OPTIMIZATION OF INOCULUM SIZE & AGITATION RATE ON EXPRESSION OF RECOMBINANT CHITINASE IN *Escherichia coli*.

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ABSTRACT

Chitinase generally referred as endochitinases is randomly hydrolyzing chitin to produce N-acetyl-glucosamine. In addition, chitinase play an important role in chitin metabolism of microorganism for production of carbon and energy source. Chitinase can be found in bacteria, fungi, virus and higher plant. There are a lot of applications of chitinase either in pharmaceutical, biopestisides or food industry. For this research, chitinase was expressed in recombinant bacteria using *Escherichia Coli* as a host. The optimization was carried out by using response surface methodology. Focusing on two parameters which are inoculum size and agitation rate, the optimum condition was identified to enhance the process of chitinase production. The chitinase was expressed in different inoculum size at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL. Then the experiment continued observed in different agitation rate at 50, 100, 150, 200, 250 and 300 rpm. After overall observation, the best range of inoculum size gave between 0.8 mL to 1.2 mL and the range of agitation rate was identified between 230 rpm to 270 rpm. The best range of culture conditions was used for further study using Respond Surface Methodology (RSM). The optimal set of culture conditions for high soluble recombinant chitinase was determined as an inoculum size of 0.98 mL and 244.7 rpm of agitation rate. From the experimental value, the optimal production of recombinant chitinase was achieved 0.376 U/mL. This shows the 1.57-fold increment compared to the initial experiment which produced 0.239 U/mL of chitinase activity. These conditions successfully enhance the production of chitinase by using recombinant protein in *Escherichia coli.*

ABSTRAK

Kitinase selalunye merujuk kepada endokitinase iaitu ia menghidrolisiskan chitin untuk menghasilkan *N-acetyl-glucosamine*. Tambahan pula, kitinase memain peranan penting dalam metabolisma kitin dalam organisma untuk mengasilkan karbon dan sumber tenaga. Biasanya kitinase dijumpai di dalam fungi, virus dan tumbuhan tinggi. Terdapat banyak kegunaan kitinase sama ada dalam sektor farmasi, racun serangga atau pun makanan. Merujuk kepada kajian yang telah dijalankan ini, kitinase dihasilkan di dalam rekombinan bacteria iaitu menggunakan *Escherichia Coli* sebagai host. Selepas itu pengoptimuman dilakukan menggunakan kaedah gerak balas permukaan (RSM). Dengan mengfokuskan kepada dua parameter iaitu saiz inokulum dan kelajuan goncangan, keadaan optimum dikenalpastikan untuk meningkatkan keberkesanan proses penghasilan kitinase. Kitinase dihasilkan dengan berbeza keadaan saiz inokulum pada 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mL. Kemudiannya, uji kaji diteruskan dengan memerhati pada keadaan pada kelajuan goncangan yang berlainan iaitu pada 50, 100, 150, 200, 250 and 300 rpm. Selepas selesai pemerhatian, linkungan saiz inokulum terbaik adalah pada 0.8 mL hingga 1.2 mL dan untuk kelajuan goncangan pula, lingkungan yang berkesan adalah pada 230 rpm hingga 270 rpm. Kesemua lingkungan terbaik ini digunakan dalam kaedah gerak balas permukaan. Keadaan pengkulturan yang optimum untuk pengekspresan kitinase rekombinan adalah pada saiz inokulumnya 0.98 mL dan kelajuan goncangan pada 244.7 rpm. Dari uji kaji yang telah dijalankan, penghasillan aktiviti kitinase yang optimum telah di capai sebanyak 0.376 U/mL. Ini menunjukkan peningkatan 1.57-kali ganda berbanding kitinase yang dihasilkan sebelum pengoptimuman iaitu aktiviti kitinase sebanyak 0.239 U/mL. Pada keadaan ini menujukkan keberkesanan pengoptimuman penghasilan kitinase dengan mengunakan rekombinan kitinase dalam *Escherichia coli*.

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LIST OF SYMBOLS/ABBREVIATIONS

ANOVA	-	Analysis of Variance
CCD	-	Central composite design
DNS	-	Di-Nitro Salicylic Acid
g	-	Gram
g/L	-	Gram per litre
hr	-	Hour
IPTG	-	Isopropyl-β-D-thiogalactoside
mg	-	milligram
mg/L	-	milligram per liter
Min	-	minutes
mL	-	mililiter
OD	-	Optical density
PMSF	-	Phenylmethylsufonylfluoride
R	-	Correlation coefficient
R^2	-	Regression coefficient
RSM	-	Response surface methodology
rpm	-	Round per minute
U/mL	-	Unit enzyme activily per mililiter
v/v	-	volume per volume
v/w	-	volume per weight
w/v	-	weight per volume
w/w	-	weight per weight
µg/mL	-	microgram per mililiter
%	-	percentage
°C	-	degree Celsius
µL	-	microliter
µmol	-	micromole

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Chitin is the main constituent of shrimp wastes with β-(1–4)-linked N-acetyl Dglucosamine (GluNac) as the chief building block. Having high hydrophobic property, chitin is fundamentally inert to natural degradation and is insoluble in aqueous solutions. Owing to its abundant and cheap resource and biocompatibility, chitin has the potential for bioconversion to simpler molecules of GluNac monomers and chito-oligosaccharides by means of enzyme-catalyzed reactions or chemical procedures with the ease in production coming from the former procedure (Tharanathan *et al*., 2003).

Nowadays, there are a lot of important role of enzyme. Enzymes are naturally produced by organism. As for chitinase, this enzyme will be used to hydrolysed chitin, commonly this process occur in viruses, fungi, insect, higher plant and also in animal (Flach *et al*., 1992; Felse and Panda, 2000). Therefore potential of chitinase to degrade chitin will make it more valuable in industrial market (Felse and Panda, 1999). Chitinase is widely used for example in preparation of pharmaceutically, biopesticides and mosquito control. New founded research says that chitinase has potential as a serum for human (Reetari *et al.*, 1999).

In general, chitinase is a glycosyl hydrolase that catalyzes the hydrolytic degradation of chitin, a fibrous insoluble polysaccharide made of -1,4-N-acetyl-D-glucosamine residues. Chitinases are found in a wide variety of organisms that possess chitin as a constituent in fungi, insects, crustaceans and organisms that do not possess chitin as well in the bacteria, plants and vertebrates (Reetarani *et al.,* 1999).

The roles of chitinases in these organisms are diverse (Jolles *et al.,* 1999). Invertebrates require chitinases for partial degradation of old exoskeletons. Fungi produce chitinases to modify chitin, which is used as an important cell wall component. Bacteria produce chitinases to digest chitin and utilize it as carbon and energy sources. It is suggested that the production of chitinases by higher plants is a part of defense mechanisms against fungal pathogens (Boller *et al.,* 1985).

Trichoderma sp. possesses a chitinolytic system formed by several endochitinases, several exochitinases, and several N-acetyl-glucosaminidases, so that the measured specific chitinase activity is the result of the hydrolytic activity of those chitinolytic enzymes that are functional under specific condition. Recently there are significant studies about chitinolytic enzymes from *Trichoderma* spp., especially on *Escherichia coli*.

The production of recombinant protein has been established in various host organisms. Bacteria, especially *Escherichia coli*, remain important to production either in laboratory or in industrial scale (Hjorth *et al.,* 1992). *Escherichia coli* have been “workhorse” for the production of recombinant protein and also it is the best characterised host with many available expression systems (Lee and Makrides, 1996).

There are several previous studies about production of chitinase by different microorganism. Reported by Kole and Altosaar (1985), using non-pigmented, stable mutant *Serratia mercescens* showed the increases about 167 % of chitinase activity over the wild type strain under similar condition. The mutant produced 184 U of chitinase while the wild type produced 76 U of chitinase. Other researcher founded

Streptomyces has ability to produce chitinase (Tagawa and Okazaki, 1991). The most competent strain, *Streptomyces cinereoru*ber revealed that colloidal chitin was the best substrate produced 20.7 U of chitinase activity while *Aspergillus niger* cell wall gave about 16.4 U of chitinase. However, *Alcaligenes xylosoxydans* reported as novel and highest chitinase producing isolate to be active against fungal phytophatogens like *Fusarium sp*. and *Rhizoctonia bataticola* (Vaidya *et al.*,2001)

Advanced in genetic engineering and biotechnology enhance the productivity of chitinase. Nowadays, there are a lot of applications by using chitinase. Chitinase were used in the field of pest control, pollution abatement and basically in commercial biology (Felse and Panda, 1999). Due to the important of chitanase, many researcher try to finding solution to increase the production chitinase while there are highly demanding in manufacturing industry.

The new ideal produced the expression chitinase in recombinant technology that will be able to produce highly bioconversion of enzyme in industry sector. Due to the technology, bacteria as a host for example *Escherichia coli* potentially given the good respond for this matter. Development of chitinase user in industry especially in large scale production will make demand for chitinase enzyme is increases. Therefore in this study, response surface methodology based on rotatable central composite design was applied to identify and optimise the critical, crucial and significant cultivation conditions that will maximize the expression of chitinase enzyme in recombinant *Escherichia coli*.

1.2 Problem statement

The production of chitinase in general is expensive and complicated. This is because the process to extract the chitinase from the inside of the bacteria is a complex process. Nevertheless, the best condition of the fermentation process must be carried out and optimize to produce maximum yield. For this study the parameter of inoculum size and agitation rate was selected on expression of chitinase in recombinant *E. coli*. Also optimization of those parameters will be enhancing the production yield. When more chitinase is produced, there is more chitin that can be degraded naturally and save the environment.

1.3 Objective of Research

The main objective in this study is to optimize the effect of inoculum size and agitation rate on expression of recombinant chitinase gene from *Trichoderma virens* UKM-1 in *Escherichia Coli.*

1.4 Scopes of Research

This research consists of four scopes of study, which are:

a) To determine the best inoculum size on expression of chitinase activity in recombinant *Escherichia Coli* by different inoculum size at 0.5, 1, 1.5, 2, 2.5 and 3 mL.
b) To identify the best agitation rate for chitinase to be expressed in varies of agitation rate at 50, 100, 150, 200, 250 and 300 rpm.
c) To observe the optimum effect of agitation rate and inoculum size on expression chitinase in recombinant *Escherichia coli* by using response surface methodology.

CHAPTER 2

LITERATURE REVIEW

2.1 Chitinase

Currently, enzyme has played an important role in term of naturally behaviour in the environment. Enzyme like chitinase is found in bacteria, fungi, higher plant, insect and some vertebrates (Flach *et al.,* 1992; Felse and Panda, 2000)**.** Generally chitinase refer as an endochitinase, which is enzyme. Chitinase as role a play in hydrolyse chitin into monomer of N-acetylglucosamine linked. Besides, chitinase also compose of multiple protein modules and it is belong to glycoside hydrolyse (GH) families 18 and 19. There are two important enzymes in chitinase system which is endochitinase and acetylhexosaminidase (Patil *et al.,* 2000).

2.1.1 Type of Chitinase

Endochitinase and excochitinase are the types of chitinase enzyme. According to the endochitinase activity that can be defined as “the random cleavage at internal points in the chitin chain” (Felse and Panda, 1999). It also generates low molecular mass multimers of GlcNAc, such as chitotetraose, chitotriose and diacetylchitobiose (Dahiya *et al.,* 2006).

The activity of excochitinase is defined as “the progressive action starting at the non-reducing ends of chitin with the release of successive diacetyl chitobiose unit” (Felse and Panda, 1999). Under excochitinase there are two subcategories. First is chitobiosides, which can catalyze the progressive release of diacetylchitobiose starting at the nonreducing end of chitin microfibril. Then the second subcategories are N-acetyl glucosaminidase, which cleave oligomeric product of endochitinase and chitobiodases generating monomers of GlcNAc (Dahiya *et al.,* 2006).

To identify the structures of enzyme, at least three chitinases have already been determined (Ikegami and Watanabe *et al.,* 2000). They are endochitinase from barley seeds, *Hordeum vulgare* (Hart *et al.,* 1995), hevamine with combined chitinase and lysozyme activities from a plant, *Hevea brasiliensis* (Scheltinga *et al.,* 1996), and the third chitinase is from a gram-negative soil bacterium which is *Serratia marcescens* (Perrakis *et al.,* 1994). Therefore, the three-dimensional structure of a chitinase whose function has clearly been identified is necessary for elucidation of its binding mechanism with chitin and its role in the subsequent catalytic activity (Ikegami *et al.,* 2000). Figure 2.1 shows the three dimension of chitinase enzyme structure.

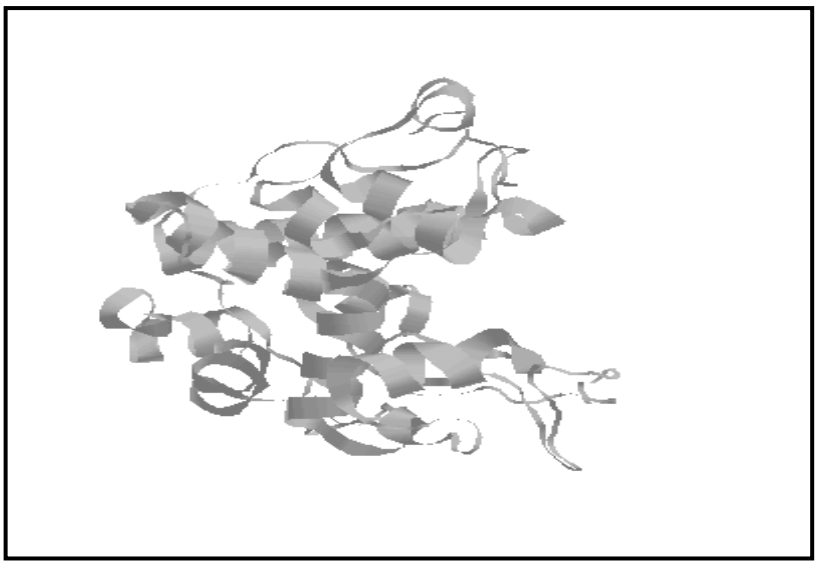

Figure 2.1: Structure of chitinase enzyme

2.1.2 Classification of Chitinase

From previous studies due to amino acid sequence similarity, families 18, 19 and 20 of glycosyl hydrolases can refer to group of chitinolytic enzyme. Chitinase contain from bacteria, fungi, viruses, animals and some plant chitinases are referring to evolutionary term of family 18. While according to family 19, there are consist of plant chitinases (class I, II and IV) (Hart *et al.,* 1995). Both of two families are not share amino acid sequence similarity. However, family 20 is includes the β-*N*-acetylhexosaminidases from human, bacteria and *Streptomycetes* (Dahiya *et al.,* 2006).

For instance, the characteristic of the chitinase can classify by the sequences in six classes (Iseli *et al.,* 1996). The classes are referring to the information of their N-terminal sequence, localisation of the enzyme, isoelectric pH, signal peptide and the inducers. Class I is consist of cysteine rich N terminal, leucine or valine-rich signal peptide and vacuolar localization. Furthermore subdivided as sub Class Ia and Ib based on the basic and acidic nature, respectively of chitinases. This Class I chitinases was found to be restricted to the plant sources, whereas Class II enzymes were present in plants, fungi and bacteria (Patil *et al.,* 1999).

Due to Class II chitinases that were pathogen induced, lack the cysteinerich N- terminal domain, but had sequence similarity with Class I chitinases. Most of the Class I chitinases were endo-chitinases and Class II chitinases had exo-action. Class III chitinases did not show any sequence similarity to enzymes of Class I or II (Collinge *et al.,* 1993). Even though Class IV chitinases had similar characteristics as Class I chitinases including immunological properties, but they were significantly smaller than Class I chitinases. In Classes V and VI there included single examples (Iseli *et al.,* 1996). The nettle lectin precursor (Class V) showed two chitin binding domains in tandem (Reetarani *et al.,* 1999).

2.1.3 Chitinase Production

Generally chitinase is produced by microorganism where it is inducible in nature. But in industrial sector, production of chitinase had been produce in the large scale amount. Commonly fermentation method is usually used in industry. There are several fermentation methods that had been used to produce microbial chitinase which are liquid batch fermentation, continuous fermentation, fed-batch fermentation, solid-state fermentation, cell immobilization and biphasic cell system (Dahiya *et al.*, 2006).

In bioconversion, extracellular chitinase production is reported to be influenced by media components such as carbon and nitrogen sources, and agriculture residues such as rice bran and wheat bran (Bhusan, 1998; Dahiya *et al.*, 1995). Reported by Bhusan (1998) that effect glucose can an enhancing chitinase production since glucose was used within chitin in production medium. However, a suppressing effect of glucose on chitinase production was reported by Miyashita (1991).

Some other factors such as aeration, pH, and incubation temperature also affect chitinase production. The other several methods, such as immobilization (Bhushan and Hoondal, 1998), biphasic cell systems (Chen and Lee, 1995) and solid-state fermentations have been used for improving chitinase production in different microorganism (Bhushan and Hoondal, 1998). In an immobilization system, whole cell immobilization of an organism to a solid support such as polyurethane foam was applied. Chitinase production was enhance up to 4.8-fold over a period of 72 hours in submerged fermentation.

Production of extracellular chitinase can enhance by *S. Marcescens* in an aqueous two-phase system (ATP) of PEG and dextran was reported by Chen and Lee (1995). They reported that a maximum chitinase achieve into 14.5 unit compared with 13.6 units in a polymer-free system. While in solid-state fermentation by using flake chitin as the solid substrate, the higher chitinase yield was produce from

Enterobacter sp. NGR4 was 616 U/g of solid substrate after 168 h of growth at 30^{o}C and 75% moisture level. In combination of wheat bran with chitinous substrate, founded that the increases of chitinase yield which is 1475 U/g at wheat bran to flake ratio, 1; moisture, 80%; and inoculums, 2.6 ml after 168 h (Dahiya *et al.,* 2005).

2.1.4 Function of Chitinase

In order production of chitinase from organism, there are several function that can be used by organism itself and also industrial sectors. In bacteria, chitinase play roles in nutrition and parasitism while involving morphogenesis in fungi, protozoa and invertebrates (Reetarani *et al.,* 1999). In addition, chitinase are also involved in the defence mechanism of plant and vertebrates (Reetarani *et al.,* 1999).

Baculoviruses, which are used for biological control of insect pests, also produce chitinases for pathogenesis (Deising *et al.,* 1995). Whereas in human, chitinase activity is play role in serum that has recently been describe (Reetarani *et al.,* 1999). The possible role is a defence against fungal pathogen (Reetarani *et al.,* 1999). Fungal chitinases are required for hyphal growth.

Furthermore, chitinases are enzyme that capable to hydrolysing chitin to its monomer N-acetyl glucosamine (GlcNAc) (Felse and Panda, 1999). In bacteria chitinase are considered primarily to digest and hydrolyse chitin into a carbon and nitrogen nutrient (Cohen and Chet, 1998). On the other hand, chitinase act like catalyst in chitin degradation (Cohen and Chet, 1998).

Fungal chitinase are important for surviving of producing organism itself. This is because they are involved in important morphogenic processes such as spore germination, hyphal elongation and hyphal branching (Kuranda and Robbins, 1991; Takaya *et al.,* 1998).

2.2 Expression System

2.2.1 *Escherichia coli* Expression System

A number of bacteria endochitinases have been cloned and over expressed by using recombinant DNA technology (Felse and Panda, 1999). *Escherichia coli* is one of the most widely used host for production of recombinant protein as it is the best characteristic host with many available expression systems (Lee *et al.,* 1996). However, *Escherichia coli* cannot produce some protein containing complex disulfide bonds, or mammalian proteins that require post-translation modification for activity. Although, many recombinant have been successfully produced by using *Escherichia coli*.

Overexpressed protein are often produce in the form of inclusion bodies, from which biologically active protein can only recovered by complicated and costly denaturation and refolding process. The final yield of this soluble refolded protein are usually low, according to the mainly to protein aggregation resulting from interaction between hydrophobic region of the proteins (Choi and Lee, 2004).

In command cases expression is induced using isopropyl-β-D-thiogalactopyranoside (IPTG) at a defined concentration and the reasons for using this pre-determined IPTG concentration are seldom presented. The establishment of optimal inducer concentration is essential since it has been reported that in some recombinant *Escherichia coli* cultures induced with a high IPTG concentration, this has lead to a severe reduction in specific growth rate (Davis *et al.,* 1991) or the expression of some proteases thus reduction the yield of the foreign.

There are the number of attempts have been made to clone and express genes from several organism to produce chitinase such as *B. circulans* WL-12 (Mitsutomi *et al.,* 1998), *Enterobacter aggalomerans* (Chernin *et al.,* 1997) and *S. marcescens* 2170 (Suzuki *et al.,* 1998) into *E. coli*. Reported by Fuch *et al.,* (1986), the gene of chitinase was expressed more efficiently in *E. coli* compared with *Pseudomonas*.

2.2.2 Fungi Expression System

There are a lot of expression systems as a host towards production of recombinant protein. Optimization of recombinant processes was mainly attained by genetics-based solution and in some case by modification of the fermentation strategies (Cserjan *et al.*, 1999). However, only limited information on the heterologous expression of fungal chitinases is available (Antonio *et al.*, 2003).

Mycoparasitism, a general term to describe the multi-step degradation and final assimilation of phytopathogenic fungi, has been proposed as a major mechanism supporting the antagonistic activity displayed by *Trichoderma* (Kubicek *et al.*, 2001). Since this process requires the degradation of the cell wall of the fungus host, cell wall hydrolases by *Trichoderma*.

Some other finding has been supported by the analysis of gene expression. The expression of *T. atroviride nag.1* is triggered by fungal *B. cinerea* cell wall (Mach *et al.*, 1999). There are commercially available chitin monomer N-acetyl-glucosamine and the oligomers di-N-acetyl-chitobiose. However, *ech42* expression in *T. atroviride* was observed during growth on fungal cell wall, but could not be triggered by those chitin degradation products (Mach *et al.*, 1999).

Interesting in *T. harzianum chit33* expression, there are triggered by carbon starvation, nitrogen starvation and physiological stress (Kubicek *et al.*, 2001). That stress-mediated regulation suggested a general phenomenon involve in chitinase gene expression of *Trichoderma* spp.

2.2.3 Yeast Expression System

Saccharomyces cerevisiae which is yeast has been establish used as a host for production of heterogeneous protein (Brake, 1990). Although, yeast contains sub-cellular organelles, the carbohydrate modifications performed by yeast often differ from those higher eukaryotic cells.

Recently, chitinase A from *Entrobactor* sp. G-1 and chitosanease A from *Matsuebacter chitosanotabidus* were cloned in the yeast *Schizosaccharomyces pombe* to study the function expression of these enzymes and their effect on morphogenesis in *S. pombe* (Shimono *et al.*, 2002). In this study of host cell, the chitinase was expressed inside the cells while the chitosanase was expressed as a secretion product.

Reported by Revah-Moiseev and Carrod (1981) was suggested the use of shellfish waste for the bioconversion of chitin to yeast single cell protein using chitinolytic enzymes. They used *S. marcescens* chitinase system to hydrolyse the chitin and *Pichia kudriavazeii* to yield single cell protein. *M. Verrucaria* and *S cerevisiae* was utilised chitinolytic enzymes for production of single cell protein from chitinous waste (Vyas and Deshpande, 1991).

2.3 Factor Affecting the Expression of Recombinant Protein

2.3.1 Effect of Inoculum Size on Expression of Recombinant Protein

In fermentation process there are several factor may be affect during the processing. Inoculum size is one of the parameter must have to discover to optimize the production of enzyme in recombinant protein. Inoculum size could have ability to effect the duration of the lag phase (Agustin, 2000). Their studies have examined low inoculum size effects when populations are exposed to harsh conditions. The lag time

of *Listeria monocytogenes* was extended when the cells were severely stressed by starvation (Augustin *et al.* 2000). This was observed at very low cell densities and explained by an increase in the variation of individual cells' lag time.

Indeed, the low inoculum size effects are quite general and reflect the distribution of injury in a microbial population, which becomes apparent when such low inoculum studies were performed (Pin *et al.*, 2006). Furthermore, the effect of various stresses on the distribution of individual lag times of *L. Monocytogenes* (Guillier *et al.* 2005) and the evolution of the injury distribution of small populations of *L. innocua* as the concentration of acetic acid in the medium is increased shown by Metris (2006).

The initial application of these ‘single-cell kinetic’ studies in foods has also been reported (D'Arrigo *et al.*, 2006). The use of automated turbidometry in these studies has proven very useful and one point is consistently made that at higher inoculum size, the time to detection is the time taken for the ‘fittest’ organism to complete repair and divide. Hence the time to detection of higher inoculum size by using turbidometry and are those organisms found on one-side of the distribution tail.

Masana and Baranyi (2000) explained the inoculum size effect of *B. thermosphacta* on the probability of growth and the location of the growth/no growth boundary by invoking the hypothesis that population differences in resistance to environmental factors were responsible.