergoes with a filtration process to gain the cake whereas for SSF another specific process is needed. For SSF, the use of filter as barrier on the surface of substrate will increase the feasibility of recovering fungal biomass. However, this barrier will reduce the access of fungi to penetrate into substrate during incubation time.

Commonly, the crude chitin and chitosan can be converted into GlcN or *N*-GlcNAc through chemical extraction (acid, alkaline) and enzymatic hydrolysis of the β-(1,4)-glycosidic bonds (Cousin, 1996). Both of chemical and enzymatic extraction in terms of AIM (alkali insoluble material) is produced by treating the fungal biomass with NaOH (1.0-11.0 M) overnight (Nwe and Stevens, 2004; Mario *et al.*, 2008). The AIM is then separated by centrifugation. For enzymatic extraction, AIM was treated with acetic acid and then dissolved into β-glucanase or thermamyl at pH 7.0 to remove glucan bound (Nwe and Stevens, 2004).

A common method for chemical extraction is acid hydrolysis using hydrochloric acid at dilute concentrations. *N*-GlcNAc can be deacetylated into GlcN units by addition of NaOH (Nwe and Stevens, 2002a). Conversion of fungal biomass directly to GlcN monomers using a strong acid solution reacted at high temperatures followed by base neutralization has been performed by Hsieh *et al.* (2007) and Sitanggang *et al.* (2009, 2010). It is necessary to control the acid hydrolysis of chitin and chitosan because excessive amounts of acid can result in GlcN degradation (Zhu *et al.*, 2005).

Another recently developed technique for chemical extraction of GlcN and *N*-GlcNAc extraction from chitosan and chitin is a process involving two sulfuric acid hydrolyses and one nitrous acid treatment. Reaction of chitosan and chitin with sulfuric acid causes the depolymerization and deamination of the chitinous material and results in the conversion to anhydromannose (Zamani *et al.*, 2008). The mixture of GlcN and *N*-GlcNAc forms a colored complex upon the addition of 3-methyl-2-benzothiozolone-hydrazone-hydrochloride (MBTH) and $FeCl_3$ This method also utilizes colorimetric analysis of GlcN and N-GlcNAc (Plassard *et al.*, 1982).

Wu *et al.* analysed the chitinous content from the fungal species *A. niger* and *M. rouxii*, which were grown under submerged conditions. Separation of cell biomass from the broth was carried out via a vacuum filter and the biomass refluxed with a base to remove proteins and produce AIM. Chitosan is insoluble under basic conditions. AIM was refluxed with acetic acid for further purification and chitosan was then precipitated out by the addition of a strong base (White *et al.*, 1979; Wu *et al.*, 2005; Nwe *et al.*, 2008a). This method of chitosan extraction from fungal biomass is commonly seen in literature.

Enzymatic hydrolysis of chitosan and chitin from fungal biomass is not as commonly practiced as acid hydrolysis because it is more costly. Enzymatic hydrolysis of chitinous materials derived from fungal mycelia is researched by Nwe *et al.* (2008b). A combination of acid and enzyme is used for chitosan extraction from *G. butleri*. The enzyme α-amylase is used to further purify the extraction of β-glucan. This method is able to produce a greater amount of chitosan when compared to only using acid hydrolysis (Nwe and Stevens, 2002b; Nwe and Stevens, 2004).

Cai *et al.* (2006) tested the ability of the enzymes lysozyme, snailase, neutral protease and chitin deacetylase to extract chitosan from *A. niger*. The general process involved disruption of the cell walls, deproteination, and deacetylation using enzymes. The chitosan was extracted using acetic acid and base and GlcN content was analysed using the modified Elson-Morgan procedure. Both the quality and the

quantity of GlcN extracted were reported to be higher than that extracted via chemical methods (Cai *et al.*, 2006).

It has been reported that enzymatic hydrolysis of chitosan yields a more uniform distribution of GlcN oligomers than chemical hydrolysis. The use of enzymes is more costly, however Xie and West (2009) looked into the ability of cellulase to hydrolyze chitosan of *A. niger*. Cellulase is less costly than specific enzymes such as chitinase, chitosanase, and lysozymes, and it was reported that the chitosan was purifed so that when treated with alkali, no AIM resulted. The method of depolymerization of chitosan involved first dissolving the fungal mycelium in a weak acid and isolation of chitosan via reflux with concentrated base. Table 2 shows several methods for determination of GlcN, acetyl GlcN and chitosan from microbial fermentations.

(Machová *et al.*, 1999).

For separation and purification process of fungal GlcN, our laboratory is developing simple procedure to purify GlcN resulted from fungal fermentation of *Aspergillus sp* BCRC31742. Utilized WF medium consists of white fine granulated sugar (33.9 g l^{-1}), peptone (40.6 g l^{-1}), $MgSO_4$ (0.5 g l^{-1}), KH_2PO_4 (0.5 g l^{-1}), $CaCl_2$ (0.1 g l^{-1}). Fermentation was carried out in 5 L fermenter and could produce GlcN concentration averagely 4.0 g l^{-1}. The general procedure can be seen in Figure 2. The main steps of this separation process are (1) chemical extraction using hydrochloric acid (30% wt) for 4 h at 100ºC, (2) precipitation of GlcN using ethanol and (3) crystallization of GlcN using rotary evaporator. For maintaining the color of GlcN crystal (colorless), crystallization process has to be done at temperature of below 60ºC.

Table 2. Determination methods of fungal chitosan, GlcNAc and GlcN from fungal fermentation

No	Method	Parameter	Anal.time	Results	Reference
1	Combination of chemical extraction and modification of Elson-Morgan procedure	GlcN	small scale: 14 h large scale: 12 h	1. GlcN: 35-40% of hyphal wall	White *et al.*, 1979
2	Enzymatic extraction	GlcN	40 h	Recovery of chitosan from *A. niger*: 50.0%.	Cai *et al.*, 2006
	Chemical extraction	chitosan	15 h	Recovery of chitosan from *A. niger*: 41.7%.	
	Combination of chemical extraction and modification of Elson-Morgan procedure	GlcN	18 h	GlcN contenty from *A. niger* (dry basis): 0.20 g/gdw cells	
3	Chemical extraction: acidic hydrolysis	GlcN	thermal method: 5 h	GlcN content of *Aspergillus sp* BCRC 31742: 0.22 g/gdw cells	Sitanggang *et al.*, 2009
5	Chemical extraction	chitosan	69 h	1. Chitosan of *Cunninghamella blakesleena*: 9.4%/gdw 2. Chitosan of *Absidia coerulea*: 10.4%/gdw	Hiroshi *et al.*, 1992
6	Combination of enzymatic extraction and Chemical extraction	chitosan	23 h	1. Chitosan yield of SSF :11.7% of dry mycelia (basic medium + $(NH_4)_2SO_4$) 2. Chitosan yield of SmF :11.6% of dry mycelia (basic medium + $(NH_4)_2SO_4$)	Nwe *et al.*, 2002
7	Chemical extraction	GlcN N- acetyl GlcN	8 h	1. GlcN and GlcNAc of *R. oryzae*: 69.2±3.6% of AIM 2. GlcN and GlcNAc of *R. pusillus* : 80.7±2.6% of AIM	Zamani *et al.*, 2008
8	Chemical extraction	GlcN	25 h	GlcN content of *Aspergillus sp*: 0.19 g/gdw cells	Hsieh *et al.*, 2007

In addition to the acid and alkaline hydrolysis and enzymatic extraction of chitinous material from the fungal cell wall, ultrasonication has also been looked at as a technique to depolymerize the chitin-glucan complex found in the *A. niger*. The isolation of chitin-glucan complex is by precipitating out the AIM with base. The carboxymethylated chitin-glucan complex (CM-CG) is undergoes derivatization using the base, acids, and organic solvents, and the resulting sample is then treated with ultrasonication in an ice bath. Aliquots of the sample at various time intervals were analysed using HPLC analysis, gel filtration, and C-NMR spectroscopy and it was discovered that there are two predominant categories of chitin

Figure 2. General steps of fungal GlcN purification process

The purity of GlcN resulted from this process could reach up to 96.8% with the recovery was about 67.1%. For determination of fungal GlcN, HPLC was utilized.

Since GlcN does not have strong chromophore, it needs to react with a derivatizing agent to enhance its absorptivity (Hsieh *et al*., 2007; Sitanggang *et al*., 2009; Sitanggang *et al*., 2010, Chang *et al*., 2011). Sample was derivatized using 40 mol/m^3 of 1-napthyl isothiocyanate in pyridine solution for 1 h at 50ºC and shaken at 100 rpm. Internal standard was 0.1 %wt of 3,5-dinitrobenzoacetonitrile in acetonitrile. Detection was performed at a wavelength (λ) of 230 nm, mobile phase flow rate of 1.3 mL/min with an analytical time of 40 min.

Conclusions

The production of GlcN using microorganism has increased due to the increased limitations of GlcN production using both of chemical and enzymatic extractions of shellfish derived chitin. Micoorganisms–bacteria, fungi can be used to produce GlcN. *E. coli* has been engineered to improve the secretion of *N*-GlcNAc into fermentation broth whereas for fungi medium fermentation, strain, and fermentation system play an important role to yield high biomass concentration and high GlcN content. Separation and purification process of GlcN between bacteria and fungi are different since in bacteria, GlcN is secreted into the broth whereas for fungi GlcN is building block of polymeric chitin-chitosan that located in the cell walls of fungi. Different fermentation system–SSF and SmF also give different separation processes of GlcN at the initial state of recovering fungal biomass. Energy expenditure in SmF and the difficulty for recovering fungal biomass in SSF can be taken into consideration. Extraction of GlcN from fungal biomass can be carried on by means of chemical and enzymatic extraction. Commonly, chemical extraction is preferred since the enzymatic one is costly.

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