CRANFIELD UNIVERSITY

SUPUNNIKA SOMJAIPENG

# ECOPHYSIOLOGICAL APPROACHES TO ENHANCE PRODUCTION OF THE ANTI-CANCER DRUG TAXOL BY *Paraconiothyrium variabile* AND *Epiccocum nigrum* AND LYSOZYME BY *Pichia pastoris*

CRANFIELD HEALTH/SCHOOL OF APPLIED SCIENCES

APPLIED MYCOLOGY

PhD Thesis

Academic Year: 2009 - 2013

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This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

# ABSTRACT

This study has investigated the interaction of ecophysiological factors such as water activity ($a_w$), temperature, pH and solute types on (a) the production of the anti-cancer drug taxol by the endophytic fungi *Parachoniothyrium variabile* and *Epcoccum nigrum*, and (b) the production of human lysozyme by a recombinant strain of the methanogenic yeast *Pichia pastoris*.

Of more than 200 isolates from fresh health twigs of *Taxus baccato* trees on the Cranfield University Campus, only two strains of endophytic fungal species, *P. variabile* and *E. nigrum,* were able to produce taxol. *P. variabile* could produce 0.53 to 1.75 μg/l taxol and *E. nigrum* could produce up to 1.32 μg/l in a defined M1D liquid medium. Ecological studies showed the profiles for growth and taxol production. The growth rate of *P. variabile* were generally higher at 0.99 $a_w$ and 25$^{o}$C, but optimal at 0.99-0.98 $a_w$. The average growth rate was faster when sorbitol was used as the $a_w$ depressor when compared with glycerol, glucose and salt-amended media. Statistical analysis indicated that all the studied stress factors significantly affected radial growth rate of *P. variabile* on a taxol conducive M1D agar ($P<0.05$). The optimum pH was 5.0 regardless of the $a_w$ x temperature conditions ($P<0.05$). The combined effects of $a_w$, solute types and temperature on taxol production by *P. variabile* was determined using a 5x5x3 factorial design. The maximum amount of taxol was 7.11 μg/l when cultured with M1D using KCl to modified media to 0.98 $a_w$ and 25 $^{o}$C. In contrast, on un-amended M1D medium at 25°C yielded about 1.75 μg/l of taxol.

For *E. nigrum*, optimal conditions for growth were observed at 0.99-0.98 $a_w$ and 20-25$^{o}$C. Growth on medium imposed with sorbitol was significantly faster ($P<0.05$). All three stress factors significantly affected radial growth rate of this strain ($P<0.05$). This strain grew faster at pH 5.0. The attenuation of taxol for this strain was observed during examination of the combined effects on taxol yield. Because of the attenuation and unstable production of taxol by the strain of *E. nigrum*, it was decided to try and use different elicitors to enhance production of taxol.

Various elicitors were examined in order to stimulate or reverse attenuation in taxol production. For *P. variabile*, this was successful with the use of salicylic acid at 50 mg/l as an elicitor with the taxol yield of 14.74±4.80 µg/l. The study of synergetic effects between elicitor, $a_w$ and pH on taxol production showed that highest amount of taxol (68.92±11.86 µg/l) was produced under modified $a_w$ with KCl (0.98 $a_w$) at pH 5 and supplemented with 20 mg/l of salicylic acid.

For *E, nigrum*, when supplementing the media with serine, the most pronounced effect on increasing yield was observed (29.55 fold). Under modified $a_w$ conditions with KCl (0.98 $a_w$) at pH 5, the production of taxol was significantly increased when serine was supplemented at 30 mg/l. The maximum taxol was 57.13±11.84 µg/l. In contrast, on un-amended M1D medium with serine at 30 mg/l, resulted in 8.50 times lower yield.

Response surface methodology was used to evaluate the combined effects of different temperatures (20, 25, 30°C) and $a_w$ (0.98, 0.94, 0.90) on radial growth rate of *P. variabile* and *E. nigrum* on M1D agar when sorbitol was selected as $a_w$ depressor. The established model provided better prediction of radial growth rate of *P. variabile* ($R^2$=0.92), while the predictive model of *E. nigrum* performed to an acceptable accuracy for radial growth rate ($R^2$=0.86). These predictive models are a useful tool which can be applied to predict the growth under solid state fermentation for taxol production.

For human lysozyme production, factorial designs and a screening experiment were employed to identify significant factors affecting growth and human lysozyme production by *P. pastoris* on YPD agar and in shake flask cultivation, respectively. The results showed that the lowest temperature (15°C) and with glycerol as the $a_w$ depressor (0.97 $a_w$) gave the maximum cell numbers of *P. pastoris* (3.06 x $10^3$ cfu/ml) when examining interaction effects between $a_w$ x temperature x types of solutes. In interactions between pH x $a_w$ x type of solutes at 30°C, this non-ionic solute also resulted in the highest cells numbers (1.97 x $10^3$ cfu/ml) at pH 7 and 0.97 $a_w$. A decrease of $a_w$ and pH significantly reduced and delayed *P. pastoris* growth on YPD; the effects were more drastic when the ionic solute NaCl was used to decrease $a_w$. Statistical analysis indicated that $a_w$ had a significant effect on *P. pastoris* growth ($P < 0.05$).

The screening experiment revealed that temperature had the most significant effects on lysozyme production ($P < 0.05$). A maximum yield at 100 hrs was 75.06 U/mg at pH 5.8, 20°C, methanol 1.5 %, expression method type 1 without $a_w$ modification and was increased by 2.28 times compared with control conditions. Influence of different buffering systems on lysozyme production was investigated. MES-buffered medium resulted in the highest enzyme production (53.79 U/mg) at pH 6.8, 25°C, a methanol concentration of 0.6%, and 0.97 $a_w$.

These studies suggest that the ecological factors including temperature, $a_w$, types of solutes and pH play a role in the productivity not only in fungal secondary metabolites, but also can impact on the production of recombinant proteins.

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Supunnika Somjaipeng

สุพรรณิการ์ สมใจเพ็ง

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| **$a_w$** | water activity |
| **AOX** | Alcohol oxidase |
| **BMGY** | Buffered minimal glycerol complex medium |
| **BMMY** | Buffered methanol minimal complex medium |
| **C** | Clavicipitaceous |
| **CFUs** | Colony forming units |
| **cm** | Centimetreorn Meal Agar |
| **CMA** | Corn meal Agar |
| **ESI** | Electrospray ionization |
| **g** | Gram |
| **h** | Hour |
| **HPLC** | High Pressure Liquid Chromatography |
| **IgE** | Immunoglobulin E |
| **IR** | Infrared radiation |
| **ITS** | Internal transcribed spacer region |
| **kg** | Kilogram |
| **L** | Litre |
| **LC** | Liquid chromatography |
| **LC-MS** | Liquid chromatography mass spectrometry |
| **LC-MS/LS** | Liquid chromatography tandem mass spectrometry |
| **λmax** | Lambda maximum |
| **$LD_{min}$** | Minimum lethal dose |
| **µ** | Micro ($10^{-6}$) |
| **m** | Milli ($10^{-3}$) |
| **MEA** | Malt Extract Agar |
| **MeOH** | Methanol |
| **mg** | Milligram |
| **mM** | Milimolar |
| **mp** | Melting point |
| **m/z** | Mass to charge ratio |
| **NAG** | N-acetyl glucosamine |
| **NAM** | N-acetyl muramic acid |
| **NC** | Non- Clavicipitaceous |
| **ng** | Nanogram |
| **OMA** | Oat Meal Agar |
| **PDA** | Potato Dextrose Agar |

| | |
|---|---|
| **Rf** | Retention factor |
| **RMSE** | Root mean squares error |
| **RSEP** | Relative standard error of prediction |
| **TLC** | Thin layer chromatography |
| **Vmax** | Maximum velocity |
| **WHO** | World Health Organization |
| **YPD** | Yeast Extract-Peptone-Dextrose |

# 1 Literature review

## 1.1 General introduction

Biopharmaceuticals and biotechnology-based products, have become increasingly important in recent years. Products include vaccines, proteins and enzymes. There has been a gradual increase in the market share, approximately 8% of the total pharmaceutical market by 2004 and this might rise to 15% by 2015. The global biopharmaceuticals market was worth around 64.5 billion dollars (Walsh, 2003; Kambhammettu, 2009). Bioactive microbial products are also good candidates for the discovery of new pharmaceuticals. Many of these compounds such as antibiotics and anti-cancer agents play an important role in all aspects of human life (Strobel, 2002; Guo et al., 2008; Suryanarayanan et al., 2009; Tenguria, 2011; Prado et al., 2012).

The biodiversity of microorganisms provides useful source of high value natural products, especially in the kingdom fungi. There are a variety of products that play important roles in the treatment of several human diseases. Historically, the best example is the production of penicillin by *Penicillium chrysogenum*. Although within the fungal kingdom there are estimated to be about 1.6 million species. There are numerous unexplored species and undiscovered potential pharmaceutical products (Carlile et al., 2001).

By definition, secondary metabolism represents synthesis of compounds which are not directly required for normal growth. These compounds are low molecular weight molecules and are produced from metabolic intermediates by specific secondary pathways. The production of secondary metabolites seems to be genus or species specific. For instance, taxol, an anti-cancer compound is found in the tree *Taxomyces andreanae,* but is also produced by a wide range of fungi including *Fusarium lateritium* and *Pestalotiopsis microspore* (Griffin, 1994; Carlile et al., 2001; Deacon, 2006; Shwab and Keller, 2008). Many fungal secondary metabolites have been used as pharmaceuticals due to their bioactive properties such as antibiotics, tumor

inhibitors and cholesterol lowering drugs. Today, there is much interest in the production of such compounds naturally by using cell factories as opposed to chemical synthesis.

## 1.2 Anti-cancer drug taxol

### 1.2.1 *Taxus*:The common yew

Yews (genus *Taxus*), small evergreen coniferous trees or shrubs, are one of the members of the family *Taxaceae*. They are often considered not to be conifers because their seeds are held in berrylike fleshy cup instead of seed cones (Figure 1.1). There are common species members of this genus including the Common yew (*Taxus baccata*), the Pacific yew (*T. brevifolia*), the Canadian yew (*T. canadensis*) and the Japanese yew (*T. cuspidate*) (Phillip, 1978; Sutton, 1990; More and White, 2003).

Common yew or English yew (*T. baccata*) occurs naturally in most of Europe, Iran and Algeria (Figure 1.1). The fossils from Maiden Castle (Iron age hill fort) in Dorset (UK) have revealed evidence of the occurrence of this species in England dating back to both Neolithic age (4000 - 2500 BC) and Early Iron age. This tolerant specie is widely spread in England, Wales, southern Scotland and Ireland. It can survive in a wide range of soils (mainly on limestone and chalk), climate conditions and can grow under a forest canopy in woodland (Turrill, 1971). Although yews are generally small and spreading trees, they can exceed 20 m in height and able to attain thousands years of age (More and White, 2003; Sutton, 1990). Individual trees growing under favourable conditions can achieve remarkable ages; however, these ages have little relevance to natural woodland where individual trees face the competition for resources (Peterken, 1996). The trunks of old trees are massive and hollow. The bark of yew is reddish brown, scaly and peeling in thin strips.

The male and female flowers develop on separate plants (dioecious) which start blooming in March or April. The developed seed (size 1.2 cm) is partially surrounded by the red fleshy cup and the fruiting time is between August and September.

(a) (b)

Figure 1.1 (a) English yew (*Taxus baccata*) (b) yew berries

The English yew tree has long been regarded as a poisonous plant, both foliage and bark, especially the dried needles, which are poisonous to most domestic livestock and people (More and White, 2003). In the past, the majority of yew trees were planted in the enclosed areas of the village, especially in churchyards. The reason for this was to avoid contact with children and livestock. The toxicity of yew has been attributed to taxine alkaloids present in all parts of tree, except the red berry-like structure called the aril. The first taxoid of toxic components, named taxine, was isolated from yew trees in 1856.

Based on their chemical structure, there are two categories including taxines A and taxines B. The amount of taxines can vary depending on the variety of *Taxus* sp. It has been noted that *T. baccata* and *T. cuspidate* contain high amount of taxines (Wilson et al., 2001). Moreover, their toxic levels of taxines remain in plant parts throughout the year whether fresh or dried. Livestock are more likely to be poisoned accidentally by taxines than humans. Clinical signs of poisoning develop after ingestion of plant parts which act in different ways. Death is a clinical sign of acute poisoning in animals that eat parts of the Yew tree. Additionally, the minimum lethal dose ($LD_{min}$) of taxines depends largely on the species of animal. This is shown by the toxicity studies of taxines in animals. The lowest levels of estimated $LD_{min}$ can be seen in horses at 1.0-2.0 (mg of taxines/kg body weight) or 100-200 g of yew leaves, while chickens showed least sensitive ($LD_{min}$ 82.5 mg/kg body weight). As a result of this, toxic outbreaks occur most frequently in horses (Clarke and Clarke, 1988; Cooper and Johnson, 1998; Molyneux and Panter, 2009).

Acute poisoning in animals can cause death. In sub-acute poisoning, common symptoms of an animal suffering from exposure to toxins are ataxia (staggering, unsteady gait), bradycardia (slow heart rate), dyspnea (labored breathing), muscle tremors, recumbency, convulsion and eventually death. This is because the taxines can be absorbed through the digestive tract rapidly (Cooper and Johnson, 1998; Parton and Bruerc, 2002; Molyneux and Panter, 2009). Accidental ingestion of this toxin by humans can result in: dizziness, pupil dilation, nausea, vomiting, diffuse abdominal pain, muscle weakness and convulsion (Burke et al., 1979; Sinn et al., 1990).

Besides taxine, other taxane alkaloids also have been discovered from another *Taxus* species. One of the most interesting discoveries has been the presence of taxol in the bark of the Pacific yew tree (*Taxus brevifolia*) with mitotic spindle poison. In the meantime, there was also experimental evidence to suggest other toxicities of this compound. Cardiotoxicity is one of the side effects that has been of concern. Donehower and Rowinsky (1993) noted that during clinical phase I and II studies of taxol, asymptomatic bradycardia occurred in a large number of patients. Moreover, at the maximum tolerated doses, 29 % of patients with ovarian cancer also showed this

cardiotoxicity. The other cardiac disturbances observed were: atrioventricular blocks, left bundle branch block, ventricular tachycardia (VT), and manifestations of cardiac ischemia.

Additionally, the occurrence of other cases of taxol toxicity also documented includes hypersensitivity reactions, hematological toxicity and neurotoxicity. For hypersensitivity reactions, type I hypersensitivity reactions have been observed among treated patients. These reactions appeared at a variety of doses which included an adverse reaction: dyspnea, hypotension, bronchospasm, urticaria, and erythematous rashes and a rare case of fatal effects. The mechanism of taxol-associated hypersensitivity reactions has been postulated that an IgE-mediated mast-cell degranulation induced by taxol (Weis et al., 1990). However, in some patients, the sudden onset of an overwhelming observed hypersensitivity reaction was compatible with other mechanisms such as a non-IgE-mediated idiosyncratic mast-cell degranulation by taxol or by Cremophor EL (Gelderblom et al., 2001) and complement activation (Szebeni et al., 2001).

As mention above, taxol has been shown to have a spindle poison activity. This was not enough to encourage scientists to consider this compound as a new anti-cancer drug. The finding that taxol has an unique mechanism of action in 1979 showed that this drug promoted the assembly of microtubules rather than acting as inhibitors of tubulin polymerization (Schiff et al., 1979). This new mechanism was a significant additional factor in increasing the interest in this compound, resulting in a wide range of studies to enhance production.

## 1.3 Taxol from discovery to clinic

Cancer has been cited in the top ten leading causes of all deaths worldwide. This disease affects people at all ages with the risk for most types of cancer increasing with age. Globally, the five most frequency cancers in both sexes are lung, breast, colorectum, stomach and prostate cancer (The International Agency for Research on Cancer, 2011).

The highest incidence rate of all types of cancer in both females and males was in the World Health Organization (WHO) regions for Europe and the Americas. Furthermore, the number of new cancer cases globally is increasing. An estimated more than 11.5 million people are expected to die from cancer in 2030 (WHO, 2011).

Researchers have been investigating a wide range of issues relating not only to cancer treatments, but also the development of new anti-cancer drugs. Although drugs can be produced through chemical synthesis, natural products still play an important role as new sources of effective drugs. For example, in the Kingdom Plantae alone, more than 3000 plant species have been reported with anti-cancer properties (Kaur et al., 2011). Before 1960, there was no information about cytotoxic properties isolated from plants. The discovery of taxol dates back to 1960. A collection program was established in the United States under the National Cancer Institute (NCI) with the strategy to isolate new anti-tumor drugs in plants. One of the 650 collected plant samples included plant parts of *Taxus brevifolia* such as barks, twigs, leaves and fruits which were examined in detail. The plant extracts were tested with 9KB (human nasopharyngeal cancer cell line) and this led to the discovery of taxol. The chemical structure of this compound was reported in 1971 using a Nuclear Magnetic Resonance (NMR) method for structural determination (Figure 1.2). From needle extracts, the product showed the following physical properties: mp 213-216°C, λ-max: 227 nm (MeOH), IR (nujol):Vmax = 3300-3500 (OH,NH) 1730 (ester) 1710 (ketone) 1650 (amide) $cm^{-1}$. The chemical formula is $C_{47}H_{51}NO_{14}$ with molecular weight of 853 (Wall and Wani, 1996; Wani, et al., 1971).

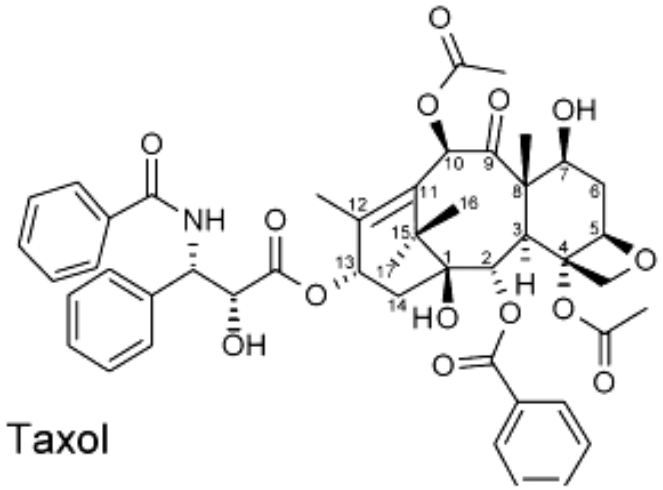



Figure 1.2 The chemical structure of taxol

Scientists have spent a lot of time in the laboratory carrying out, firstly, drug discovery and development, and then in testing through experiments with model human diseases. For taxol, initial clinical trials began with a preclinical testing phase in 1977 and went on progressively through the phase 1 and 2 clinical trials between 1983–1986 (Table 1.1; Wall, 1998). Moreover, the revelation of the effectiveness of taxol against ovarian cancer by McGuire and collogues (1989) fascinated researchers which then accelerated the intensive studies of this compound. They reported that 30% of patients with ovarian cancer responded to taxol treatment, whereas there was no effect with other cancer drugs. Recently, taxol has been reported as a potential agent for treating lung cancer, neck cancer and other diseases including rheumatoid arthritis, malaria, Alzheimer's disease (Adlard et al., 2000; Arsenault et al., 2000; Koka et al., 2009).

Table 1.1 Development timeline for taxol (Wall, 1998; Brown, 2003; Obeslies and Kroll, 2004).

| Development | Year |
|---|---|
| Collection of bark from *Taxus brevifolia* as part of the NCI/USDA screening | 1962 |
| Cytotoxicity of crude extract demonstrated against KB cell in vitro | 1964 |
| First isolation of paclitaxel and confirmation of cytotoxicity in KB cells | 1966 |
| Isolation of paclitaxel reported at 0.02% concentration | 1967 |
| Chemical structure of paclitaxel published | 1971 |
| Activity in a panel of tumor systems | 1975-1976 |
| Preclinical development | 1977 |
| Mechanism of action published | 1979 |
| Animal toxicology | 1982 |
| Phase I clinical trails | 1983-1984 |
| Phase II clinical trails/activity in ovarian cancer | 1985-1986 |
| Synthesis of taxol side chain | 1986 |
| Semisynthesis of taxol | 1988 |
| Improved syntheses of taxol side chain | 1990-1993 |
| Bristol-Myers Squibb receives CRADA from NCI | January 1991 |
| NDA field with FDA | December 1992 |
| Total synthesis of taxol/Holton-Nicolaou | 1994 |
| Approved for use against breast cancer | 1994 |
| Approved for AIDS related Kaposi's sarcoma in the U.S. | 1997 |
| Taxol/cisplatin approved in U.S. for non-small cell lung cancer | 1999 |
| Historical marker dedicated to commemorate original collection of *Taxus brevifolia* 40 year earlier | 2002 |
| RTI's Natural Products Laboratory bestwed National Historic Chemical Landmark | 2003 |

## 1.4 Fungal endophytes

### 1.4.1 Types of fungal endophytes

The term endophytic fungus is defined as a species that lives in the mycelial form in close association with the living plant tissue but does not create any plant disease or symptoms (Carlile et al., 2001; Strobel and Daisy, 2003; Maheshwari, 2006). The fungal endophytes are dominated by the class of Ascomycetes fungi (Huang et al., 2001; Rodriguez et al., 2009; Tenguria et al., 2011). Endophytic fungi have been classified into two major groups namely Clavicipitaceous (C-endophytes) and

Non-clavicipitaceous (NC-endophytes) (Carroll, 1988). Recently, NC-endophytes have been categorized into three classes with the different criteria including tissues colonization, host range and mode of transmission, for example (Table 1.2; Rodriguez et al., 2009).

Table 1.2 Symbiotic criteria used to characterize fungal endophytic classes (Rodriguez et al., 2009).

| | Clavicipitaceous | Nonclavicipitaceous | | |
|---|---|---|---|---|
| Criteria | Class 1 | Class 2 | Class 3 | Class 4 |
| Host range | Narrow | Broad | Broad | Broad |
| Tissues colonizrd | Shoot and rhizome | Shoot, root and rhizome | Shoot | Root |
| *In planta* colonization | Extensive | Extensive | Limited | Extensive |
| *In planta* biodiversity | Low | Low | High | Unknown |
| Transmission | Vertical and horizontal | Vertical and horizontal | Horizontal | Horizontal |
| Fitness benefits* | NHA | NHA and HA | NHA | NHA |

*Nonhabitat-adapted (NHA) benefits such as drought tolerance and growth enhancement are common among endophytes regardless of the habitat origin. Habitat-adapted (HA) benefits results from habitat-specific selective pressure such as pH, temperature and salinity.

C-endophytes are known as the grass endophytes with the mutualistic association and are transmitted vertically through seeds. These endophytes colonize plant shoots and rhizomes. The role in the host plants have been known particularly from protection host plants against insect feeding and mammalian herbivores. However, the defence strategies depend on the host plants and their endophytes. The accumulation of toxic alkaloids in host plant tissues may contribute to proecting the plant from insect infection (Clay et al., 1985; Cheplick and Clay, 1988; Powell and Petroski, 1992; Shiba et al., 2011; Matsukura et al., 2012). The classes of bioactive alkaloids include ergot alkaloids, lolines, indole-diterpenes and paramine (Panaccione et al., 2013). Grass infected with such systemic endophytes also provide the positive impact on the physiology of host plants, resulting in increased tolerance to abiotic stresses such as drought and metal concentration. Under drought conditions, infected perennial ryegrass with the endophytic fungus *Neotyphodium lolii* had more tillers and greater tiller

length than non-infected grass (Kane, 2011). Enhanced salinity stress tolerance in tall fescue (*Festuca arundinacea*) has been shown when infected with *Neotyphodium coenophialum* by reducing the concentration of $Na^+$ and $Cl^-$ in host roots (Sabzalian and Mirlohi, 2010). Increased tolerance to heavy metal has been reported from endophyte-infected tall fescue, resulting in higher cadmium accumulation than endophyte-free plants (Soleimani et al., 2010; Ren et al., 2011).

NC-endophytes are diverse and can infect a wide range of host plants with a symbiotic relationship. Based on the difference in the life history and ecological interactions, NC-endophytes have been classified into three classes (Rodriguez et al., 2009). The intensive studies in such endophytes have been started after the discovery of anti-cancer drug taxol from taxol-producing endophytic fungus, *Taxomyces andreanae* (Petrini et al., 1992). The roles of NC-endophytes in host plants have been reported the fitness benefits such as stresses tolerance and increased growth. For example, in the presence sugarcane endophytic fungus, *Epiccoccum nigrum*, enhanced the root system biomass was obtained. Further, the antifungal activity of this endophyte against sugarcane pathogen namely *Fusarium verticillioides*, *Colletotrichum falcatum* and *Ceratocystis paradoxa* was reported (Fávaro et al., 2012). The evidences of enhancing the resistance of host plant against insect herbivores by endophytes were provided when endophytes were inoculated into the plants. For example, endophytic isolates from medical plant, *Nigrospora* sp. and *Cladosporium* sp., enhanced the mortality of the polyphagous pest *Spodoptera litura* (Fab.) in cauliflower (Thankur et al., 2013). Some endophytes are able to produce secondary metabolites as their host plants, the defence strategies against insect herbivores can be associated with the host plants and endophyte secondary metabolites (Kusari et al., 2013). Other studies have showed the effect of NC-endophytes on nematodes. Endophyte-inoculated plants by the strain *Fusarium oxysporum* reduced root damage from nematodes (Dababat and Sikora, 2007; Kurtz and Schouten, 2009; Waweru et al., 2013). The defence against fungal pathogens in host plants with NC-endophytes also reported. For example, the reduction of leaf death and leaf necrosis caused by pathogen (*Phytophthora* sp.) in tropical tree (*Theobroma cacao*, Malvaceae) were observed, when the endophyte-free leaves were inoculated with endophytes (Armold et al., 2003). To date, the studies of the

NC-endophytes have been focused on the bioactive metabolites (Schulz et al., 2002; Strobel, 2002; Aly et al., 2010; Tenguria et al., 2011; Wang et al., 2011; Zhao et al., 2011; Chandra, 2012; Prado et al., 2012).

### 1.4.2 Isolation of fungal endophytes

As the endophytes reside within living host plant tissues, the strategies for selection and collection the host plants for endophytic fungi should be considered. It can be summarized as suggested by Strobel (2002) and Tenguria et al. (2011): (i) Host plants from the unique habitats which possess unusual biology and the adaptive and survival strategies are appropriate to study. For example, the plants from the coastal beach are selected in which the plants are constantly exposed to various stresses such as salinity and drought. Nevertheless, the plants are still alive under such extreme conditions. This was studied with *Fusarium culmorum* a fungal endophyte which conferred salt tolerance on host dune grass (*Leymus mollis*) plants on coastal beaches (Rodriguez et al., 2008); (ii) The host plants with the ethnobotanical history are the choice for exploring endophytes for bioactive compounds. Many novel bioactive metabolites have been isolated from endophytes associated with medicinal plants including anti-cancer agents, antioxidant compounds and immunomodulatory agents (Kaul et al., 2012). In traditional medical practice, the medical plants have been used by native people for treatments for many centuries. The plant-associated endophytes may contribute to the production of these compounds for such treatments; (iii) Endemic plants, plants with unusually long lifespans, plants that spread across the ancient land mass and ancient plants have the tendency to be sources of interesting endophytes. The colonization rates of endophytic fungi in young bark (1-year-old bark) were lower than 2 and 3-year-old bark during summer when investigating endophytes from *Pinus tabulaeformis* Carr. in the Dongling Mountains of Beijing, China (Guo et al., 2008); (iv) Plants in an area of high biodiversity, have more diverse endophytic fungi. The individual leave may contain up to 90 species in tropical tree leaves (Arnold et al., 2003; Bayman, 2006).

For locating the plant samples geographically, the Global Positioning System (GPS) devices are generally used to locate the position. Plant sample are collected from stem, leave, root and bark which then are placed in the sealed plastic bag preventing water loss. After the plants are collected, they are usually stored at 4°C until isolation can be performed. For endophyte isolation, the surface sterilization of plant materials is the critical step to remove the surface-contaminating microorganisms. The use of running tap water to remove debris and some surfaced microbes from plant materials is a common first step followed by chemical surface sterilization. The most frequent choice for surface sterilization is sodium hypochlorite (Collado et al., 1999; Larran et al., 2002; Tomita, 2003; Madki et al., 2010). The concentration and the contact time with the solution is important to not have toxicity effects (Ramakrishna et al., 1991). A powerful sterilizing agent, such as 70% ethanol, is sometimes used for seconds or minutes due to extreme toxicity. Generally, ethanol is used alone or used prior to treatment with another compound (Strobel et al., 1996; Li et al., 1998ab; Wang et al., 2000; Kumaran et al., 2008; Li et al., 2008; Kumaran et al., 2009ab).

After plant materials are sterilized, they are air dried under laminar flow or rinsed thoroughly with sterile water. The outer tissues of the surface-sterilized plant samples are then removed with sterilized blade and the excised inner tissue placed on the surface of the agar medium. Potato dextrose agar (PDA) is used for general purpose isolation media. However, malt extract agar (MEA), Murashige and Skoog agar (MS agar) and water agar have also been used (Chakravarthi et al., 2008; Padhi and Tayung, 2013; Tejesvi et al., 2013).

## 1.5 Endopyte and taxol production: an additional resource

Not surprisingly, after the introduction of taxol to the market by Bristol-Myers Squibb, over-harvesting of Pacific Yew for this drug occurred. The taxol content of Pacific Yew bark ranges from 0.001-0.08 % and requires approximately 10,000 kilogram of bark to produce 1 kg of taxol (Vidensek et al., 1990). This could lead to the extinction of a species.

Today, taxol can be chemically synthesized using a semi-synthetic process. However, some intermediates of this multistep chemical process are still required from the Yew tree (Patel, 1998). Therefore, taxol is an expensive product and thus not very accessible to many people worldwide. Moreover, a full course of the treatment for one patient will vary depending on other factors such as the type of cancer, height and weight of the patient. As a result of the expensive process required to produce taxol, scientists have investigated alternative methods in order to produce taxol more cheaply and ensure wider availability. For example, tissue and cell cultures of *Taxus* spp. have been used. This method has been one of the most interesting areas to try and produce taxol because of the scarcity of the Yew tree. Unfortunately, commercial scale production of *Taxus* species in culture is still limited by the cell viability and high operating costs (Hirasuna et al., 1996; Nguyen et al., 2001; Yuan et al., 2006; Frense, 2007; Exposito et al., 2009).

In 1993, a novel taxol-producing endophytic fungus, *Taxomyces andreanae*, was isolated from the Yew tree (*Taxus brevifolia)*. This discovery led to interest in the potential for producing paclitaxel at a lower price by using fermentation systems. Many studies have examined novel endophytic fungi to screen for the production of taxol (Petrini et al., 1992). Strains of species such as *Pestalotiosis* sp., *Fusarium* sp., *Phyllosticta* sp., *Bartalinia robllardoides, Terminalia arjuna, Taxomycos andreanae, Colletotrichum gloeosporioides* have been isolated from various host plants which have the ability to produce taxol (Tan and Zou, 2001; Kumala et al., 2007; Gangadevi and Muthumary, 2008; Li et al., 2008; Kumaran et al., 2009; Gangadevi and Muthumary, 2009). However, the relative yield of taxol from such fungi is small ranging from ng to μg/l (Table 1.3).

Table 1.3 The yield of taxol reported from different fungal endophytes and their hosts (adapted from Kumaran et al., 2009).

| Fungi | Host plants | Taxol yield ($L^{-1}$) |
|---|---|---|
| *Taxomyces andreanae* | *Taxus brevifolia* | 24-50 ng |
| *Monochaetia* sp. | *T. baccata* | 102 ng |
| *Fusarium lateritium* | | 130 ng |
| *F. arthrosporioides* | | 131 μg |
| *Pestalotia bicilia* | *T. cuspidate* | 1081 ng |
| *Altermaria* sp. | | 157 ng |
| *Pestalotiopsis microspora* | *T. wallachiana* | 268 ng |
| *P. microspora* | *T. sumatrana* | 500 ng |
| *Pithomyces* sp. | *T. walachiana* | 95 ng |
| *P. microspora* | *Taxodium distichum* | 60-70 μg |
| *P. microspora* | *Wollemia nobilis* | 1487 ng |
| *P. guepinii* | | 485 ng |
| *Pestalotiopsis* sp. (W-1f-1) | | 172 ng |
| *Pestalotiopsis* sp. (W-x-3) | | 127 ng |
| *P. microspora* | *T. walachiana* | 0.31-1.8 μg |
| *P. vericolor* | | 478 μg |
| *Periconia* sp. | *Torreya grandifolia* | 30-831 ng |
| *Altemaria* sp. | *Ginko biloba* | 115-260 ng |
| *Pestalotia heterocomis* | *From yew forest soil* | 31 μg |
| *Ozonium* sp. (BT2) | *T. chinensis* | 50-1487 ng |
| *Phyllosticta spinarum* | *Cupressus sp.* | 235 μg |
| *Phyllosticta citricarpa* | *Citrus medica* | 265 μg |
| *Phyllosticta dioscoreae* | *Hibiscus rosa-sinensis* | 298 μg |
| *Phyllosticta melochiae* | | 274 μg |
| *Phyllosticta tabernaemontanae* | | 461 μg |
| *Phomopsis* sp. | | 418 μg |

The taxol yield from endophytic fungi isolated from *Taxus* spp. has generally been low. Scientists have been searching for new strains from other plants. Consequently, angiosperms have become a fascinating source of endophytic fungi. For instance, *Bartalinia robillardoides* Tassi is an endophytic fungus that was isolated from the medical plant *Aegle marmelor* Correa ex Roxb in India. This strain is able to produce taxol in liquid medium at 187.6 μg/l and has also shown cytotoxicity effects after testing by using apoptotic assays on various cancer cells line including human breast, human colon and human intestinal cells (Gangadevi and Muthumary, 2008b). Moreover, a new strain *Phyllosticta melochiae* Yate was isolated from *Melochia corchorifolia* L. The record maximum yield of taxol was 274 μg/l in M1D medium and production rate of this strain also increased to 55000 fold when compared with *Taxomyces andreanae* (Kumaran et al., 2008). This group has also isolated and identified a new strain, *Phyllosticta tabernaemontanae*, a leaf spot fungus of an angiosperm, *Wrightia tinctonia.* It was able to produce 461 μg/l on M1D medium and 150 μg/l on PDB medium. (Kumaran et al., 2009).

### 1.5.1 Environmental factors, growth and secondary metabolite production

Although the amount of taxol that is produced by endophytic fungi is relatively small compared to semi-synthetic processes or cell culture of *Taxus* spp., the short generation time and possibility for scale up provides a route to developing fungi as cell factories. Until now, there has been a lot of interest in ways to improve the culturing to improve the taxol yields. As taxol is a secondary metabolite, the production of such a product is regulated by various factors including precursors, carbon source, nitrogen source, phosphate, trace element and environmental growth conditions (Griffin, 1994; Li et al., 1998a,b; Parra et al., 2005; Xu et al., 2006). This is supported by previous studies of several factors in order to optimize the media of taxol-producing fungi such as *Fusarium maire.* Media with $NH_4NO_3$, $MgSO_4$ and Sodium acetate had a significant effect, increasing taxol from 20 to 225.2 μg/l after strain improvement using UV radiation treatment (Xu et al., 2006).

A number of studies have been published describing several strategies to improve growth and productivity of filamentous fungi (Gqaleni et al., 1997; Baxter et al., 1998; Parra et al., 2005). Media design and environmental conditions are often examined. Ecological niches in which fungal groups grow need to be considered in trying to optimize production systems using fungi. There are several abiotic factors affecting growth and yield of secondary metabolites such as temperature, pH, water activity ($a_w$) and light (Deacon, 2006; Carlile et al., 2001; Magan and Aldred, 2007). Temperature and water are described as critical factors for fungal growth and metabolite production, especially secondary metabolites (Magan, 2007).

Water is needed by fungi for taking up nutrients, releasing extracellular enzymes and essential for metabolic reactions. However, there are two types of water which make up the total water content. That which is bound to organelles and cells and that which is freely available (Carlile et al., 2001; Deacon, 2006; Magan, 2007). This freely available water is that which is available for microbial growth. The minimum $a_w$ for fungal activity varies with species (Table 1.4; Mugnier and Jung, 1984; Grant, 2004).

It has been shown that optimum and marginal conditions of $a_w$ and temperature on growth and secondary metabolite production can be very different (Sanchis *et al*., 2005). Thus single and combined environmental parameters can influence primary and secondary metabolism. For example, a *Phoma* species known to produce squalestatins, had an optimum temperature for growth of 25°C, whereas 20°C was optimum for squalestatin production (Baxter et al., 1998). Gqaleni et al. (1997) showed that *Aspergillus flavus* produced alfatoxin at an $a_w$ of 0.996 and 30°C, and cyclopiazonic acid at the same $a_w$ and 25°C. This report suggested that the combined effect of temperature and $a_w$ is highly significant at $P<0.01$ affecting cyclopiazonic acid and aflatoxin production. These interactions were also shown to be statistically significant ($P<0.01$) for production of squalestations by a *Phoma* sp. (Baxter et al., 1998; Parra et al., 2005). Mycelial growth of *Penicillum verrucosum* and ochratoxin A production are affected by both $a_w$ and temperature interactions with very different optima for metabolite production from that for growth (Cairns et al., 2005 Pardo et al., 2006). Lahlali and coworkers (2005) suggested that variance analysis of three interacting

factors: $a_w$, temperature and solute on growth rate of *Penicillum expansum* confirmed that this was highly significant ($P<0.01$). The growth rate decreased as temperature and $a_w$ decreased. Adding glycerol, sorbital and glucose in the culture medium resulted in growth at low levels of $a_w$ at 0.89. In contrast, there was no growth at 0.93 $a_w$ when using the ionic solute NaCl.

Table 1.4 Minimum inhibitory water activity values for growth of fungi. (adapted from Grant, 2004)

| $a_w$ value | Yeast | Mould |
|---|---|---|
| 0.95-0.90 | *Rhodotorula* spp. | *Rhizopus* spp. |
| | *Pichia* spp. | *Mucor* spp. |
| | *Canida* spp. | |
| | *Trichoderma* spp. | |
| 0.90-0.85 | *Saccharomyces* spp. | *Cladosporium* spp. |
| | *Hansenula* spp. | |
| | *Torulopsis* spp. | |
| 0.85-0.80 | *Zygosaccharomyces bailii* | *Aspergillus patulum* |
| | | *Aspergillus glacus* |
| 0.80-0.75 | | *Aspergillus conicus* |
| <0.70 | *Zygosaccharomyces rouxii* | *Xeromyces bisporus* |

Surprisingly, no studies have been carried out on the ecology of taxol producing fungi and whether temperature and $a_w$ interactions may affect the yield of taxol produced. This is surprising as previous studies have demonstrated that changes in environmental stress physiology may significantly increase the titres of enzyme and secondary metabolites produced in fermentation and immobilized systems (Parra et al., 2005).

### 1.5.2 Elicitation and secondary metabolite production

Under optimized fermentation conditions, the production of plant-derived compounds by endophytic fungi would be independent from the host plants. The attenuation and the reduction of secondary product production on repeated subculture is one of the key challenges to address. Observations on those problems have been noted among taxol-producing endophytic fungi. Plant pathogenic microorganisms are likely to possess virulence factors that are countered by plant secondary metabolites synthesizing in response to an infection. Elicitation can promote the productivity of secondary metabolites by mimicking the defense responses in plants. The term Elicitation can be defined as an approach to induce the secondary metabolite production under *in vitro* conditions by mocelues or treatments called elicitors (Kurz and Constabel, 1998; Singh,1999; Namdeo, 2007). Elicitors can be biotic such as fragments from fungi, pathogens, oligosaccharides and organic acids or abiotic such as salt of heavy metal (Zhong, 2002; Heldt, 2005; Angelova et al., 2006; Vanhulle et al., 2007; Kusari et al., 2012). In addition, elicitors can be classified into 2 types based on their origin including exogenous and endogenous elicitors. The exogenous elicitors are substances originated outside the cell, whereas endogenous elicitors have originated inside the cell. Table 1.5 represents the classification of elicitors.

The application of biotic and abiotic elicitors to activate secondary metabolite productions have been documented commonly in plant cell culture showing a dramatic increase in yield (DiCosmo and Misawa, 1985; Kessmann et al., 1990; Gundlach et al., 1992; Godoy-Hernández and Loyola-Vargas, 1997; Zhao et al., 2005; Schreiner et al., 2011; Ch et al., 2012). Moreover, this elicitation strategy has been applied to enhance taxol yield from plant cell cultures. The effects of some elicitors such as methyl jasmonate, silver nitrate, fungal fragments, salicylic acid and abscisic acid on taxol yield have also been studied (Yu et al., 2001; Zhong, 2002; Khosroushahi et al., 2006; Rezaei et al., 2011; Xu et al., 2011; Asghari et al., 2012).

Table 1.5 The classification of elicitors for the production of secondary metabolites (Namdeo, 2007).

| A. Nature of elicitor | |
|---|---|
| Biotic elicitors | Abiotic elicitors |
| - Directly released by microorganisms and recognized by the plant cell (enzyme, cell wall fragments)<br>- Formed by action of microorganisms on plant cell wall (fragments of pectins etc.)<br>- Formed by the action of plant enzymes on microbial cell walls (chitosan, glucans)<br>- Compounds, endogenous and constitutive in nature, formed or released by the plnat cell in response to various stimuli | - Of physical or chemical nature working via endogenously formed biotic elicitors<br>- UV light<br>- Windfall<br>- Denatured proteins (RNase)<br>- Freezing and thawing cycles<br>- Non essential components of media (agarose, tin, etc.)<br>- Heavy metals<br>- Chemicals<br>- with high affinity to DNA<br>- with membrane-destroying activities like detergents: xenobiochemicals<br>- Fumicides (Maneb, Butylamine, Benomyl)<br>- Herbicides (Aciflurofen) |

| B. Origin of elicitor | |
|---|---|
| Exogenous elicitors | Endogenous elicitors |
| - Originated outside the cell, including the reaction immediately or via endogenous mediators<br>- Polysaccharides: Glucomannose, Glucans, Chitosan<br>- Peptides as poly cations: Monilicolin, Poly-L-lysine, Polyamines, Glycoproteins<br>- As enzymes: Polygalacturonase, Endopolygalacturonic acid lyase, Cellulase<br>- Fatty acids: Arachidonic acid, Eicosapentanoic acid | - formed via secondary reactions induced by a signal of biotic or abiotic nature in the cell - dodeca-β-1,4-D-galacturonide<br>- hepta-β-glucosides<br>- alginate oligomers |

With some elicitor compounds, the possibility to increase and restore taxol biosynthesis have been reported. For example, Li et al. (1998a) suggested that the production of taxol by *Pestalotiopsis microspora* was significantly stimulated by adding benzoate and lowering inorganic phosphate in culture media. Furthermore, benzoate at 0.01 mM also activated taxol production by *Periconia* sp. isolated from *Torreya grandifolia* which increased taxol production 8 fold from 118 ng/l in the control to 831 ng/l (Li et al, 1998b). Significant enhancement in the level of fungal taxol was noted in a strain *Paraconiothyrium* SSM001 by supplementation with salicylic acid and benzoic acid. Moreover, co-culturing this strain with one isolate (*Phomopsis* sp.) from *Texus* (yew) needles, resulted in a 3.8 fold increase in fungal taxol (Soliman and Raizada, 2013). Thus, the successful application of elicitation seemed to be species-dependent which may be promising as it showed a dramatic increase in yield by fermentation process. This provides the challenge for intensive research to enhance the synthesis of secondary metabolites from fungal endophytes.

## 1.6 Lysozyme and *Pichia pastoris*

### 1.6.1 Lysozyme

In 1921, lysozyme was discovered by Alexander Fleming. This enzyme is able to catalyze hydrolysis reaction cleaving β, 1-4 glycosidic linkage between N- acetylmuramic acid (NAM) and N- acetylglucosamine NAG) of peptidoglycan in the cell wall of gram positive bacteria such as *Staphylococcus aureus, Micrococcus luteus, Bacillus stearothermophilus, Clostridium tyrobutyricum* (Figure 1.3; Hughey and Johnson, 1987; Salazar and Asenjo, 2007; Maidment et al., 2009; Huang and Demirci, 2009). Although it is not possible to access the peptidoglycan layer in gram negative bacteria as easily as gram positive bacteria, the broad-spectrum antibacterial activity of lysozyme can be extended widely to lyse this bacteria after modification. Several strategies have been investigated to enhance production: for instance, denaturation of lysozyme, modification of lysozyme with perilladehyde (Ibrahim et a.l, 1994; Masschalck and Michiels, 2003; Touch et al., 2003; Lesnierowski et al., 2004).

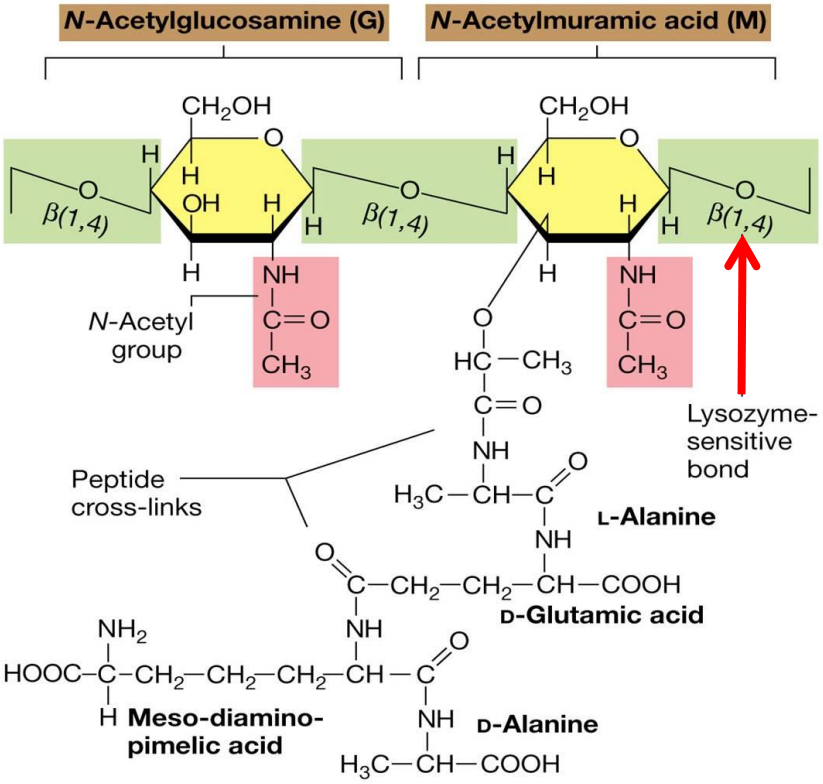



Figure 1.3 Lysozyme specificity. Structure shown is the example of one of the repeating units of peptidoglycan cell wall of bacteria. The red arrows indicate points of lysozyme hydrolysis (From Madigan et al., 2003).

Additionally, lysozyme also exhibits another function in antiviral, immune modulation, anti-inflammatory and antitumor effects. In medicine, it is used as a model protein in order to determine and address some human diseases such as amyloid disease caused by protein misfolding (Goda et al., 2000). With its antibacterial properties, it has also been used as a preservative in the food industry, especially in cheese manufacturing to prevent late blowing caused by outgrowth of clostridial spores and to control lactic acid bacteria in alcoholic fermentation (Tucker and Woods, 1995).

Human lysozyme and hen egg-white lysozyme are classified into C-type lysozyme. Although hen egg-white lysozyme is available commercially and used in various applications, human lysozyme shows higher antimicrobial activity. Furthermore, several studies provide evidence that hen egg-white lysozyme is allergenic (Fremont et al., 1997; Sampson, 2004; Sicherer and Sampson, 2006; Mine and Yang, 2008). With regard to a wide range of applications in lysozyme, many studies have investigated how to enhance yields of the product using genetic engineering.

### 1.6.2 *Pichia pastoris*

The methylotrophic yeast, *Pichia pastoris* has become remarkable host cell for the expression of recombinant products. It can be grown to very high cell density and express high levels of product. Moreover, there are many advantages of such yeasts overcoming the problems with *Saccaromyces cerevisae* including protein processing, protein folding and post-translational modification. Therefore, the recombinant product from *P. pastoris* is more similar to human protein rather than using other host cells (Siegel and Brierley, 1989; Wegner, 1990; Chen et al., 1997; Stratton et al., 1998; Cereghino and Cregg, 1999).

There are a variety of expression vectors of *P. pastoris*; however, the most wildly used promoter system is a strongly inducible promoter AOX1 (Macauley-Patrick et al., 2005). This gene is induced by methanol encoding enzyme alcohol oxidase (AOX) which is used in methanol metabolism in the compartmentalized region, called peroxisomes. There are several enzymes involve in this pathway such as alcohol oxidase, catalase and formaldehyde dehydrogenase. The first step is catalyzed by alcohol oxidase generating hydrogen peroxide that can be degraded to oxygen and water by catalase (Figure 1.4). The final product of this metabolism is formaldehyde that is able to leave the peroxisome becoming the energy for the growing cell on methanol.

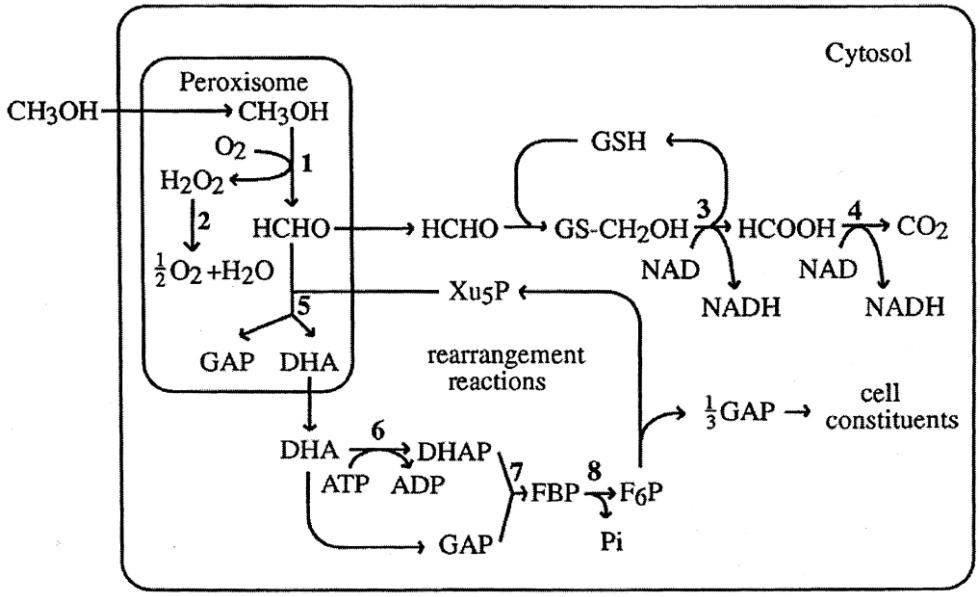



Figure 1.4 Methanol metabolism in *P. pastoris* (Cereghino and Cregg, 2000)

1. alcohol oxidase; 2. catalase; 3. formaldehyde dehydrogenase; 4. formate dehydrogenase; 5. dihydroxyacetone synthase; 6. dihydroxy acetone kinase; 7. fructose 1,6-bi-phosphate aldolase; 8. fructose 1,6-bisphosphatase

In construction of expression systems with *P. pastoris* when the AOX1 is selected as the promoter, the foreign gene is inserted in the AOX1 position. Then, this expression vector is introduced into the yeast genome (Figure 1.5).This strong promoter provides the control at a transcriptional level. There is a possibility to obtain high yields of product by controlling expression after reaching high cell density in a culture medium. This promoter appears to be repressed while yeast cells grow on another carbon source such as glycerol and subsequently expressed after presenting methanol in the culture medium (Cregg et al., 2000; Daly and Hearn, 2005; Macauley-Patrick et al., 2005).

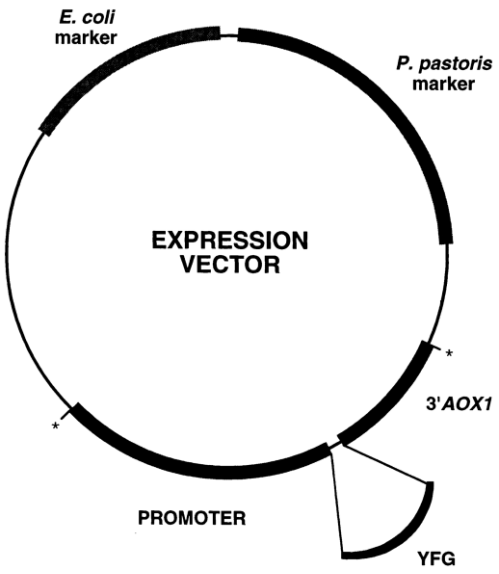



Figure 1.5 General diagram of a *P. pastoris* expression vetor. YFG refers "Your Favorite Gene" and * for cassette amplification (Cereghino and Cregg, 2000).

*P. pastoris* also contains another promoter namely AOX2. It is not a strong promoter as AOX1 and exhibits only 97% homologous to AOX1. Regarding to methanol utilization, there are three phenotypes of this yeast strain. If this strain has only AOX2, called $Mut^{s}$ (Methanol utilization slow), this cell exhibits slow growth in methanol medium but is able to tolerate only low levels of methanol. As AOX1 of this strain is deleted, therefore the level of enzyme alcohol oxidase is low. The $Mut^{+}$ (Methanol utilization plus) strain contains both of the AOX promoters and able to metabolize methanol at the wild-type rate. The $Mut^{-}$ (Methanol utilization minus), is the strain without both AOX promoters, therefore is unable to metabolize methanol as a carbon source (Zhang et al 2000; Macauley-Patrick et al. 2005).

There are several types of lysozyme that are produced/expressed in this host system. For instance, Digan et al. (1989) reported bovine lysozyme concentration exceeded 550 mg/l and a cell density of 120 dry cell/l by using fed-batch fermentation. In continuous fermentation processes the lysozyme product obtained is approximately 350 mg/l and 100 dry cells/l. There are also the same characteristics of recombinant product compared with purified lysozyme that was confirmed by immunoblot and polyacrylamide gel analyses.

Bovine lysozyme was produced in methanol fed-batch fermentation at 450 mg/l using the $Mut^+$ strain. Using high cell density, fed-batch fermentation and mixed-substrate are the suggestion to improve productivity (Brierly et al., 1990). To achieve a high level of human lysozyme from *P. pastoris*, alfa-factor pro-sequence (MF-α) was constructed to human lysozyme gene. This construction secreted product at about 17.0 μg/ml that was more than 20 fold lyzozyme than other signal sequences (Oka, et al., 1999). The Hen egg lysozyme, H5-lysozyme, was investigated in relation to effects of fermentation conditions on expression. The results showed high productivity in BMM medium with a more than 422 fold increase in lysozyme than previous reported data for *Saccharomyces cerevisiae* as the host cell (0.005 mg/l). The medium composition, induction time and cultivation method affected the expression directly (Liu et al., 2003). In the medical areas, *P. pastoris* strains have been constructed for the expression of various variants of human lysozyme. For example, human lysozyme with an extra N-terminal residues, so-called EAEA-lysozyme. The expression yield was about 300 mg/l (Goda et al., 2000). Another study showed high yield of sweet-tasting protein lysozyme, hen egg lysozyme (400 mg/l). The temperature was controlled at 28$^{o}$C, pH 5.0 and agitation speed at 900 rpm during fermentation (Masuda et al., 2005). Kumita et al. (2006) reported the secreted yield of the F57I and W64R variant human lysozyme as 0.04 mg/l and 0.3 mg/l, respectively.

### 1.6.3 Environmental stress in recombinant protein producing yeasts

Today, there is more information which has shown that different stress conditions of host cells can influence the overexpression of foreign genes including metabolic stress and environmental stress. Metabolic stress clearly interferes with the gene copy number and transcription process, environmental stress such as temperature, osmolarity, pH and oxygenation is employed in cultivation conditions with the aim of maximizing fermentation products (Minning et al., 2001, Shi et al., 2003, Mattanovich et al., 2004, Wang et al., 2009, Murasugi, 2010).

One of the most important factors affecting recombinant protein production is temperature. At lower cultivation temperatures, *P. pastoris* is able to improve recombinant productivity, even though optimum temperature for growth is 28-30°C (Mattanovich et al., 2004, Zhao et al., 2008). As a result of proteolytic activity of host cell secreted into the fermentation medium, Jahic et al (2003) used temperature limited fed-batch technique at 12°C increasing yield and successfully to reducing extracellular proteolysis with lower cell death rates compared with traditional methanol limited fed-batch technique at 30°C. Wang et al (2009) reported various effects of temperature on polygalacturonate lyase production. Higher yield of enzyme (931 U/ml) was obtained at the reduced temperature condition (22°C) and showed higher cell viability. These similar positive effects were also obtained from another experimental result by lowering growth temperature from 30 to 25°C. The specific productivity of the 3H6 Fab fragment was increased 3-fold and significantly increased biomass yield (Dragosits et al., 2009) Additionally, this experiment also revealed the temperature effect on the cellular pathway. There were several physiological parameters involved in higher expression levels at low temperature, including energy metabolism, oxidative stress response, protein folding, amino acid metabolism and RNA/ribosomal biogenesis. These affected are able to be an additional explanation. At lower cultivation temperatures, flux of the TCA cycle, chaperone molecules and protein levels in oxidative stress response were reduced.

For the production of heterologous proteins in yeasts, adjusting the pH, is important as it differs from optimum pH for growth. Thus from a practical perspective the aim should be to minimize the proteolytic effect (Scorer et al., 1993, Jahic et al., 2003) or enhance the expression level (Shi et al., 2003). This depends on the type of heterologous protein. *P. pastoris* expresses several types of extracellular protease such as aspartic, cysteine and serine-type which their enzyme kinetics is influenced strongly by pH (Shi et al., 2003). In this context, several strategies have been investigated. Jahic et al (2003) successfully utilized the combinations of low pH (pH 4) and low temperature (22°C) which can functionally stabilize protein product by increasing concentration of full-length product 3.3 times. The concentration of final product also increased (1.5 g/l) when compared with the original conditions (pH 5, 30°C). However, Hohenblum et al., (2003) indicated that at low pH (3.0) during the induction phase strongly influenced and decreased cell viability. Thus, it can be implied that this fermentation condition results in stress induction in the cells. Clearly, optimum pH is product dependent not only identified by stability properties of product, but also by proteolytic activity.

*P. pastoris* is a xerotolerant yeast and thus is able to grow over a wide range of water availability conditions. To overcome osmotic stress problem, the yeast cell produces various compatible solutes; for example, glycerol, arabitol and mannitol which accumulate in different amount intracellularly. Increasing osmolarity creates significant impact on several cellular processes: in particular, protein folding, ribosome biogenesis and cell wall organization. These data indicated that *P. pastoris* showed less adaptation during cultivation in high osmolarity media (Dragosits et al., 2010). However, it has been previously reported that pre-induction osmotic stress resulted in a dramatic increase in scFv antibody production in *P. pastoris*.

Pre-induction osmotic stress with potassium acetate at low pH also resulted in a noticeable increase in productivity when compared with no aw depressor (Shi et al., 2003). Furthermore, Surribas et al. (2007) compared several strategies to improve productivity of *Rhizopus oryzae* lipase from *P. pastoris*. Using a lower salt medium with methanol non-limited fed batch and temperature limited fed-batch increased product purity 1.3 times caused by cell death reduction.

It is thus surprising that there is little knowledge of the relative impact of environmental stress ($a_w$ x temperature x pH) on growth and lysozyme production by this yeast species. It is possible that by manipulating these factors in the growth phase this may enable higher expression of lysozyme when the methanol is added in the production phase. This has not previously been studied.

## 1.7 Objectives

The aim of this study was to determine and understand the different of environmental factors influence: (a) growth and taxol production by *Paraconiothyrium variabile* and *Epicoccum nigrum* and (b) growth and human lysozyme production by *Pichia pastoris*. The research compared the production of secondary metabolite by the filamentous fungi with lysozyme production by a single cell yeast.

The following studies have been carried out:

***Taxol production***

- Isolation and screening taxol producing strains.
- Effect of ecophysiological factors on growth of taxol producing strains
    - Effect of interactions between water activity, pH, types of solutes and temperature on growth of strains of *P. variabile* and *E. nigrum*
- The application of response surface methodology to predict the radial growth rate of strains of *P. variabile* and *E. nigrum*
- Effect of ecological interacting factors on taxol production by endophytic strains
    - Effect of water activity, types of solutes and temperature on taxol production
    - Stimulation of taxol production by elicitors
    - Effect of elicitor, water activity and pH on taxol production.

***Lysozyme production***

- Screening factors for lysozyme production by *Pichia pastoris*
    - Effect of water activity, temperature and types of solutes on growth optima and ranges for *P. pastoris*
    - Effect of interaction between water activity, pH and solutes on growth of *P. pastoris*
    - Screening factors for lysozyme production by factorial design
    - Influence of buffer systems on lysozyme production

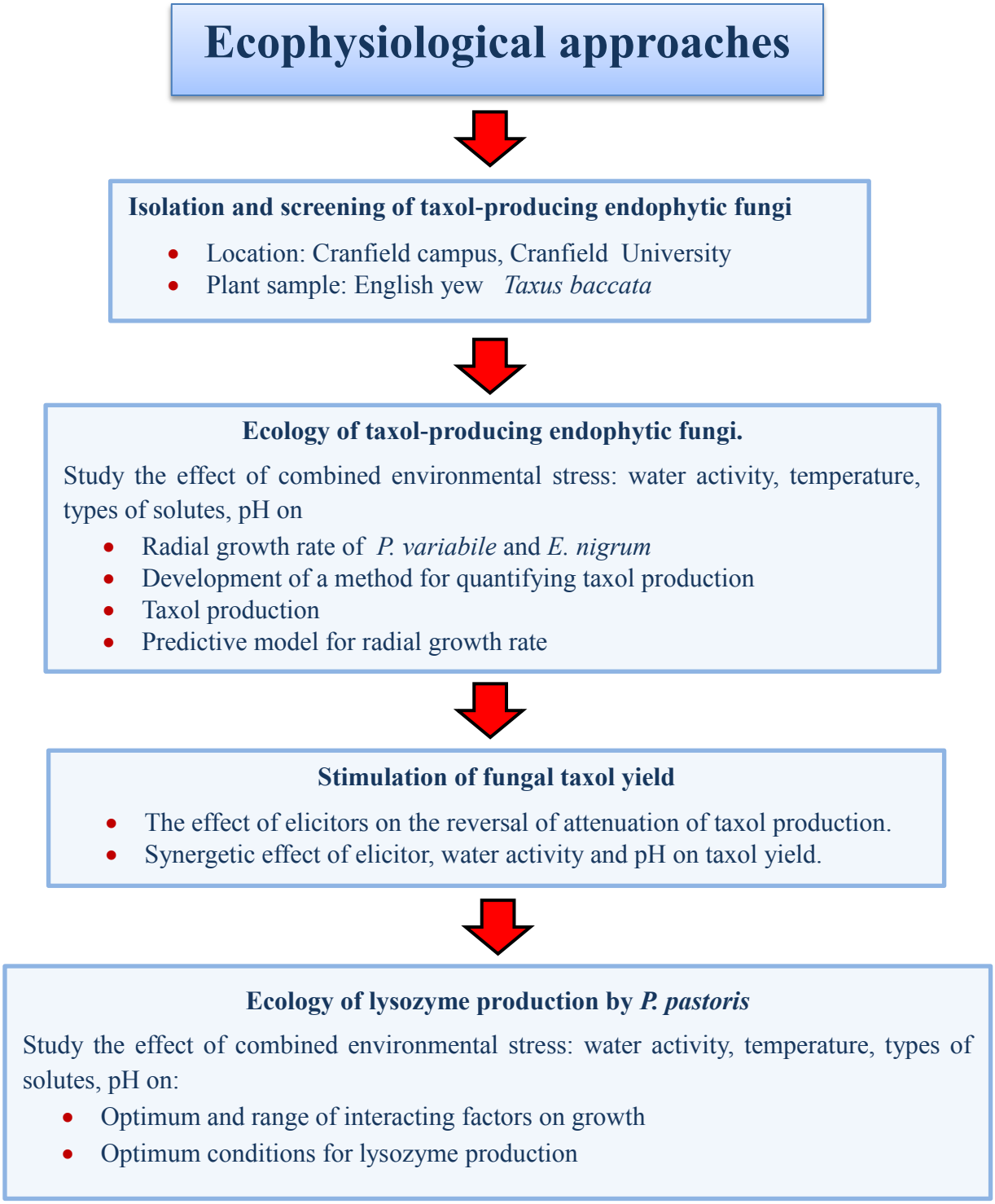



Figure 1.6. A schematic diagram represents the experimental work carried out in this thesis.

# 2 Isolation and screening of taxol producing strains

## 2.1 Introduction

Many plant species in natural ecology are infected by numerous fungal endophytes. Individual leaves can contain up to 90 species in tropical trees (Arnold et al., 2003; Bayman, 2006). These endophytic fungi can live asymptomatically within either above-ground or below-ground plant tissues including leaves, stems, bark and/or roots (Lambers et al., 1998; Carlile et al., 2001; Strobel and Daisy, 2003). The association between plants and endophytic fungi are extremely ancient which date back more than 400 million years in the early Devonian period and created selection pressure for the evolution of land plants (Krings et al., 2007).

Fungal endophytes are also well known as important producers of bioactive compounds over a long period. The discovery of new species of endophytic fungi with novel bioactive metabolites have included antimicrobial compounds, enzymes and anti-cancer drugs (Rodrigues et al., 2000; Hazalin et al., 2012; Radic and Štrukelj, 2012; Zhang et al., 2012; Zaferanloo et al., 2013). Moreover, some endophytes have the ability to produce the same or similar host plant compounds which have a high potential for medical use. The most famous natural bioactive product which created the most interest in fungal endophytes as potential bio-factories of value added secondary metabolite is taxol. A novel taxol-producing endophytic fungus, *Taxomyces andreanae,* was isolated from the Yew tree (*Taxus breviofolia*) in 1993 (Stierle et al., 1993).

Taxol, a diterpenoid potential anticancer agent, was originally isolated from the plant parts of *Taxus brevifolia* (Wani et al, 1971; Wall and Wani, 1995; Wall, 1998; Brown, 2003). Although it has significant anti-cancer properties, taxol has also been studied for its potential therapeutic properties against non-cancerous diseases, including neurodegenerative diseases and polycystic kidney disease (Woo et al., 1994; Zhang et al., 2005). Because of the increasing global demand for anti-cancer drugs, it is estimated that the global market for taxol sales was about $ 4.5 million US dollars in 2011, and

could reach $ 200 million in 2013 (Yvon, 2012). The World Health Organization (WHO) has estimated that more than 11.5 million cancer deaths worldwide is expected to occur by 2030 (WHO, 2011). Therefore demand for taxol will continue to rise. Unfortunately, taxol is an expensive drug and thus not very accessible to many people worldwide. The chemotherapy treatment depends on several factors including stage of cancer, time and concentration of the anti-cancer drug (Stephens and Aigner, 2009). For example, with newly diagnosed ovarian cancer costs around $ 4,989 US dollars for 6 cycles of standard chemotherapy per patient (Havrilesky et al., 2012).

Not surprisingly, the drug-discovery scientists have been looking for alternative sources to produce taxol with a lower price and ensure wider availability. Fungal endophytes are one of the additional resources that have attracted significant interest since they are able to grow and produce high value phytochemical products under controlled fermentation conditions (Strobel et al., 2004; Kusari et al., 2012).

The objectives of this study were to (a) isolate endophytes from temperate English Yew trees (*Taxus boccata*) and (b) screen them for their ability to produce taxol using different media.

## 2.2 Material and method

### 2.2.1 Endophytes of *Taxus baccata*

The healthy twig samples of English yew (*Taxus baccata*) were collected from Cranfield University, Cranfield campus, Bedfordshire, UK (N 52° 04.380 W 0° 37.794 and N 52° 04.307 W 0° 37.780). These samples were cleaned under running tap water to remove debris and then air-dried. Cleaned twigs were cut into small pieces approx. 6 cm long. Surface-sterilization was performed as described by Kumaran et al (2009) with some modifications. Plant fragments were immersed in 70% ethanol for 5 sec followed by rinsing with sterile distilled water for 1-2 min (three times). The surface-sterilized twig fragments were cut into small pieces in two direction (cross section,

longitudinal sections) using a sterilized blade. These were then plated on to the surface of 9 cm diameter Petri plates. The media used to isolate the endophytes included PDA (potato dextrose agar), MEA (malt extract agar), CMA (corn meal agar), MS agar (Murashige and Skoog agar) and water agar. The samples were incubated at 25°C and checked daily for hypha growth. The tips of fungal hypha were selected based on morphological characteristics cut and sub-cultured on PDA. The isolated fungi were given code numbers and stored on PDA at 4°C.

### 2.2.2 Identification of endophytic fungi

The preliminary identification was based on morphology of the fungal colony on the different types of media and characteristics of the spores. Microscopic morphology of fungi was examined and photographed by using the environmental scanning electron microscope at School of Applied Science, Cranfield University, UK. Isolated strain was grown on PDA, Oat meal agar, CMA and MEA medium at 25°C for 7 days and incubated under UV light for 2 days at 25°C to stimulate sporulation. The molecular identification of the fungi was confirmed by Professor Hania Kwaśna (Department of Forest Pathology, Poznań University of Life Sciences, Poland) by sequencing the ITS1 (internal transcribed spacer region 1) and ITS2 regions.

### 2.2.3 Culture, extraction and isolation of taxol

Three agar plugs (5 mm diameter) containing mycelia of isolated fungus were used as the inoculum. The tested strains were cultured in modified M1D ($Ca(NO_3)_2$,1.20 mM; $KNO_3$, 0.79 mM; KCl, 0.87 mM; $MgSO_4$, 3.0 mM; $NaH_2PO_4$ $H_2O$, 0.14 mM; Sucrose, 87.60 mM; Ammonium tartrate, 2.10 mM; $FeCl_3$ $H_2O$, 7.4 µM; $MnSO_4$, 30.0 µM; $ZnSO_4$ $7H_2O$, 8.7 µM; $H_3PO_4$, 2.2 µM; KI, 4.5 µM; yeast extract 0.25 g/l, pH 5.5) culture medium (250 ml) supplement with soytone (1g/l) (Pinkerton and Strobel, 1976) for 21 days at 25°C. The fungal mycelia were separated from the broth by filtration. This filtered culture was subsequently extracted by adding dichloromethane in two equal volumes of culture broth (Strobel, 1996). The extracted solvent was evaporated by

using a rotary evaporator to dryness at 35°C (Eyela, Tokyo, Japan). The dry residue was re-dissolved in 5 ml of 100% dichloromethane and then subjected to a 15 ml SPE column (silica gel; Thermo Scientific, UK) which was eluted in a stepwise elution. This SPE cartridge cleanup was developed in this study. Previously, the recommended purification method for fungal taxol was the column chromatography which required the large size of column (1.5x30 cm). This method is always time consuming, gives poor recovery and uses plenty of solvents. By the developed SPE cleanup, 20 samples can be cleaned up in less than 2 h and high recovery. Figure 2.1 shows the comparison between the traditional method and SPE cleanup method.

The elution was carried out starting with 15 ml of 100% dichloromethane and then continued with 15 ml of dichloromethane:ethyl acetate in different solvent ratios (20:1 v/v, 10:1 v/v, 6:1 v/v, 3:1 v/v, 1:1 v/v, 100% ethyl acetate). Based on the taxol standard mobility, the last four of the elution fractions were combined prior to evaporation to dryness. The dry residue was dissolved in 1 ml methanol. All samples were filtered through 0.2 µm nylon filters before further analysis with TLC, LC-MS and HPLC. The standard taxol (Paclitaxel) was purchased from SIGMA.

### 2.2.4 Thin layer chromatographic analysis

Chromatography was performed on 0.25 mm (20 cm x 20 cm) aluminum precoted silica gel plates from Merck (Germany). The authentic taxol and fungal samples were spotted on TLC plates manually, this plate was then developed in different solvent systems including: A, (chloroform/methanol, 7:1 v/v); B, (chloroform/acetonitrile, 7:3 v/v); C, (ethyl acetate/isopropanol, 95:5 v/v); D, (methylene chloride/tetrahydrofuran, 6:2 v/v) and E, (methylene chloride/methanol/dimethylformamide, 90:9:1 v/v), respectively. After gentle heating, taxol was detected after spraying the reagent consisting of 1% (w/v) vanillin in sulfuric acid. Leaving this sprayed plate for 24 h, a dark blue spot turned into a dark grey colour (Cardellina, 1991).

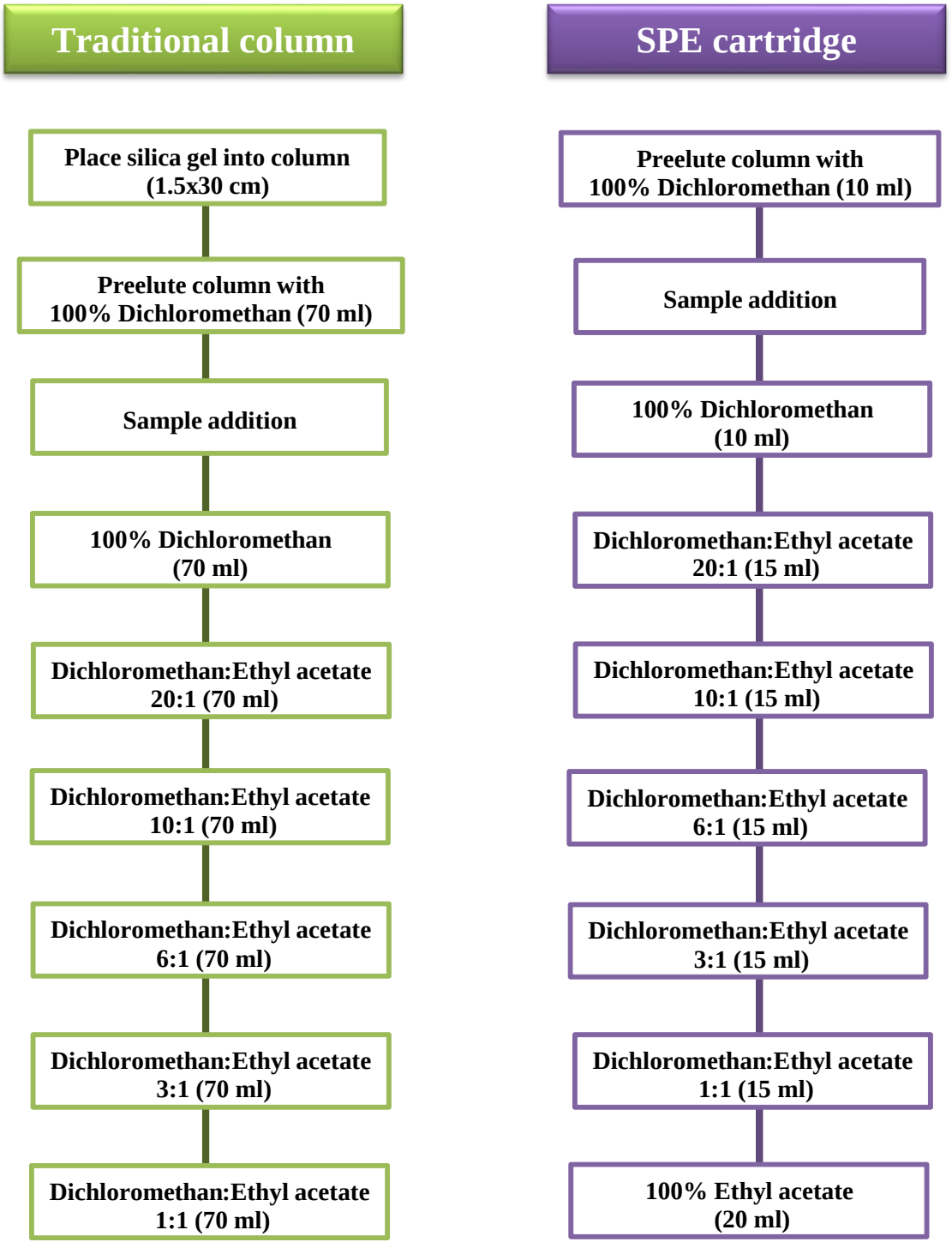



Figure 2.1 Comparison the elution scheme for purification of fungal taxol between (a) traditional column and (b) SPE cartridge.

### 2.2.5 High performance liquid chromatography analysis

Taxol in samples were analyzed by HPLC (Algilent 1200, Agilent Technologies, USA) with a C18 analysis column (Agilent Zorbax Eclipse, Part No. 990967-902). The sample (50 µl) was injected each time and detected at 230 nm. The mobile phase was methanol:water (80:20 v/v) at a flow rate of 1.0 ml/min.

### 2.2.6 Liquid Chromatography-mass spectrometry analysis (LC-MS/MS)

The instruments used in this experiment including an Agilent 1290 infinity LC system which was coupled to an Agilent 2640 quadrupole time-of-flight (Q-TOF) MS system equipped with a Jetstream$^{TM}$ electrospray ionization (ESI) source (Agilent Technologies, USA). For LC conditions, the separation was performed with an Agilent Poroshell® 120 (2.7 µm particle size, 100mm x 4.6 mm) column. The mobile phase of a mixture of (A) methanol with 0.1% formic acid and (B) water with 0.1% formic acid was used delivering at 0.5 µl/min in gradient mode as follows: 0-2 min, A/B (40/60, v/v) to 90% A from 2-18 min. A Jetstream$^{TM}$ ESI was operated in positive ionization mode and the spectra were recorded from m/z 50 to 1200 with a 1.0 s/sec scan rate. Needle voltage was set at 4 kV, drying gas and sheath gas temperature at 325 and 350°C, respectively.

## 2.3 Results

### 2.3.1 Strains and production of taxol

More than 200 endophytic strains were isolated from surface streilised segments. In addition the three strains of species from the Culture collection were also examined for taxol production. Overall only two strains were able to produce taxol. Of the two species from the CBS culture collection none were able to produce taxol although they were isolated form Yew trees (*Pestalopsis microspora* CBS790.68 and *Pestalotiopsis microspora* CBS332.76).

### 2.3.2 Morphological characterization and identification of the two taxol producing strains

The fungal endophytes were isolated from various plant parts of the English Yew trees (*Taxus baccata*) at different locations on the Cranfield Campus. Two strains were observed to produce taxol. By analysis of the sequence of ITS1 and ITS2, these isolated endophytes were identified as *Paraconiothyrium variabile* and *Epiccum nigrum*.

*P. variabile* showed excellent growth on most standard mycological media including PDA, CMA, MEA and oatmeal agar (OMA) (Figure 2.2). The highest radial growth rate of 3.37±0.04 mm $day^{-1}$ was obtained when cultured on PDA at 25°C. The aerial mycelium was floccose and white in colour on PDA. On MEA colonies grew to 44-48 mm in diameter after 7 days with a floccose surface texture grey in the centre and white in the younger outer colony area. The colony texture on OMA appeared smooth and slightly granular in the center with white mycelium. The colony margin of *P. variabile* usually was even and circular in shape.

Micromorphology observations showed that sporulation was stimulated by near UV light producing pycnidia in 3-4 weeks visible in the granular mycelium and becoming dark brown with age. The slimy masses of pycnidiospores protruded through the ostiole

of the pycnidia (Figure 2.3), which then accumulated above the pycnidia as a white droplet at the beginning and developed to dark brown over the time. The majority of pycnidiaspores were smooth, cylindrical in shape and unicellular.

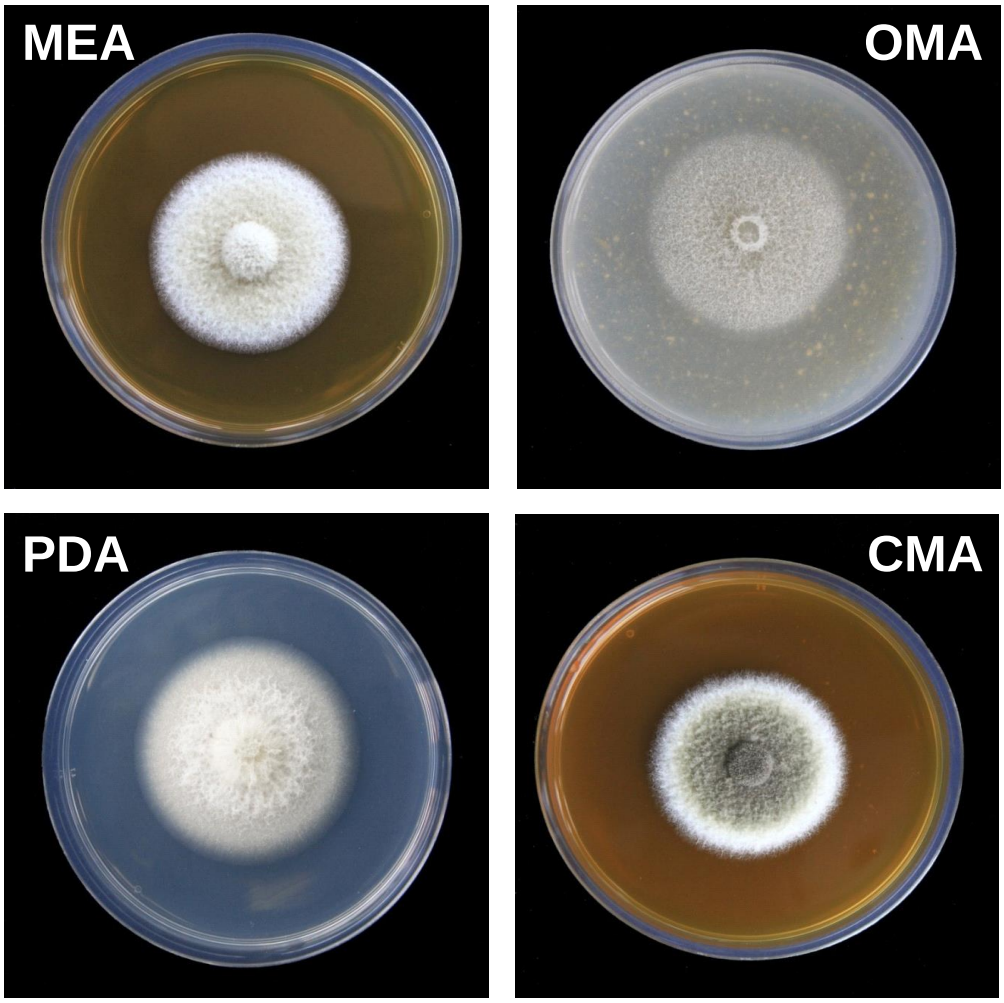



Figure 2.2 *P. variabile* colonies growth at 25°C for 7 days on MEA, OMA, PDA and CMA agar.

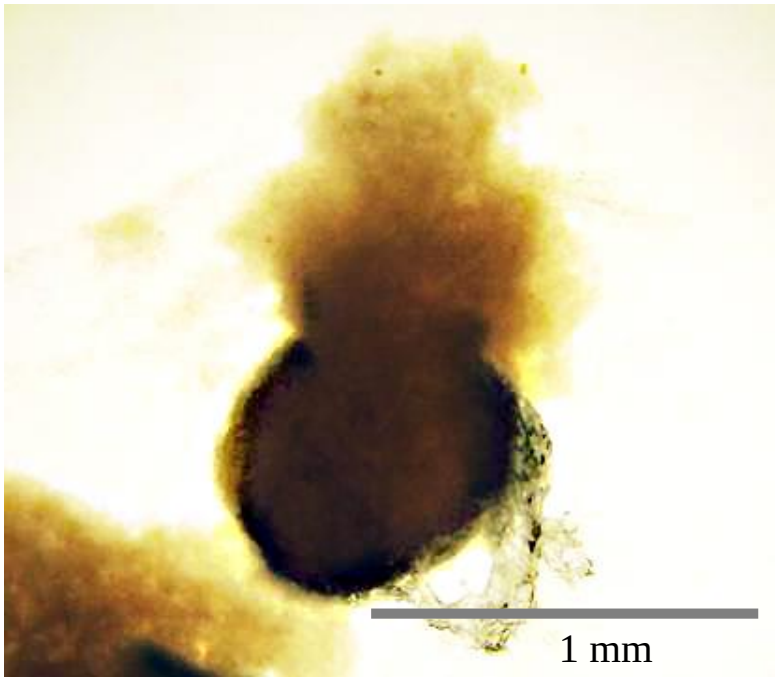



Figure 2.3 Microscopic morphology of *P. variabile* showed the pycnidia under light microscope.

Colonies of *E. nigrum* on CMA at 25°C had faster growth than on the other media tested with a rate of 3.89±0.07 mm $day^{-1}$ (diameter 70-75 mm after 7 days). Macroscopically, the colonies showed the deep radial sulcations and white to pale brown in colour with velvety to woolly texture. The undulate margin of colony was observed. This strain also exhibited rapid growth on most standard mycological media (Figure 2.4). Growth on MEA resulted in colonies with 63-67 mm in diameter after 7 days. Surface texture was woolly with white mycelium from outer and became yellow-orange coloured with age. The moderate radial sulcations were produced with a slightly undulate margin. The colonial texture on PDA appeared woolly textures, white coloured at first, which develops into various shades of orange over the time. The outer fringe remains white. Difference in surface of the colonies was distinct on OMA where granular surface texture appeared in the center with yellow in colour.

*E. nigrum* also accumulated characteristic yellow-orange exudate droplets on the mycelium (MEA and PDA) and dark brown exudates on surface in CMA. The colony reverse was coloured more intensely yellow-orange diffusible pigments (Figure 2.5). The micromorphology observation showed the sporodochia (aggregated conidiophores) which appeared in 3-4 weeks as black spots (Figure 2.6). Conidiophores were developed from a wider hypha, often branched where blastoconidia were formed in a cluster. The conidia were multi-celled, pale brown at first and covered with rough thick-wall, dark brown coloured when mature. Mostly globuse to pyriform in shape with a funnel-shape base and the truncated basal scar were observed (Figure 2.7).

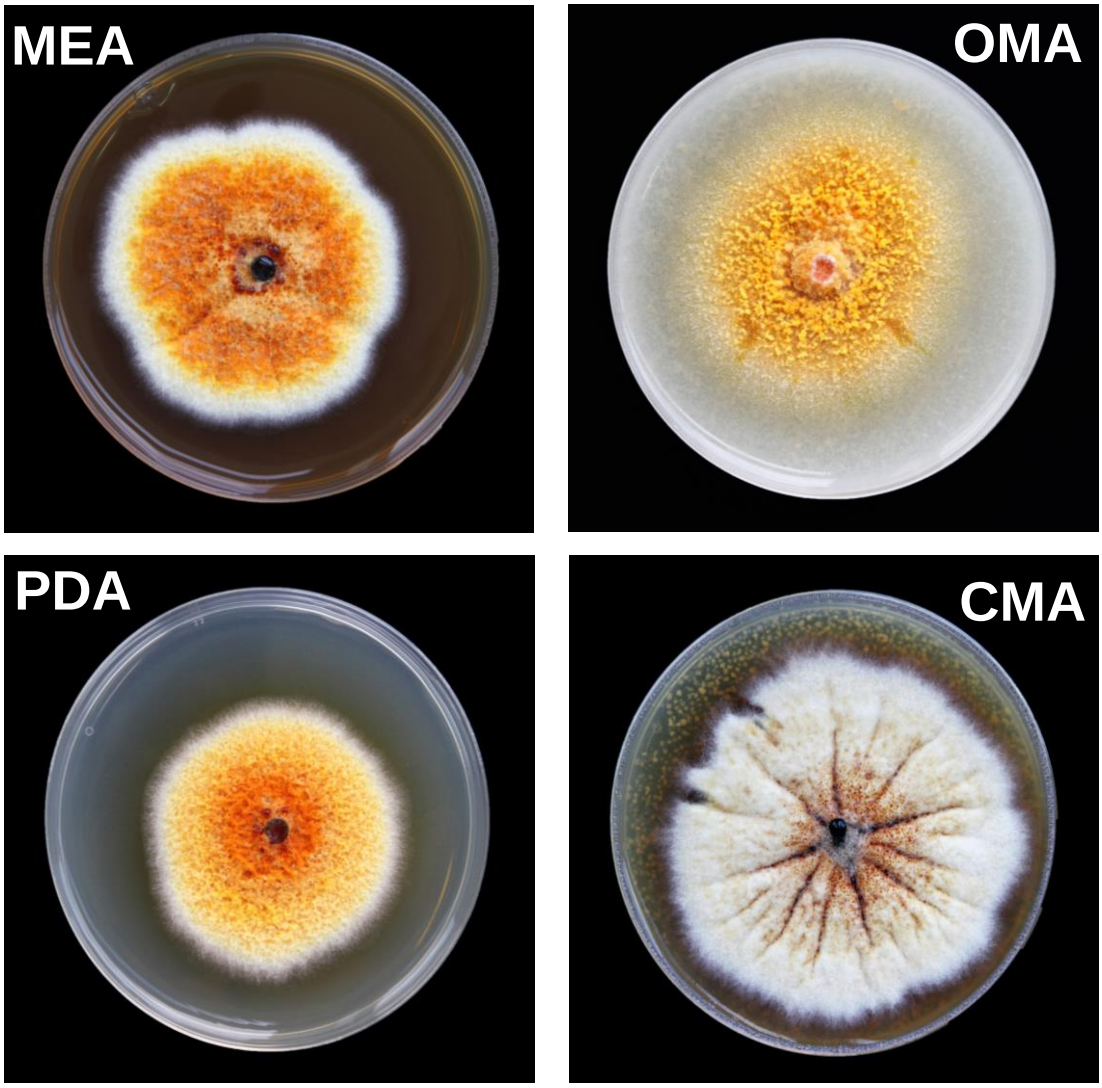



Figure 2.4 *E. nigrum* colonies growth at 25°C for 7 days on MEA, OMA, PDA and CMA agar.

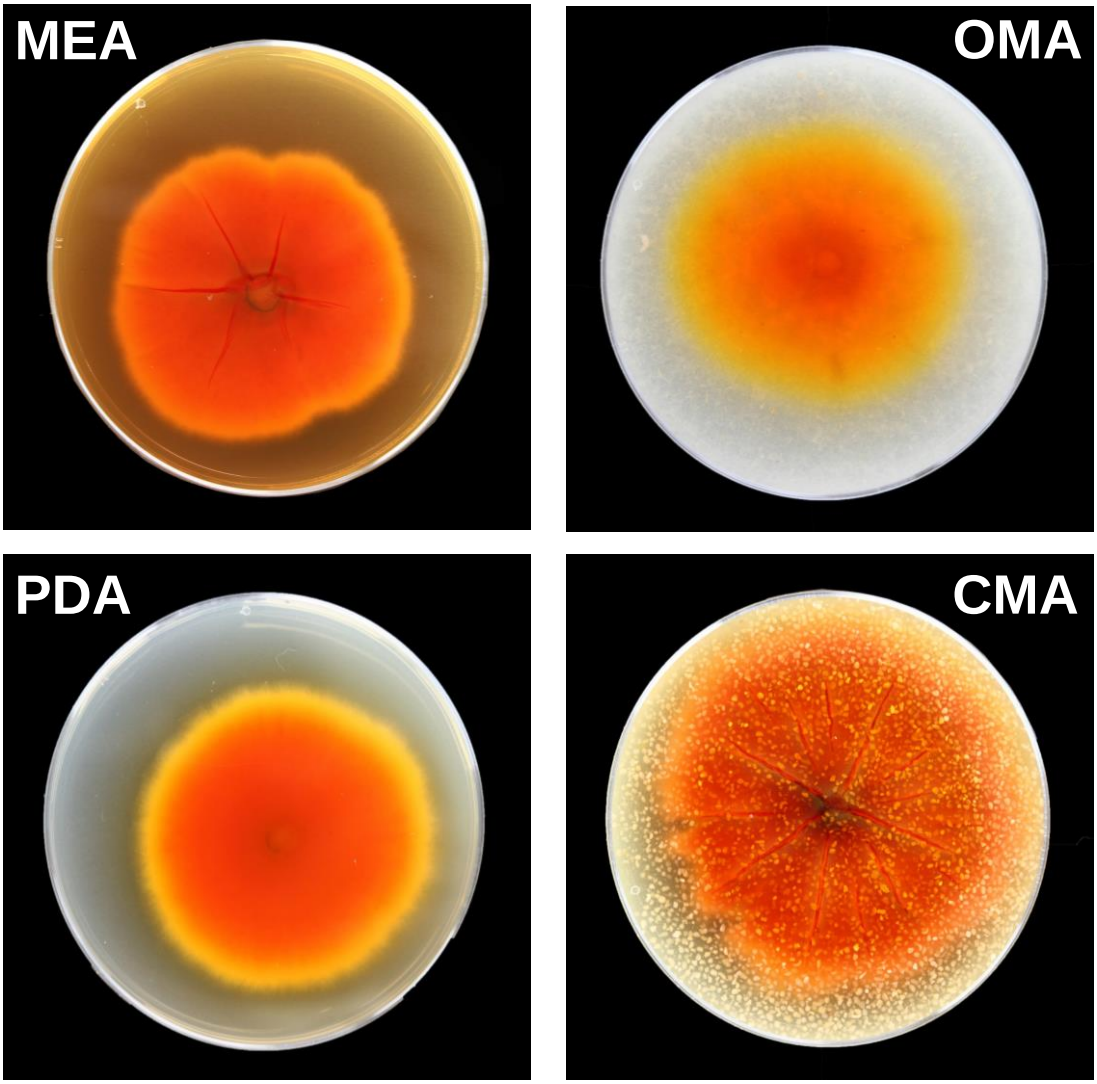
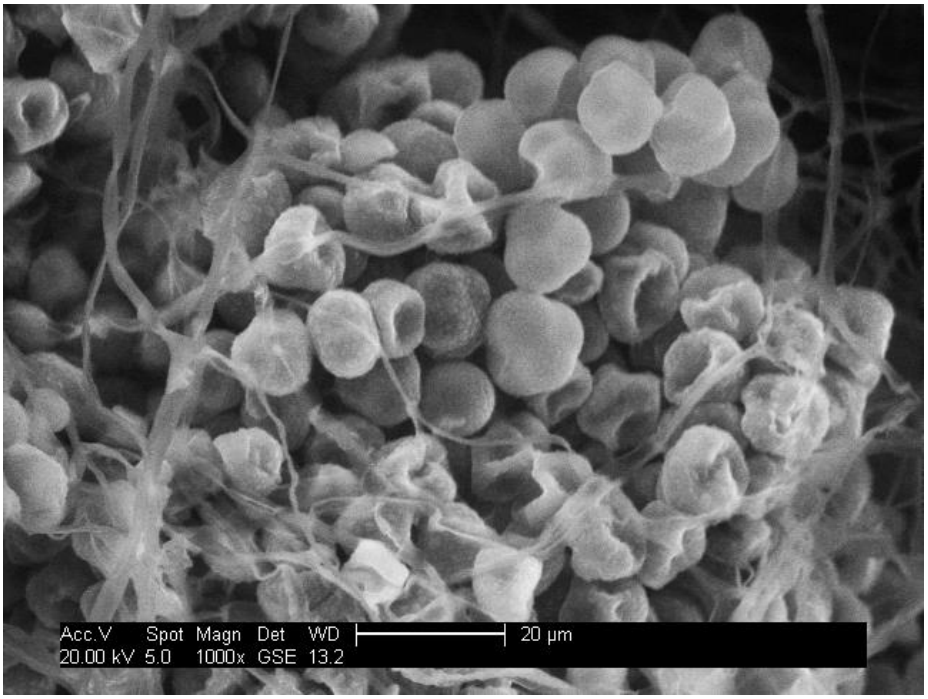



Figure 2.5 Colony reverse of *E. nigrum* growth at 25°C for 7 days on MEA, OMA, PDA and CMA agar.



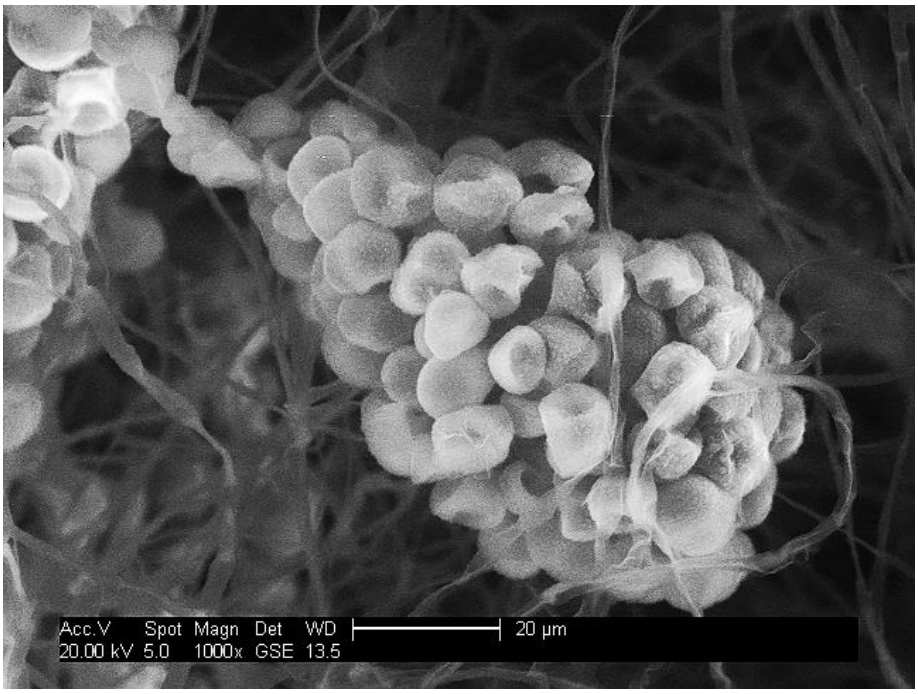




Figure 2.6 Microscopic morphology of *E. nigrum* showing the scanning electron micrograph of the mature sporodochium (aggregated conidiophores) covered with conidia.

(a)


(b)

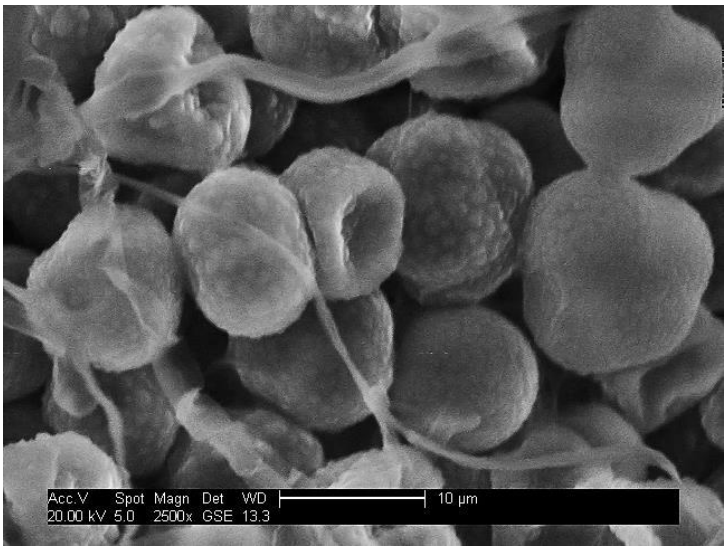



Figure 2.7 Microscopic morphology of *E. nigrum* showing (a) the blastoconidia and condia from light microscopy (b) the scanning electron micrograph of the sporodochia (aggregated conidiophores).

### 2.3.3 Chromatographic and spectroscopic study

TLC analysis was used to compare chromatographic properties of the taxol extracts with the standard which was displayed as a dark grey colour after spraying with vanillin/sulfuric and exhibited an $R_f$ value identically. Results of HPLC analysis confirmed the presence of taxol when compared with the taxol standard. The retention time of fungal taxol from *P. variabile* was 8.928 min which a retention time peak for taxol standard was 9.044 min (Figure 2.8). The amount of fungal taxol from this strain was calculated from the peak area of taxol standard. This isolated strain could produce 0.53 to 1.75 µg/l taxol in M1D liquid medium. For the *E. nigrum* strain, HPLC analysis also provided evidence of fungal taxol at a retention time peak of 9.206 min (taxol standard 9.105 min) with the amount of taxol 1.32 µg/l (Figure 2.9).

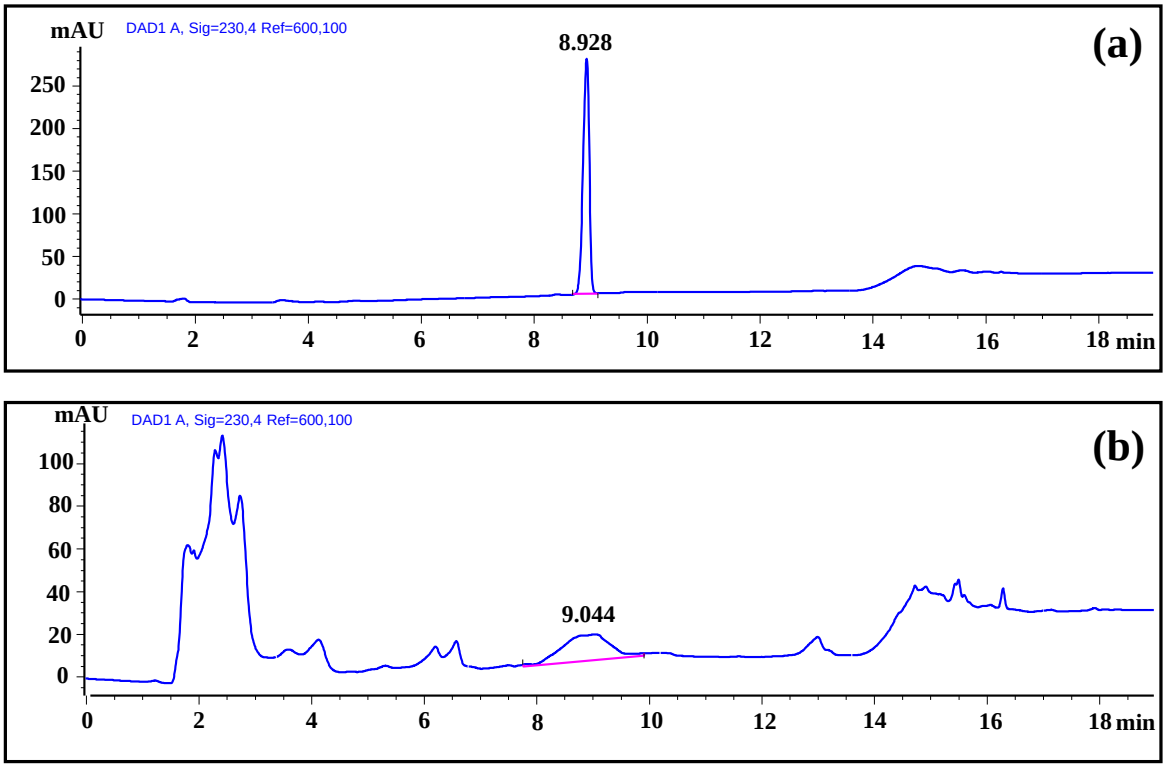



Figure 2.8 HPLC chromatogram of (a) taxol standard and (b) fungal taxol extracted from *P. variabile* grown on M1D medium for 21 days.

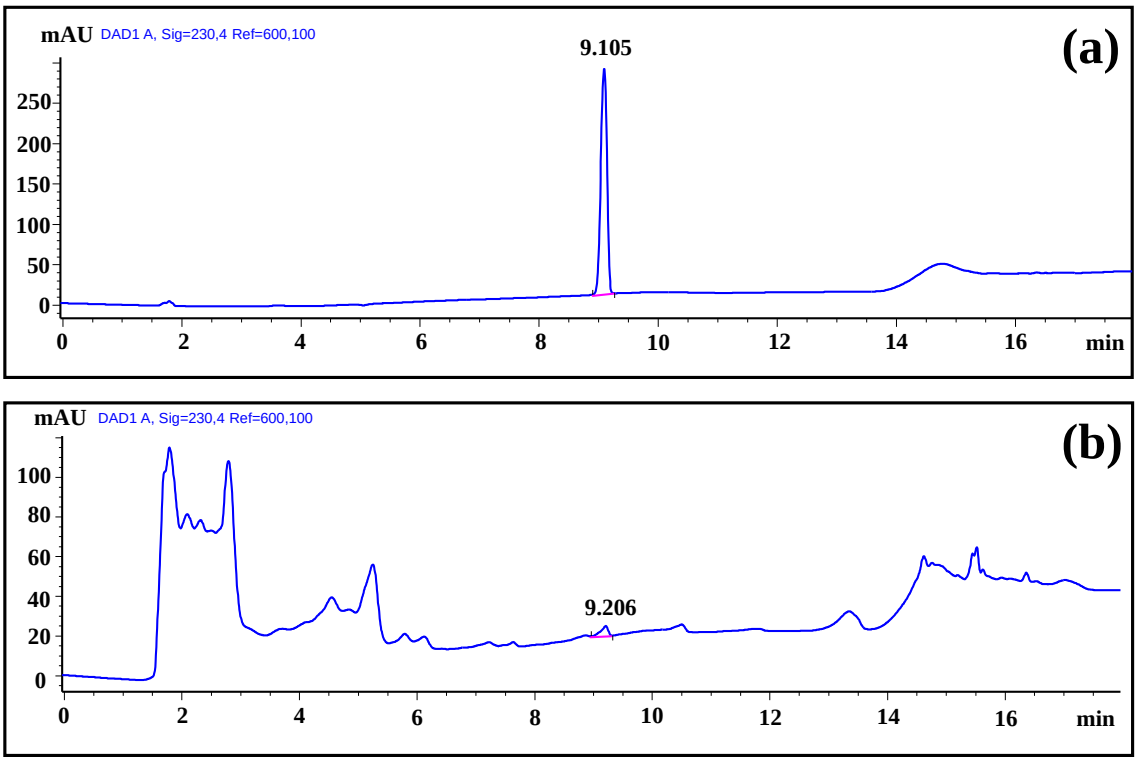
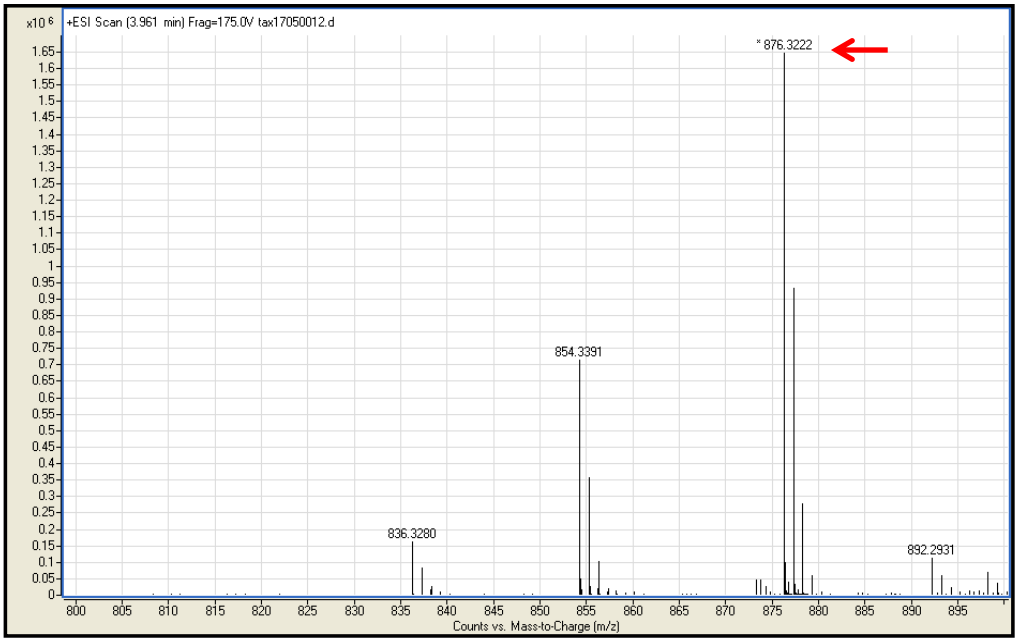



Figure 2.9 HPLC chromatogram of (a) taxol standard and (b) fungal taxol extracted from *E. nigrum* grown on M1D medium for 21 days before extraction.

As further evidence, LC-MS/MS was used to confirm the presence of fungal taxol. In LC-MS analysis with electrospray positive ionization ($ESI^+$) mode revealed that the dominant ion was an ($M+Na^+$) adduct ions at *m/z* 876.32, while an ($M+H^+$) ion with mass-to-charge ratios (*m/z*) 854.33 as the second dominant ion for taxol standard (Figure 2.10a). From this spectrum, the $M+Na^+$ adduct ions were selected as the parent ion for further passes through the first analyzer and then became fragmented in a collision cell. These daughter ions eventually analyzed by the second analyzer to produce the following MS/MS spectrum (Figure 2.10b). At $ESI^+$ mode with low energy (25 V), standard taxol and fungal taxol from *P. variabile* (Figure 2.11) displayed consistent mass spectra with *m/z* 876.3 ($M+Na^+$) adduct ions.

(a)




(b)

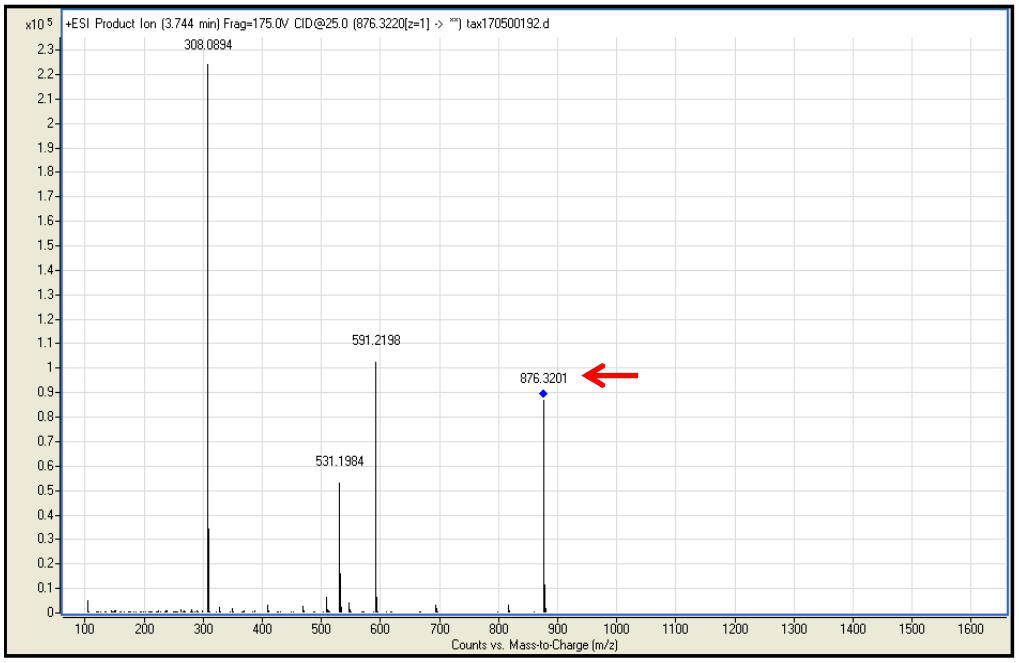



Figure 2.10 The mass spectrum of parent ions of (a) taxol standard ([M + $Na^+$] = 876.32 m/z) from LC-MS analysis, (b) parent ions and daughter ions of taxol standard from LC-MS/MS analysis by positive $ESI^+$ modes.

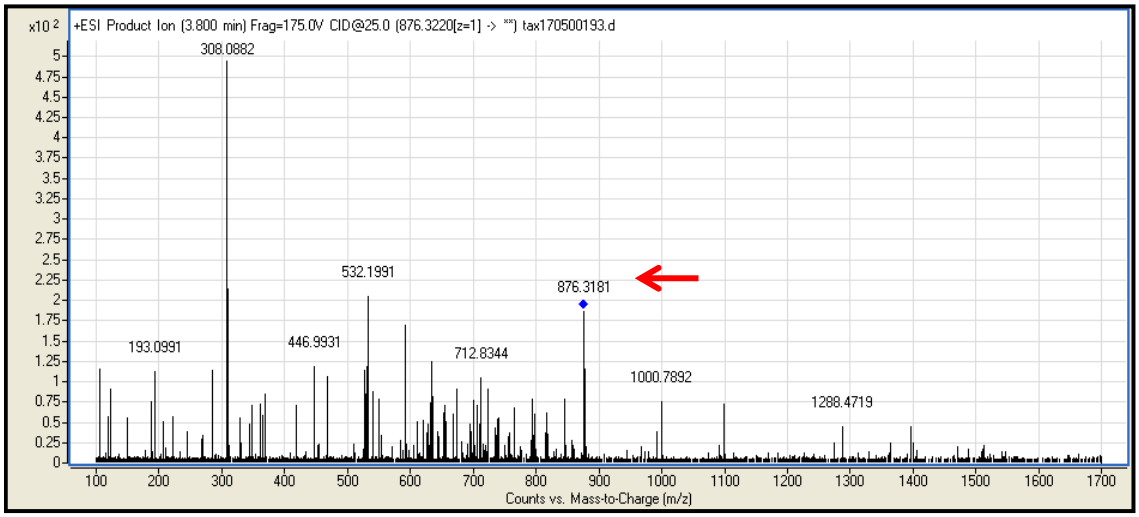



Figure 2.11 The LC MS/MS spectrum of fungal taxol from *P. variabile* showing the ($M+Na^+$) with *m/z* 876.318 by $ESI^+$ modes.

The extracted ion chromatogram (EIC) from in LC-MS with $ESI^+$ mode of fungal taxol from *E. nigrum* and taxol standard are shown in Figure 2.12 which were observed the intensity of the signal at ($M + Na^+$) m/z 876.32. This chemical analysis revealed a single peak of fungal taxol with a retention time of 13.661 min which was close to taxol standard at 13.565 min. An LC-MS chromatogram in $ESI^+$ mode of this sample also provided the valuable evidence to confirm the production of taxol by *E. nigrum* when compared with the taxol standard (Figure 2.13). The results showed the consistent mass spectra with *m/z* 876.3 ($M+Na^+$) between taxol standard and fungal taxol.

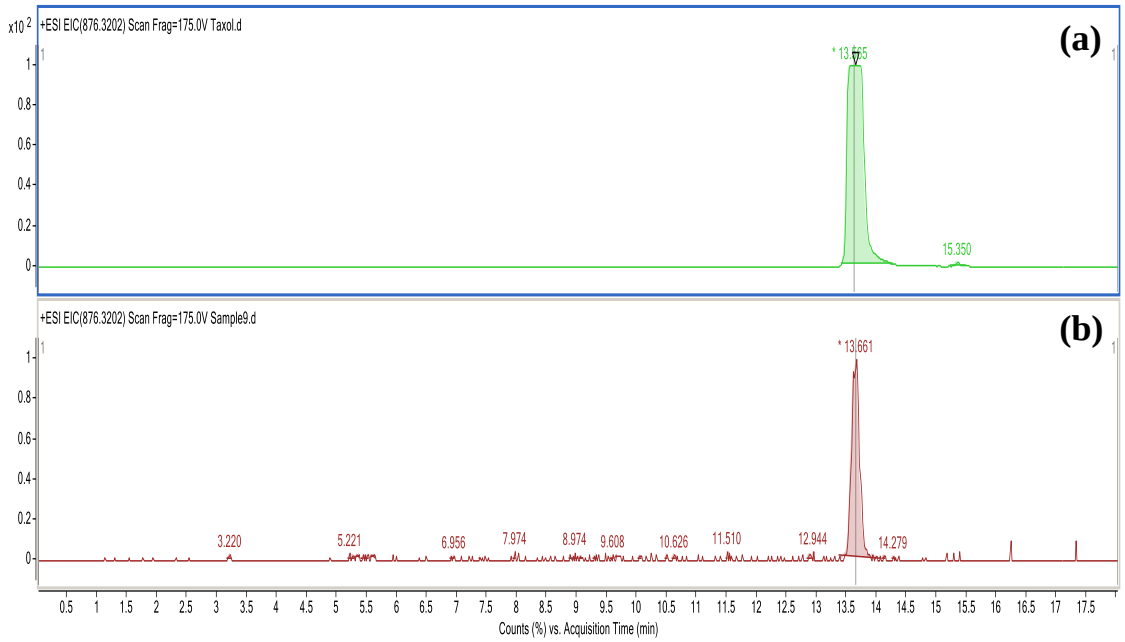



Figure 2.12 Extracted ion chromatogram from LC-MS analysis in $ESI^+$ mode of (a) taxol standard ([M + $Na^+$] = 876.32 m/z) and (b) fungal taxol from *E. nigrum.* The retention times are indicated on the top of the chromatographic peaks.

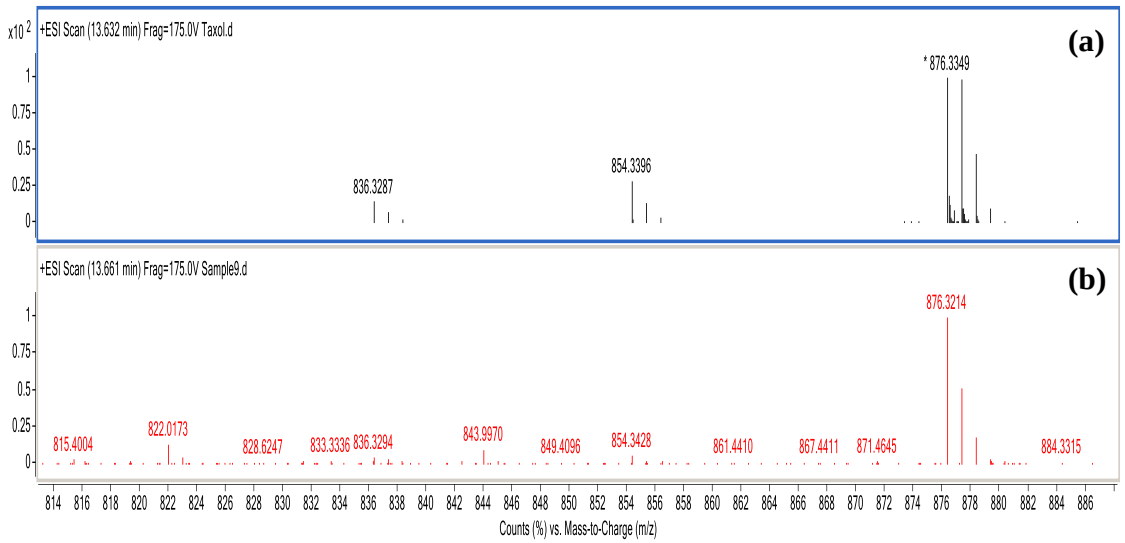



Figure 2.13 The mass spectrum from LC-MS analysis of (a) taxol standard and (b) fungal taxol from *E. nigrum* showing the ($M+Na^+$) with *m/z* 876.318 by $ESI^+$ modes.

## 2.4 Discussion

According to the observed endophytic fungi diversity inside plant tissues from a few locations in this study, it could be reflected that there are opportunities to discover novel fungal strains with interesting secondary metabolite production profiles because of the wide biodiversity of fungi in different plant types. Some tree species are likely to be hosts for hundreds of endophyte species in their tissues. From the investigation in one studied location; for example, 8 different fungal endophytic species (from twig samples only) in Yew trees were found (data not shown).

However, not all such fungal endophytic species were able to produce taxol, similar to those unique characteristic of their host plants trees (*Taxus baccata*). From this study, a number of this species of *P. variabile* were isolated and while possessing similar growth patterns they had no ability to produce taxol after screening on M1D broth medium (data not shown). Moreover, we also examined strains of endophytic fungi *Pestalopsis microspora* CBS790.68 and *Pestalotiopsis microspora* CBS332.76 from the CBS culture collection, Baarn, Holland, for the ability to produce taxol, as they were originally isolated from Yew tree. The results indicated an inability to produce any taxol (data not shown), although previous strains of these species have been reported to produce taxol (Stierle et al. 1996).

The ability to produce the same or similar host plant compounds was hypothesised the horizontal gene transfer of plant DNA into fungal genomes (Stierle et al., 1993). Recently, Xiong et al. (2013) reported a low similarity (<50%) of 2 key genes for taxol production pathway between plant and taxol-producing endophytes. They suggested that the ability of such endophytes might be involved from the initial coexistence between plants and endophytes during evolution. Thus, the horizontal gene transfer hypothesis should be further investigated where more key genes for taxol biosynthesis have been suggested to be involved in both plants and these endophytes. Moreover, 3 of 12 isolated endophytic fungi showed no ability to produce taxol, although the positive test in one key gene for taxol production was shown (Zhou et al., 2007). Additionally, some strains have been isolated from various host plants (non *Taxus spp*.)

which have the ability to produce taxol (Tan and Zou, 2001; Kumala et al., 2007; Gangadevi and Muthumary, 2008; Li et al., 2008; Kumaran et al. 2009ab; Gangadevi and Muthumary, 2009ab). Therefore, the hypothesis of taxol-producing endophytes might not be the only hypothesis to explain such a biosynthetic ability. It may be that taxol and other secondary metabolites help the endophytes remain asymptomatic in the plant tissue.

Many fungal endophytes have been found into classes of Ascomycota (Huang et al., 2001; Rodriguez et al., 2009; Tenguria et al., 2011). In this study a strain of *P. variabile* could produce taxol. It is commonly isolated from wood and leaves found across South Africa, Italy and Turkey. *Actinidia chinensis, Actinidia deliciosa, Laurus nobilis, Prunus persica* and *Prunus salicina* are the normal hosts. The maximum temperature for growth is 35°C and 20-25°C is the optimum (Damm et al., 2008). Several bioactive compounds were discovered from this endophytic genus (Shiono et al., 2011; Prado et al., 2013). Recently, a strain of *P. variabile* was shown to produce taxol. It was isolated from *Taxus media* in Canada and was found to be able to produce 40 µg/g dry weight of taxol (Soliman et al., 2011).

*E. nigrum* is very common phyllosphere species which acts as a saprophyte on crop residue and in soil on decaying plants (Samson et al., 2010; Webster and Weber, 2012). This strain can invade the living plant and become the endophyte which is commonly isolated from a wide range of plant species. Both monocotyledonous to dicotyledonous plants have been shown to have this endophyte present and strains have been found to produce a wide range of secondary metabolites (Larran et al., 2000; Da Silva Araujo et al., 2012; Wang et al., 2010). For cytotoxic activity from *E. nigrum*, there has been a report of the marine endophytes which exhibited cytotoxic activity against human bladder carcinoma cell line (Tarman et al., 2011). However, few studies have reported *Epicoccum sp*. as taxol producers with production of 0.02-0.05 ng/ml (Caruso et al., 2000). However, the impact of ecophysiological factors on the growth and taxol production by these two species has not been examined previously.

# 3 Effect of ecophysiological factors on growth of taxol producing strains

## 3.1 Introduction

The discovery of a novel-producing endophytic fungus, *Taxomyces andreanae* from the Pacific yew tree in 1993 (Stierle et al., 1993) was a significant factor that led to interest in the potential for producing taxol at a lower price by using fermentation systems. A number of studies have been published describing several strategies to improve growth and productivity of filamentous fungi (Gqaleni et al., 1997; Baxter et al., 1998; Parra et al., 2005). Media design and environmental conditions are often examined. There are several abiotic factors affecting growth and yield of secondary metabolites such as temperature, pH, $a_w$ and light (Deacon, 2006; Carlile et al., 2001; Magan and Aldred, 2007). Temperature and $a_w$ and their interactions are described as critical factors for fungal growth and metabolite production, especially secondary metabolites (Gqaleni et al. 1997; Cairns et al., 2005; Lahlali et al., 2005; Pardo et al., 2006; Magan, 2007).

The knowledge of the effect of these parameters on mycelial growth of *P. variabile* and *E. nigrum* is limited, especially those that produce taxol. Magan and Lacey (1984) reported the minimum $a_w$ (0.89 $a_w$) for growth of *E. nigrum* isolated from cereals. Growth under sub-optimal $a_w$ conditions, the decreased growth rate, decreaded resporation rate, increased oxygen uptake rate and increased lag times were the important effects (Wilson and Griffin, 1974). Moreover, metabolic activity shifted from growth to biosynthesis of compatible solutes when grew at low $a_w$. At optimal temperature for growth, the greatest tolerance to lowered $a_w$ were observed (Aldred et al., 1999; Magan, 2007).

The statistical design of experiments including response surface methodology (RSM) have been successfully applied to investigate the combined effects of these abiotic factors providing the useful information about ecological niches of different fungi

(Pardo et al., 2004; Parra et al., 2005; Setti et al., 2011). RSM is a collection of statistical techniques which includes factorial designs and regression analysis. The typical applications of RSM include determining the optimum operation conditions, demonstrate a response surface over some certain region of interest and selection of operating conditions to achieve desired specification (Myers et al., 2009).

Surprisingly, no studies have been carried out on the ecology of taxol producing fungi and whether temperature and $a_w$ interactions may affect the growth and the yield of taxol produced. This is surprising as previous studies have demonstrated that changes in environmental stress physiology may significantly increase the titres of enzyme and secondary metabolites produced in fermentation and immobilized systems (Parra et al., 2005).

The objective of this study were (1) to examine the ecophysiological factors on growth of *P. variabile* and *E. nigrum*, (2) to apply response surface methodology for establishing the second order model for radial growth rate, and (3) to verify the validity of the established model by additional experiments.

## 3.2 Material and methods

### 3.2.1 Preparation of mycelium inoculum

The inoculum was prepared from 7-day-old culture of both isolated strains (*P. variabile* and *E. nigrum*) growing on PDA at 25°C. A mycelial agar plug (5 mm diameter) was cut by using a sterile cork borer which was placed in the center of a 9 cm Petri plate of each treatment.

### 3.2.2 Effect of water activity, temperature and types of solutes on growth

The basic media, including M1D (Pinkerton and Strobel, 1976) and taxol microbial culture medium (Stierle et al., 1999) agar were used in this study. The $a_w$ of media was modified by adding non-ionic solutes (glycerol, sorbitol, glucose) and ionic solutes (NaCl, KCl). The $a_w$ were modified by adding increasing amounts of solute to obtain the following $a_w$ treatment levels of 0.99, 0.98, 0.96, 0.94, 0.92 and 0.90. These were checked with the $a_w$ meter (AquaLab, Decagon Devices, Inc., USA). Table 3.1 showed the amounts of solutes to use for modification $a_w$. The gram of solute was added into 100 ml of water. The final volume was measured to 100 ml and then was used to prepare media.

Table 3.1 Concentrations of solutes to use for modification of $a_w$ (Dallyn and Fox, 1980).

| | Water activity | | | | | |
|---|---|---|---|---|---|---|
| Solute | 0.99 | 0.98 | 0.96 | 0.94 | 0.92 | 0.90 |
| NaCl | 1.75 | 3.55 | 7.01 | 10.35 | 13.50 | 16.56 |
| KCl | 2.24 | 3.73 | 9.8 | 14.90 | 19.00 | 26.00 |
| Glycerol | 4.61 | 9.2 | 18.4 | 32.20 | 41.40 | 50.60 |
| Sorbitol | 12.0 | 19.82 | 30.75 | 50.00 | 54.66 | 85.00 |
| Glucose | 9.76 | 18.73 | 39.85 | 60.90 | 77.66 | 94.40 |

After inoculation, the Petri plates were sealed with parafilm tape and then kept in polyethylene bags at the tested incubation temperature. The diameter of the colonies were measured daily in two directions at right angles to each other (Marin et al., 1996). Radial growth rate for each studied combination treatment was obtained from linear regression slopes of the growth curves (Lahlali et al., 2006).

The experiment was designed to determine how the studied stress factors affected the radial growth rate of isolated strains. A 6x5x3x2 factorial design was applied using MINITAB version 16 (Minitab, Inc., USA). Table 3.2 shows the experimental variables and their levels. All treatments were carried out in triplicate.

Table 3.2 Experimental factors and their levels used in the 6x5x3x2 factorial design.

| Factors | Levels | | | | | |
|---|---|---|---|---|---|---|
| $a_w$ | 0.99 | 0.98 | 0.96 | 0.94 | 0.92 | 0.90 |
| Types of solutes | NaCl | KCl | Glycerol | Glucose | Sorbitol | |
| Temperature (°C) | 20 | 25 | 30 | | | |
| Media | MID | Taxol microbial culture media | | | | |

### 3.2.3 Effect of interactions between water activity, pH, types of solutes and temperature on growth

From the previous experiment, the range of studied variable levels were selected to examine the interaction between the pH on radial growth rate of the isolated fungal strains. M1D was used as the basal medium for this study. A 5x4x3x2 factorial design was applied (Table 3.3). Phosphate citrate buffer at the required pH levels was used as a solvent to modify $a_w$ with the non-ionic solutes (glycerol, glucose and sorbitol) and ionic solutes (NaCl, KCl). Then these modified solvents were used to prepare M1D. Prepared media were inoculated with a mycelial agar plug which was prepared as described in Section 3.2.1. The Petri plates were sealed with parafilm tape and kept in closed polyethylene bags at the tested incubation temperature. The diameter of the colonies were measured daily in two directions at right angles to each other (Marin et al., 1996). Radial growth rate for each studied combination treatment was obtained

from linear regression slopes of the linear parts of the growth curves (Lahlali et al., 2006). All treatments were carried out in triplicate.

Table 3.3 Experimental factors and their levels used in 5x4x3x2 factorial design.

| Factors | Levels | | | | |
|---|---|---|---|---|---|
| Types of solutes | NaCl | KCl | Glycerol | Glucose | Sorbitol |
| $a_w$ | 0.99 | 0.98 | 0.96 | 0.94 | |
| pH | 5 | 6 | 7 | | |
| Temperature (°C) | 20 | 25 | | | |

***Statistical analysis:***

When assumptions of normality and equal variance were met, ANOVA was adopted to test for significant differences in response among the studied treatments. If these assumptions of a parametric test were violated, the significant differences were assessed using the Kruskal-Wallis nonparametric ANOVA at statistical significant level of $P<0.05$. Post-hoc inter factor differences were calculated with Dunn's multiple comparison nonparametric tests with MINITAB version 16 (Minitab, Inc., USA). Bonferroni correction was used to keep the Bonferroni individual alpha=0.05. Mann-Whitney U test was adopted for statistical analyses between two samples with the significant level alpha=0.05.

### 3.2.4 The application of response surface methodology to predict the radial growth rate of strains of *P. variabile* and *E. nigrum*.

One of the experimental designs for fitting response surfaces is the central composite design (CCD), which is the most popular second-order design and very practical. The CCD for two factors involves four corners of the cube which represent the factorial points (+1/-1), four axial points ($+\alpha/-\alpha$) and replicated centre points. However, in some practical cases, the study cannot be performed outside the factorial points. In this study, a two-factor face-CCD with $\alpha=1.0$ was used to develop a response surface relating

radial growth rate (y) to temperature and $a_w$. The culture medium M1D was used as the basal medium and sorbitol was selected as the solute to modify $a_w$. Both coded and natural variable level settings for temperature and $a_w$ are shown in Table 3.4 for both strains; *P. variabile* and *E. nigrum.*

The design contained four factorial runs, four axial points with three replication and five centre runs. The statistical software package Design Expert® version 8.0.7.1 (State ease, Inc, Minneapolis, USA) was used to analyze the experimental design and generated the polynomial equation.

Table 3.4 Variables in the face-centred central composite design for *P. variabile.*

| | Range and levels | | |
|---|---|---|---|
| Variable | Low (-1) | Middle (0) | High (+1) |
| Temperature $(x_1)$ | 20 | 25 | 30 |
| $a_w$ $(x_2)$ | 0.90 | 0.94 | 0.98 |

The coded values ($X_i$) were calculated from actual values ($U_i$) using the formula;

$$X_i = \frac{\{2U_i - (U_{max} + U_{min})\}}{U_{max} - U_{min}} \quad (3.1)$$

The second-order polynomial equations were developed based on the regression analysis of the statistically significant variables. The models for the response (radial growth rate) were as follow:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_{11} x_1^2 + \beta_{22} x_{12}^2 + \beta_{12} x_1 x_2 \quad (3.2)$$

where $y$ is the predicted response (radial growth rate), $x_1$ and $x_2$ are the factors studied, $\beta_0$ is the intercept, $\beta_1$ and $\beta_2$ are linear coefficients, $\beta_{11}$ and $\beta_{22}$ are squared coefficients, $\beta_{12}$ are interaction coefficients between $x_1$ and $x_2$.

If the second-order polynomial did not fit the experimental dataset, the third-order reduced cubic model was established for a better fit.

***Model validation:***

To use the established model as the predictive model, the prediction ability should be assessed. The additional experiments within the experimental area was a two-factor face-centred central composite design with $\alpha$ = 1.0 (Table 3.5). The experimental data of the response variable and the model predicted data were used to evaluate the results mathematically. In this study, the decision criterions including the root mean squares error (RMSE); the relative standard error of prediction (RSEP) and the non-parametric Mann-Whitney U-test were used to access the performance of the proposed model. These decision criterions were defined as follows:

$$\mathrm{RMSE} = \sqrt{\frac{\sum_{i=1}^{N}(y_{pred}-y_{obs})^2}{N}} \tag{3.3}$$

$$\mathrm{RSEP} = 100 \times \sqrt{\frac{\sum_{i=1}^{N}(y_{pred}-y_{obs})^2}{\sum_{i=1}^{N}(y_{obs})^2}} \tag{3.4}$$

Where $y_{pred}$ was the model predicted value of the radial growth rate, $y_{obs}$ was the observed value of the radial growth rate and *N* was the total number of samples in the validation set.

Table 3.5 Values of coded and actual variables for the model validation

| Experiments | Factors | | | |
|---|---|---|---|---|
| | Actual vales | | Coded values | |
| | Temperature | $a_w$ | Temperature | $a_w$ |
| 1 | 20 | 0.95 | -1 | -1 |
| 2 | 30 | 0.95 | 1 | -1 |
| 3 | 20 | 0.97 | -1 | 1 |
| 4 | 30 | 0.97 | 1 | 1 |
| 5 | 20 | 0.96 | -1 | 0 |
| 6 | 30 | 0.96 | 1 | 0 |
| 7 | 25 | 0.95 | 0 | -1 |
| 8 | 25 | 0.97 | 0 | 1 |
| 9 | 25 | 0.96 | 0 | 0 |
| 10 | 25 | 0.96 | 0 | 0 |
| 11 | 25 | 0.96 | 0 | 0 |
| 12 | 25 | 0.96 | 0 | 0 |
| 13 | 25 | 0.96 | 0 | 0 |

## 3.3 Results

### 3.3.1 Effect of water activity, temperature and types of solute on growth

#### (a) *P. variabile* taxol-producing strain

The studied stress factors including $a_w$, temperature and solute types significant influenced the growth rate of *P. variabile* ($P<0.05$). No significant differences in growth rate regardless of the media used were observed ($P>0.05$). Overall, the growth rate of *P. variabile* decreased as water stress was imposed (Figure 3.1). The growth rates were generally higher at 0.99 $a_w$ ($P<0.05$) whatever the combination of experimental conditions used but was not significantly higher than 0.98 $a_w$ ($P>0.05$, Figure 3.2). Under severe $a_w$ stress (0.90), differences were observed in growth responses where in some conditions *P. variabile* was unable to grow. When sorbitol, glycerol and glucose were used as $a_w$ depressors at the lowest incubation temperature used (20°C), the growth of this strain was observed at 0.90 $a_w$. In contrast, ionic solutes such as NaCl were toxic at high concentrations to this strain. For the type of solute used, there were two statistically significant groups in relation to growth with sorbitol-modified media supporting the fastest growth rate than any of the other solutes ($P<0.05$, Figure 3.3a). Incubation at moderate temperature (25°C) provided significantly better conditions favouring the growth than 20 and 30 °C ($P<0.05$, Figure 3.3b).

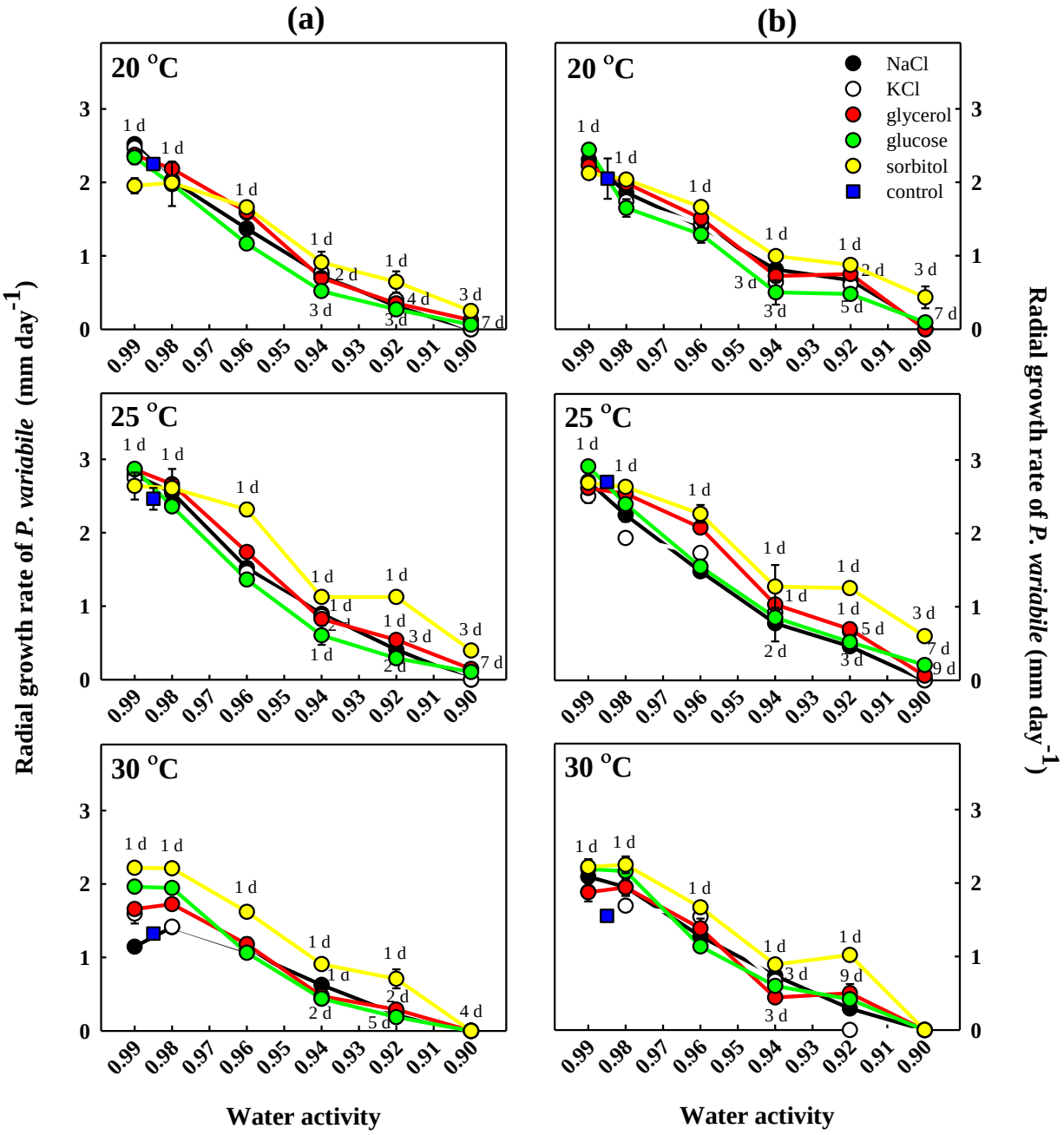



Figure 3.1 Effect of temperature, water activity and types of solutes on the radial growth rate and lag phases for growth initiation of *P. variabile* in modified media with NaCl, KCl, glycerol, glucose and sorbitol comparing with unmodified media $a_w$ 0.985 at 20, 25 and 30°C. Comparison between two type of media (a) M1D and (b) taxol microbial culture media. The number of days (d) for initiation of growth are indicated.

The lag phase prior to growth was also affected by temperature and $a_w$ treatments. Thus as temperature was changed or water stress was increased the lag phase also increased. This was dependent on the solute and temperature used. The fungus needed 1 to 3 days for initial growth at $a_w$ levels ranging from 0.94 to 0.99 at all temperatures used. At marginal conditions for growth (0.92-0.90 $a_w$) up to 9 days were required prior to growth. In the presence of sorbitol, the shortest average lag phase time ranged from 1 to 4 days at 0.99 $a_w$, regardless of temperature.

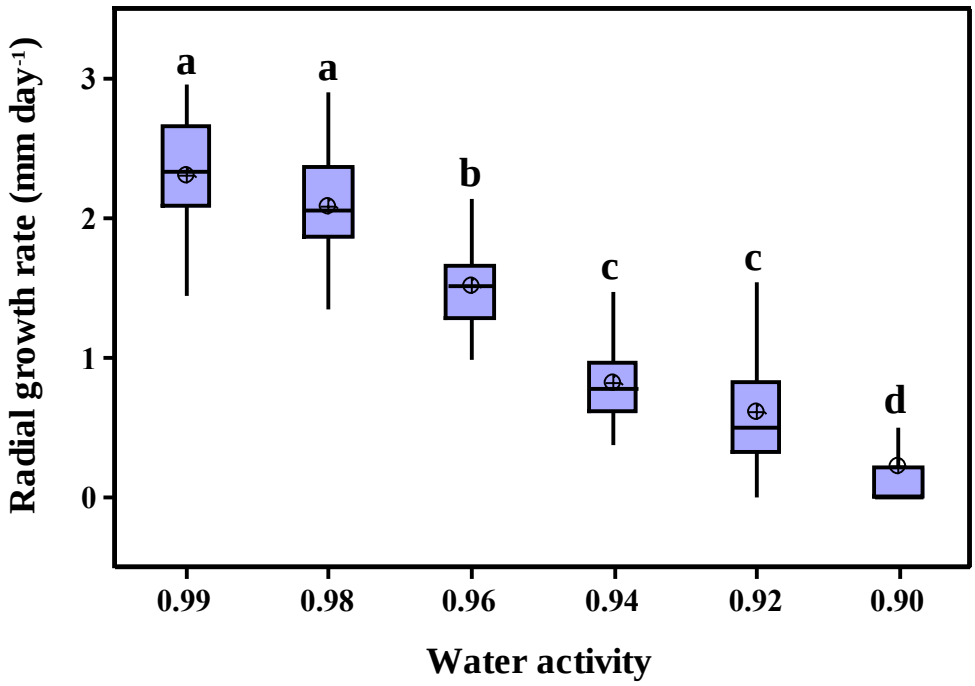
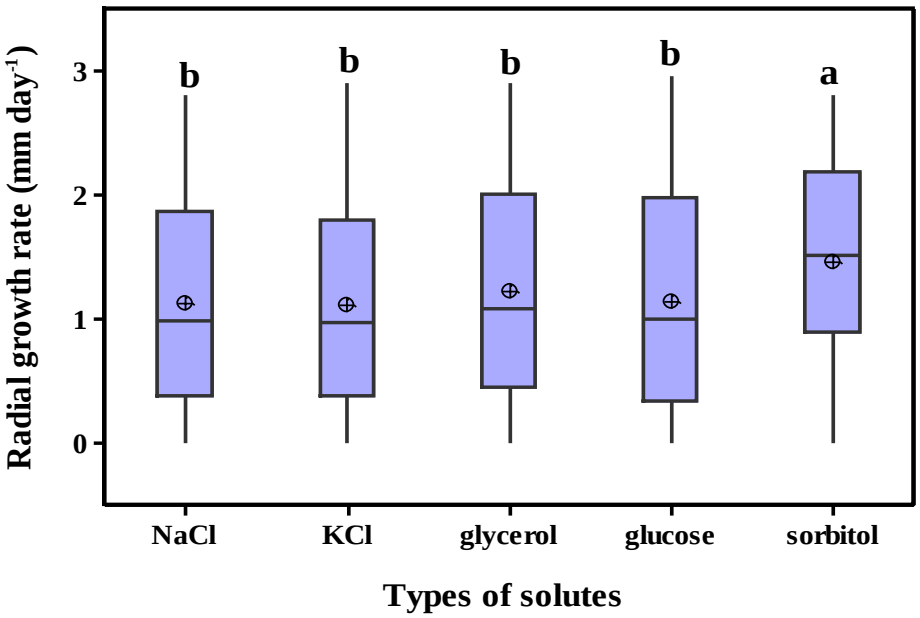



Figure 3.2 Box plots of radial growth rate of *P. variabile* according to $a_w$ stress conditions. Boxes represent the median (horizontal lines within each box), upper and lower quartiles (boxes). Extreme values are hidden. The symbol within each box represents the mean. The Bonferroni individual alpha for multiple comparisons is 0.05. Boxes labelled with the same letter shared the same group.

(a)




(b)

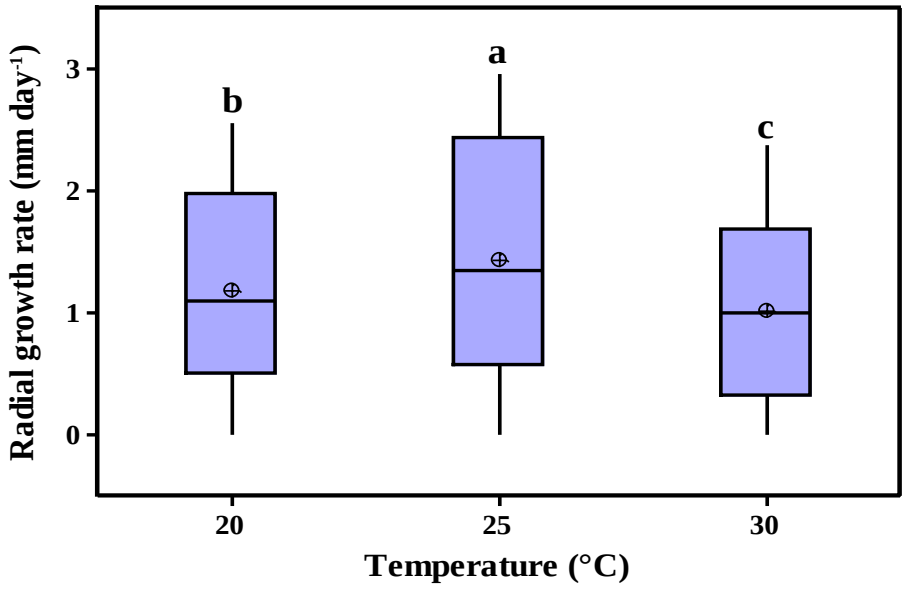



Figure 3.3 Differences in growth of *P. variabile* in response to (a) types of solutes and (b) temperature stress conditions. Boxes labelled with same letter shared the same group. The Bonferroni individual alpha for multiple comparisons is 0.05.

***(a) E. nigrum: taxol-producing strain***

For *E. nigrum*, all of three significant studied factors ($P<0.05$) including temperature, $a_w$ and solutes used affected the growth response of this strain in different ways (Figure 3.4). The decreasing trend in growth rate with increasing temperature under water stress conditions was observed. Within incubation temperature used, the growth at 20°C was higher than 25 and 30°C but the difference was not significant from 25°C ($P>0.05$, Figure 3.5a). *E. nigrum* was more sensitive to high temperature stress (30°C). Moreover, the effects on growth were similar when cultured at low $a_w$ (0.94-0.90 $a_w$) regardless of solutes used, excluding sorbitol modified media. The prolonged lag phase was obtained when cultured at high incubation temperatures (30°C) whenever such media were used.

Based on higher growth rate and shorter lag phase duration, sorbitol modified media seemed to be the most favourable for *E. nigrum* growth ($P<0.05$, Figure 3.5b). The initial growth of this strain on sorbitol-modified media required 1 to 3 days at $a_w$ level 0.99-0.92. The strong inhibitory effects of ionic solutes (NaCl and KCl) on growth were observed at high concentration and this effect seemed more pronounced when combined with high incubation temperature.

A sharp decline in growth rate was observed as water stress was increased. There was a 1.35 fold decline when compared with the lowest median growth rate under $a_w$ stress (0.90). The highest growth rate was at 0.99 $a_w$, but this was not significantly greater than at 0.98 $a_w$ ($P<0.05$) whatever the combination of conditions tested (Figure 3.6).

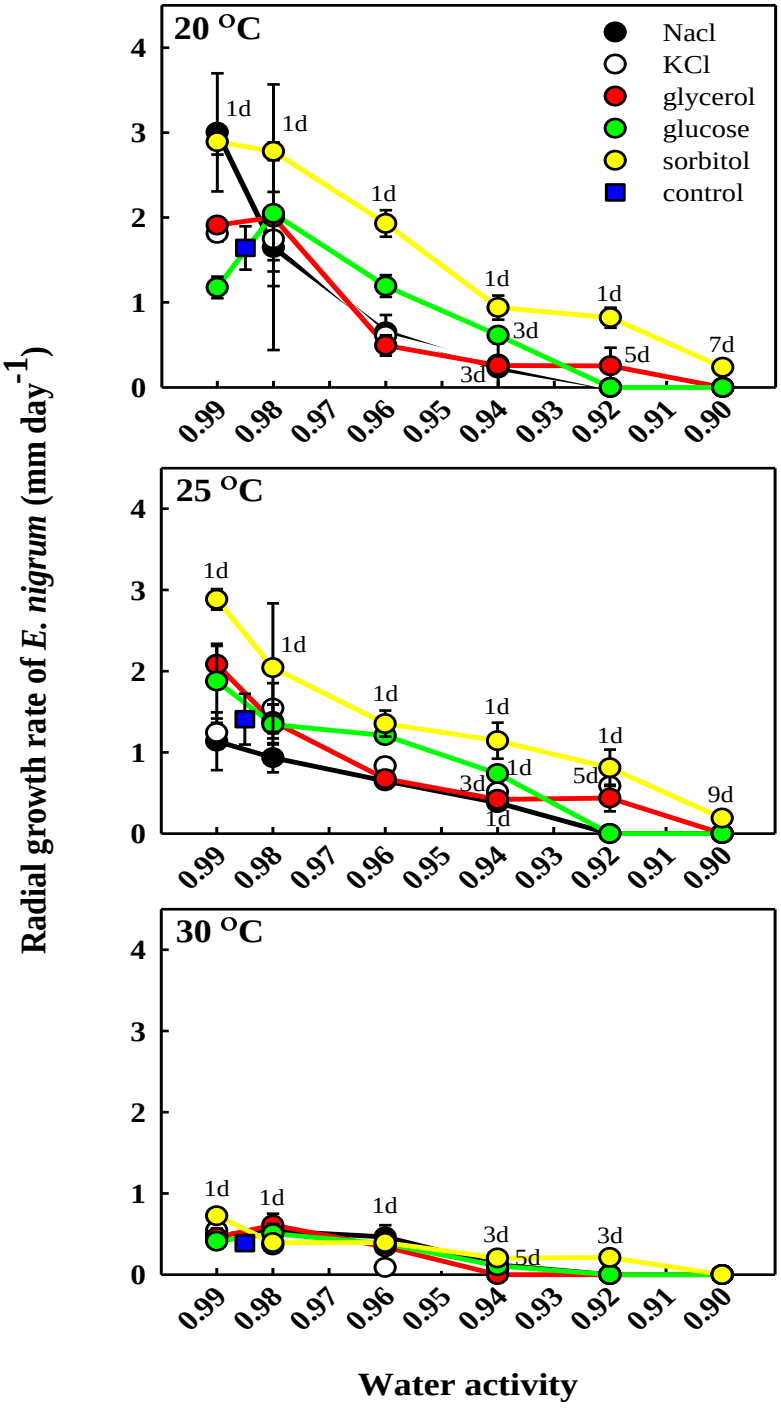
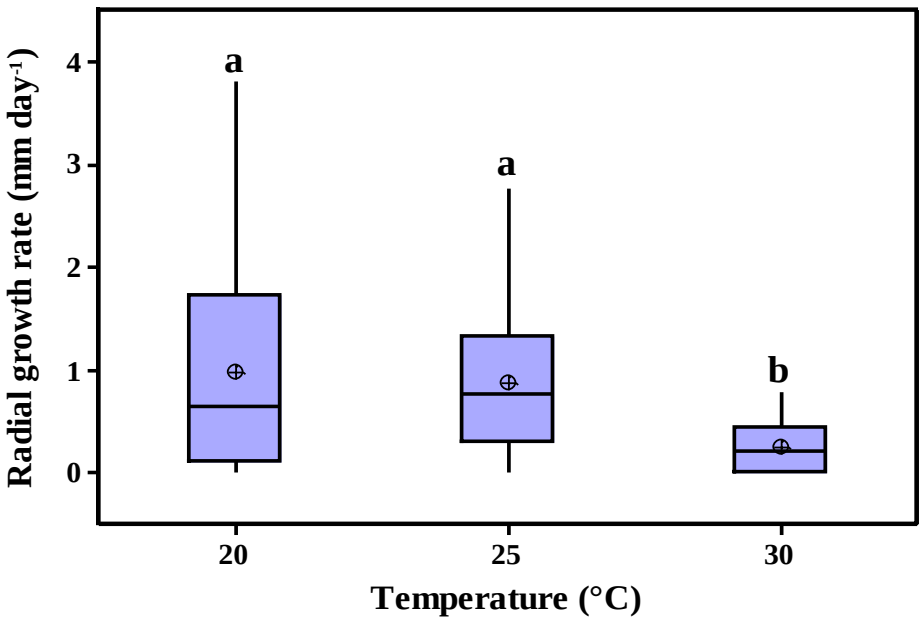



Figure 3.4 Effect of temperature, $a_w$ and types of solutes on the radial growth rate and lag phase of *E. nigrum* in modified media with NaCl, KCl, glycerol, glucose and sorbitol comparing with unmodified media $a_w$ 0.985 at 20, 25 and 30 °C. The number of days (d) for initiation growth is indicated.

(a)




(b)

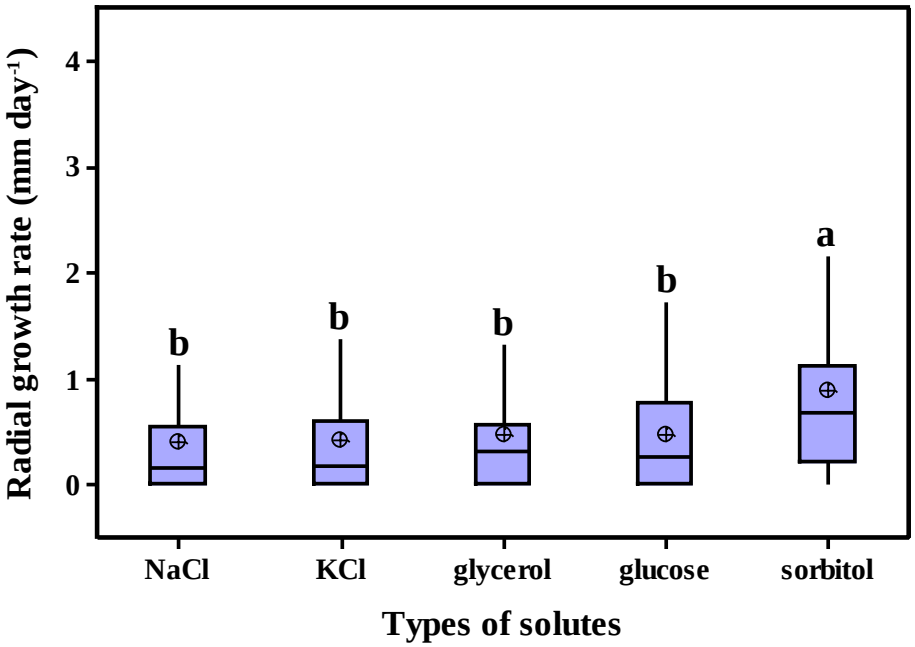



Figure 3.5 Box plot of radial growth rate of *E. nigrum* in response to (a) temperature and (b) types of solutes. The Bonferroni individual alpha for multiple comparisons is 0.05. Boxes labelled with the same letter shared the same group

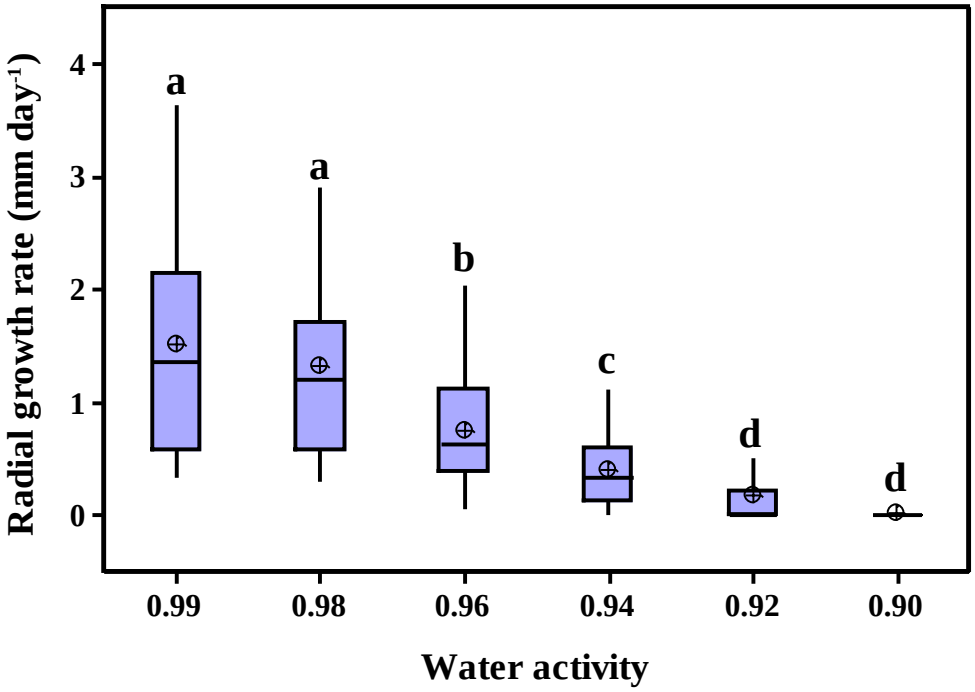



Figure 3.6 Box plot of change in radial growth rate of *E. nigrum* in response to $a_w$. The Bonferroni individual alpha for multiple comparisons is 0.05. Boxes labelled with the same letter shared the same group.

**(c) *P. variabile: non-producing strain***

From the investigation of the taxol-producing strain, one of the isolated endophytic strains was from the same species, but could not produce taxol. It was of interest to examine whether growth of a taxol-producing and non-taxol producing strain were ecologically similar or different. Overall, the stress factors examined affected growth significantly ($P<0.05$). A similar decreasing trend in growth rate was obtained with increasing water stress (Figure 3.7). The $a_w$ range for growth of this strains was 0.99 to 0.94 $a_w$ at 30°C, whereas the taxol-producing strain was inhibited at around 0.92 $a_w$ at the same incubation temperature. Growth at 25°C was optimum but not significantly different from that at 20°C ($P>0.05$, Figure 3.8). However, this strain was more sensitive to water stress x temperature conditions, regardless of the solutes used.

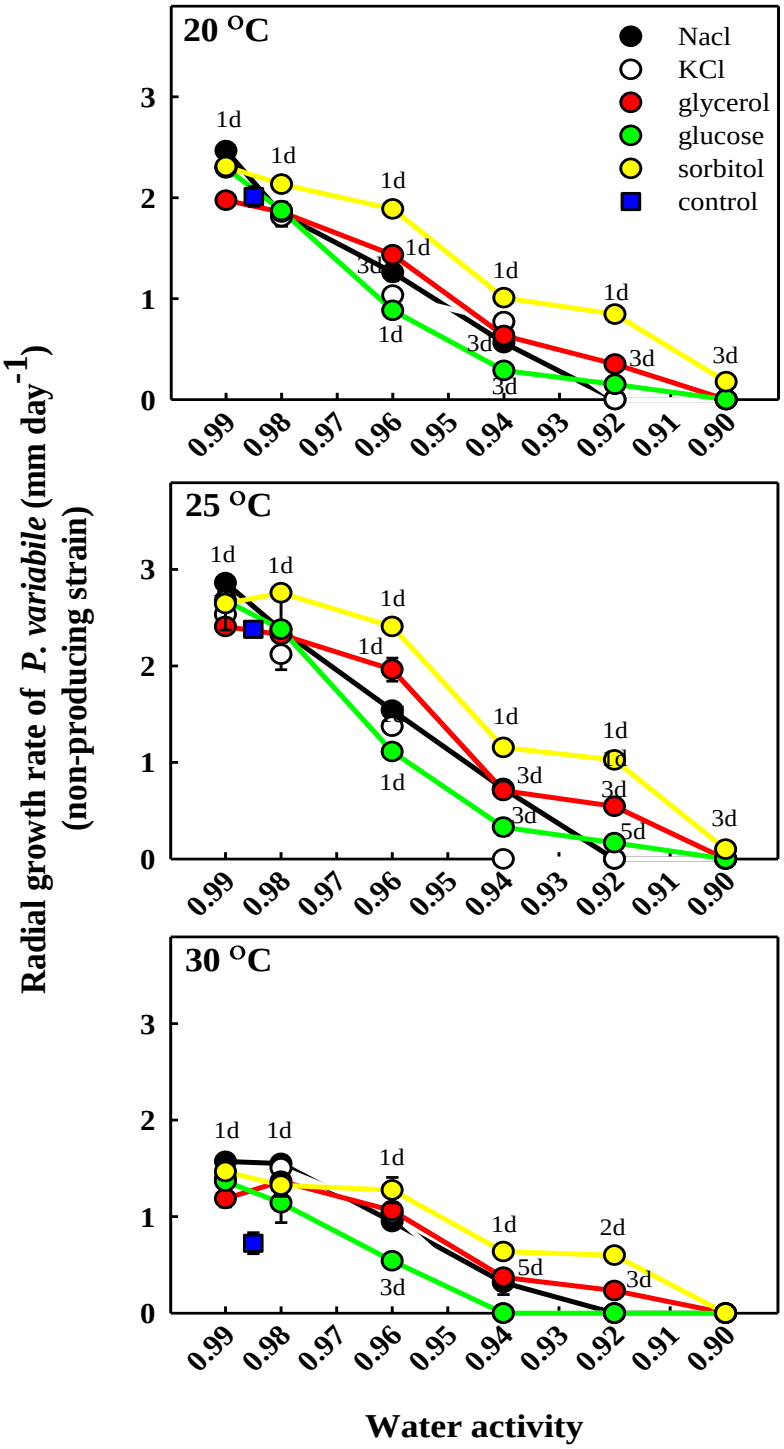



Figure 3.7 Effect of temperature, water activity and types of solutes on the radial growth rate and lag phase of *P. variabile* (non-producing strain) in modified media with NaCl, KCl, glycerol,glucose and sorbitol comparing with unmodified media $a_w$ 0.985 at 20, 25 and 30°C. The number of days (d) for initiation growth are indicated.

Within the types of solutes used, sorbitol-modified media was the best supported solute but was not significantly different from glycerol (P>0.05, Figure 3.9a). When KCl and glucose were used to impose water stress, this strain showed was more sensitive than the taxol-producing strain. Using glucose-modified media (0.92 $a_w$), five days were required for initial growth, when cultured under the most favourable temperature (25°C). Faster growth rates occurred when this strain was cultured at high water condition (0.99 $a_w$). However, at 0.98 $a_w$ there was no significant difference (P>0.05, Figure 3.9b)

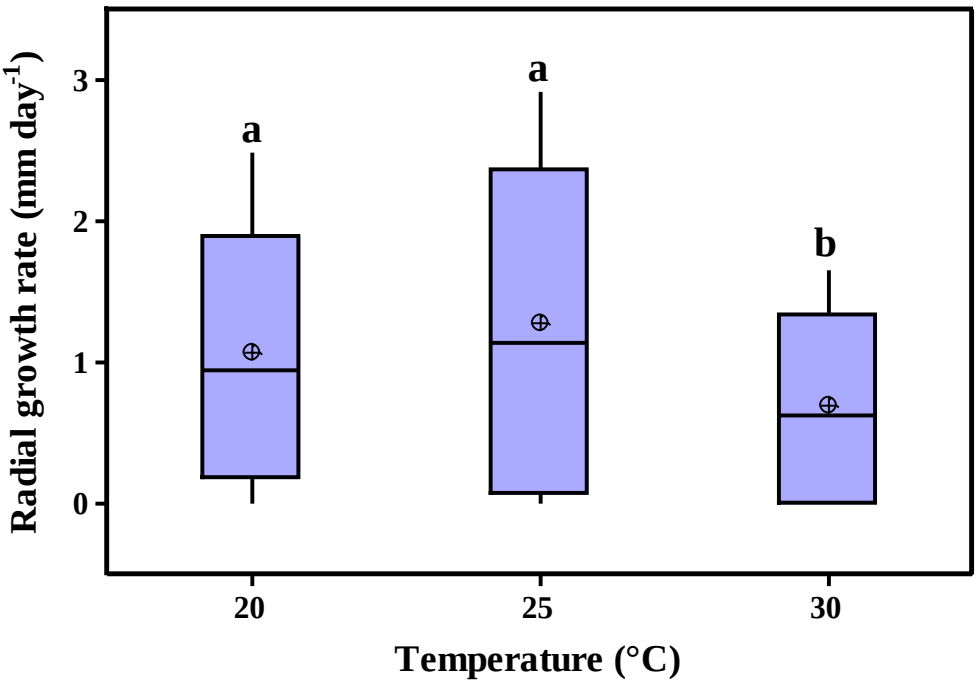
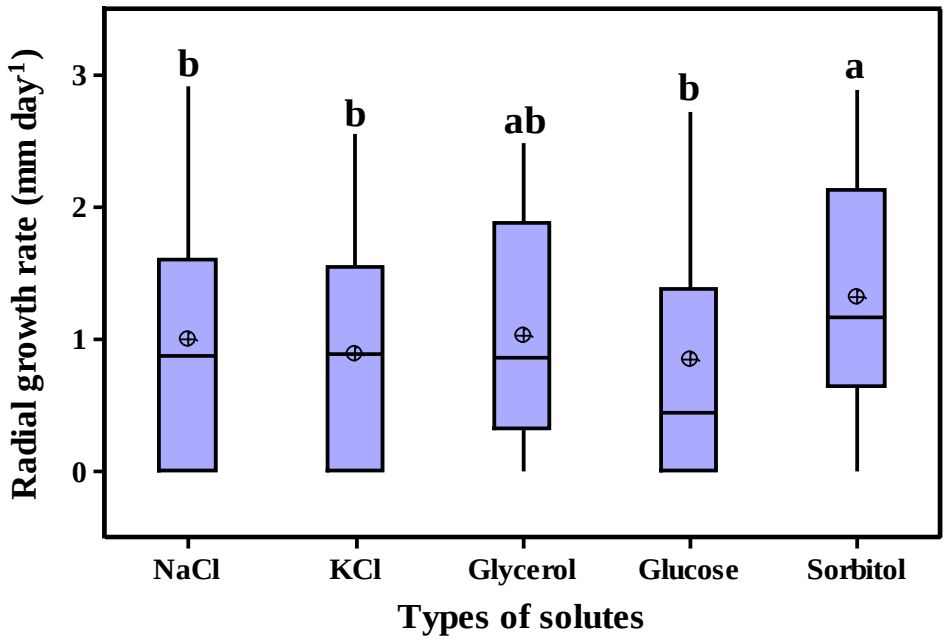



Figure 3.8 Box plot of change in radial growth rate of *P. variabile* (non-producing strain) in response to temperature. The Bonferroni individual alpha for multiple comparisons is 0.05. Boxes labelled with the same letter shared the same group.

(a)

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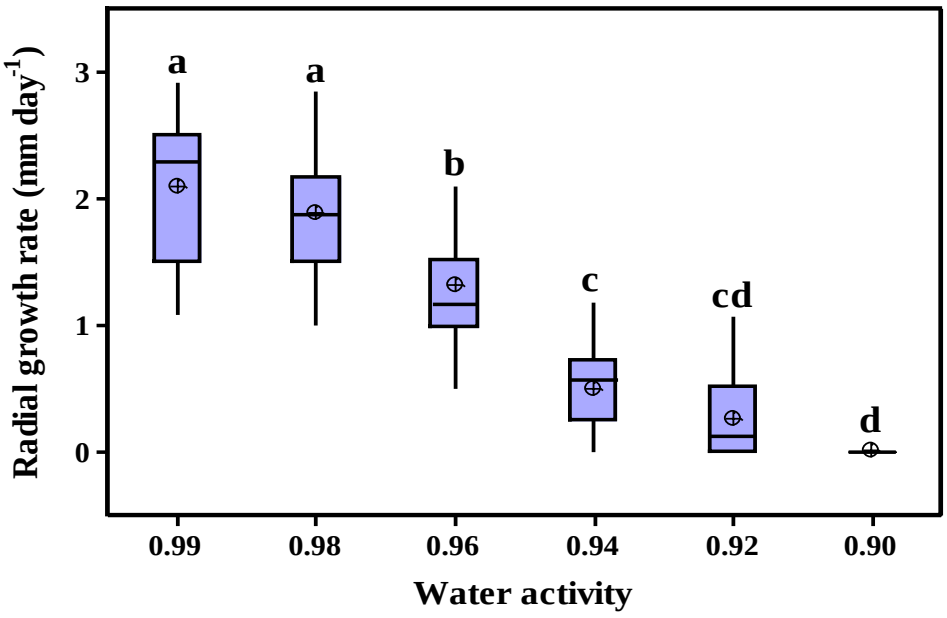



Figure 3.9 Box plot of change in radial growth rate of *P. variabile* (non-producing strain) in response to (a) types of solutes and (b) $a_w$. The Bonferroni individual alpha for multiple comparisons is 0.05. Boxes labelled with the same letter shared the same group.

### 3.3.2 Effect of water activity, temperature, types of solutes and pH on growth

#### (a) *P. variabile*: taxol producing strain

The interactions between the pH of the culture medium and radial growth rates, in modified $a_w$ media (0.99-0.94) with the different types of $a_w$ depressors which incubated at 20 and 25°C, were determined for both strains of isolated fungi. Overall, the decrease in growth rate of *P. variabile* with increasing pH under water stress conditions was observed (Figure 3.10). The highest growth rate was 2.63 mm/day which was obtained from KCl-modified medium (0.99 $a_w$, pH 5) at 25°C. The lowest growth (0.2 mm/day) was observed under the most stressed condition (0.94 $a_w$), pH 7 with NaCl-modified medium. The maximum growth of the control was 2.77 mm/day (pH 5, 25°C).

The two factors $a_w$ and pH had significant effects on growth ($P<0.05$), whereas changes in temperature and types of solutes had no significant effect ($P>0.05$). *P. variabile* exhibited a noticeable sensitive response to neutral pH conditions (pH 7). This was further increased when water stress (0.94 $a_w$) and low temperature stress condition (20°C) were imposed. The average rate of growth under such conditions declined by nearly 50% when compared with acidic conditions (pH 5). For cultivation pH, growth at pH 5 was the most favourable condition whatever combined treatments were used ($P<0.05$, Figure 3.11).

When cultured with high water availability media (0.99 $a_w$), the highest rates of growth was observed among modified $a_w$ media regardless of $a_w$ depressor used ($P<0.05$, Figure 3.12a). However, the growth was not higher than the control treatments (non-modified $a_w$) under acidic conditions (pH 5) especially with low incubation temperature (20°C). The reduction of water availability of media to 0.94 $a_w$ resulted in a significant difference between the decline in the median value (4.95 fold decline) when compared with the most favourable conditions.

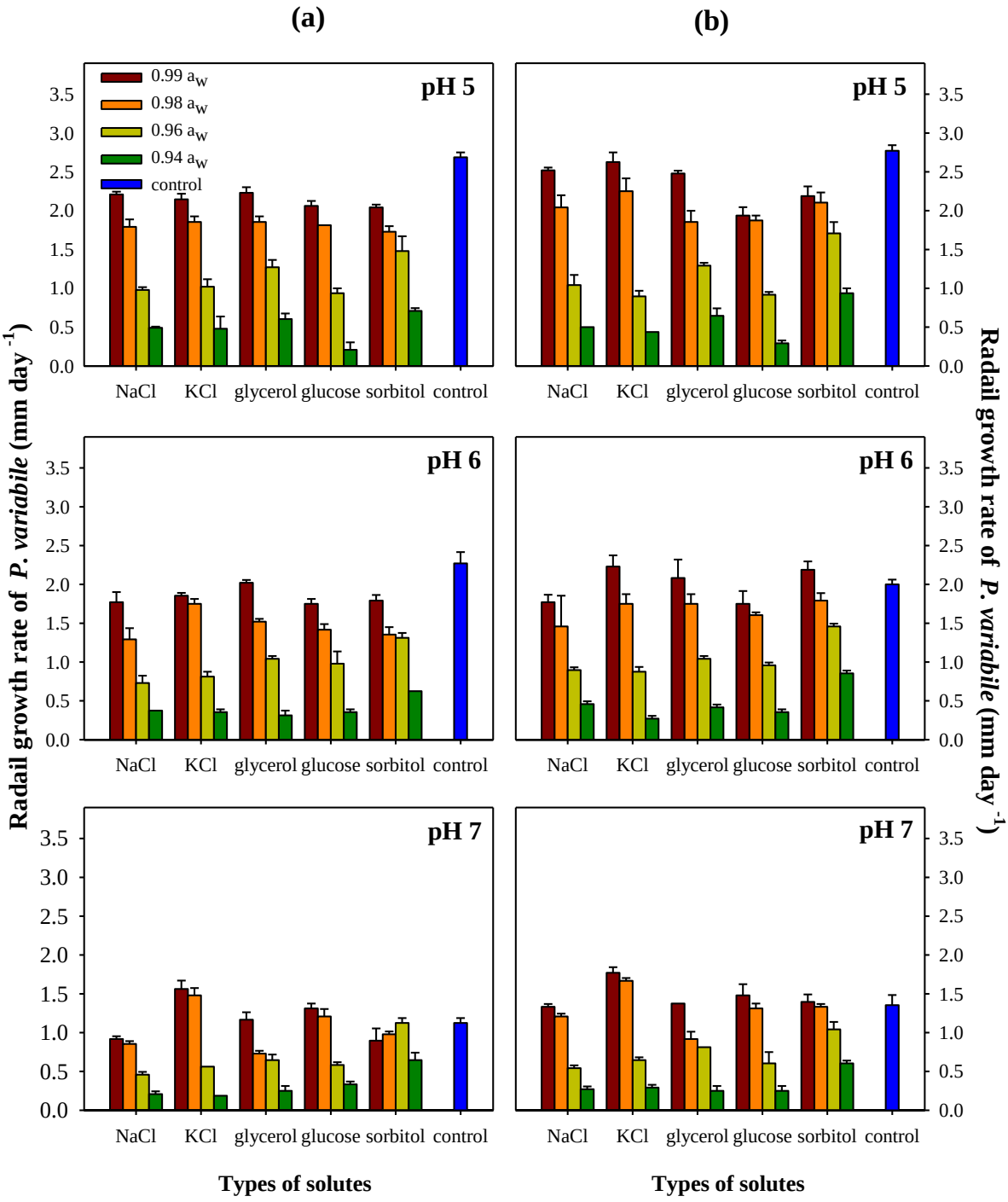



Figure 3.10 Effect of temperature, water activity, types of solutes and pH on the radial growth rate of *P. variabile* at different incubation temperatures (a) 20°C and (b) 25°C.

Although the temperature and type of solute were not significant factors (P>0.05), there was some useful information from these results. A better average growth rate could be achieved by choosing 25°C (Figure 3.12b) and modifying $a_w$ of media with KCl, sorbitol and glycerol (Figure 3.13). In addition, growth at neutral pH, and use of the ionic KCl seemed to be more supportive of the growth at both studied incubation temperatures than other $a_w$ depressors.

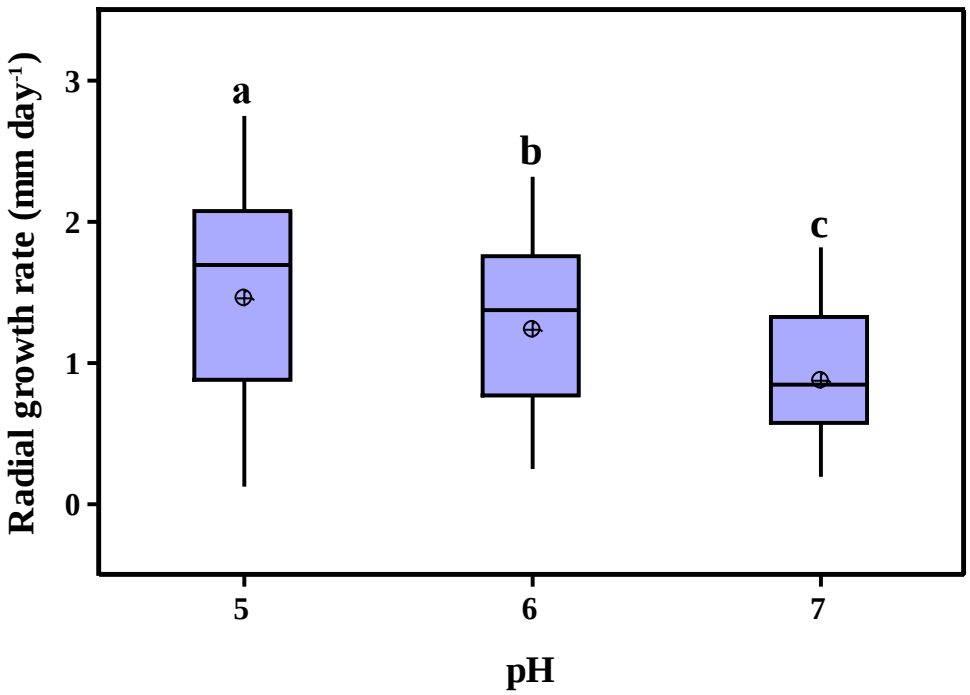
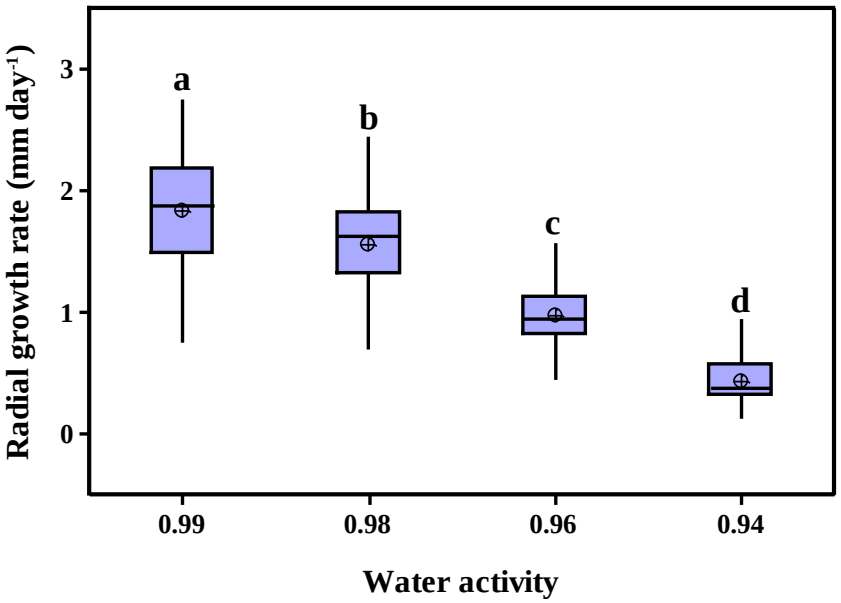



Figure 3.11 Box plot of change in radial growth rate of *P. variabile* in response to pH. The Bonferroni individual alpha for multiple comparisons is 0.05. Boxes labelled with the same letter shared the same group.

(a)




(b)

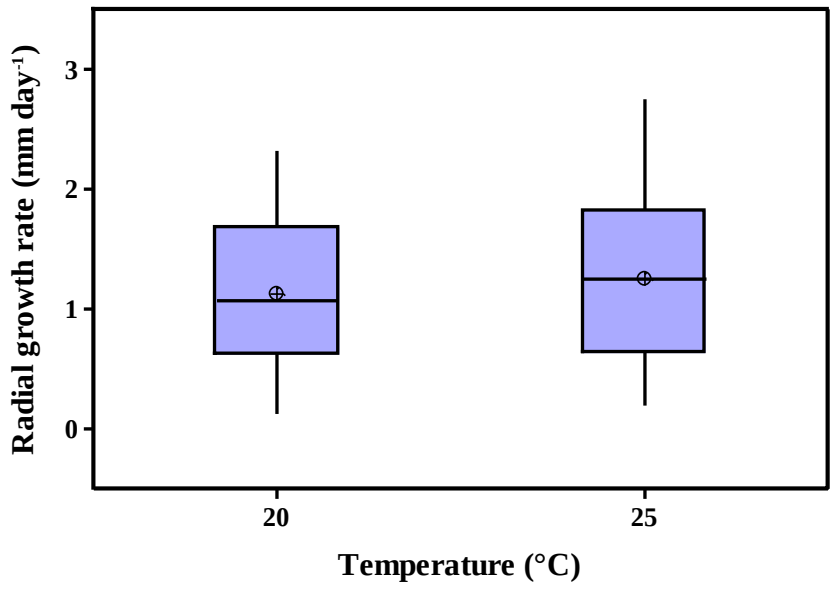



Figure 3.12 Box plot of change in radial growth rate of *P. variabile* in response to (a) $a_w$ and (b) temperature. The Bonferroni individual alpha for multiple comparisons is 0.05. Boxes labelled with the same letter shared the same group.

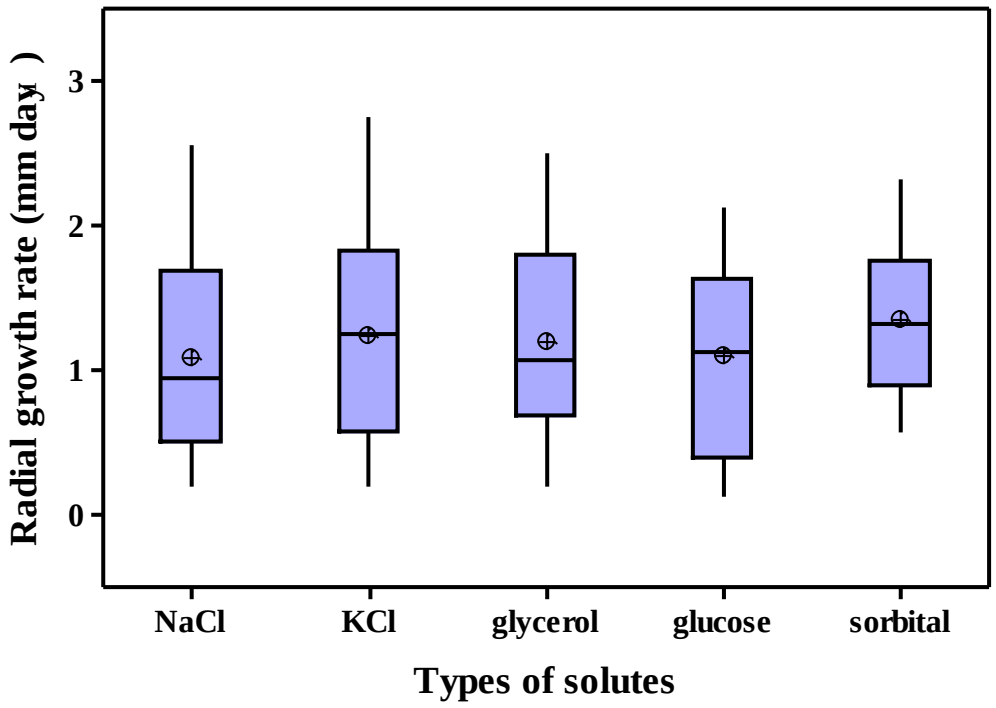



Figure 3.13Box plot of change in radial growth rate of *P. vaiabile* in response to types of solutes. Boxes labelled with the same letter shared the same group. The Bonferroni individual alpha for multiple comparisons is 0.05.

**(b) *E. nigrum***

For *E. nigrum*, the fluctuations in growth rate observed were significantly associated with all four studied factors (P<0.05,Figure 3.14). The highest growth rate when using KCl as solute was 1.23 mm day$^{-1}$ (pH 7, 0.99 $a_w$, 25°C) with no growth obtained at 0.94 $a_w$ in NaCl-modified media. The maximum growth of the control was 0.83 mm day$^{-1}$ (pH 7, 0.99 $a_w$, 25°C).

The temperature sensitivity of this strain tended to be pronounced at low temperature (20°C) in well-buffered media, whereas culture at 25°C was a more favourable condition (P<0.05, Figure 3.15a). The lowest average growth at 20°C was obtained at

pH 5 with the ionic solute NaCl, whereas adding glucose seemed to be more supportive for growth under these conditions. However, the tendency for the response of *E. nigrum* to such stress was $a_w$ depressor dependent. There were no statistically significant differences of growth rate between these solutes ($P>0.05$, Figure 3.15b).

Overall, the best growth of *E. nigrum* was at 0.99-0.98 $a_w$ ($P>0.05$, Figure 3.16a). The growth rates decreased (6.98 fold) in correlation with increasing water stress from 0.99 $a_w$ to 0.94 $a_w$. For cultivation pH, the fastest growth was obtained under acidic conditions (pH 5) ($P<0.05$, Figure 3.16b) whereas there was no significant difference between pH 6 and 7.

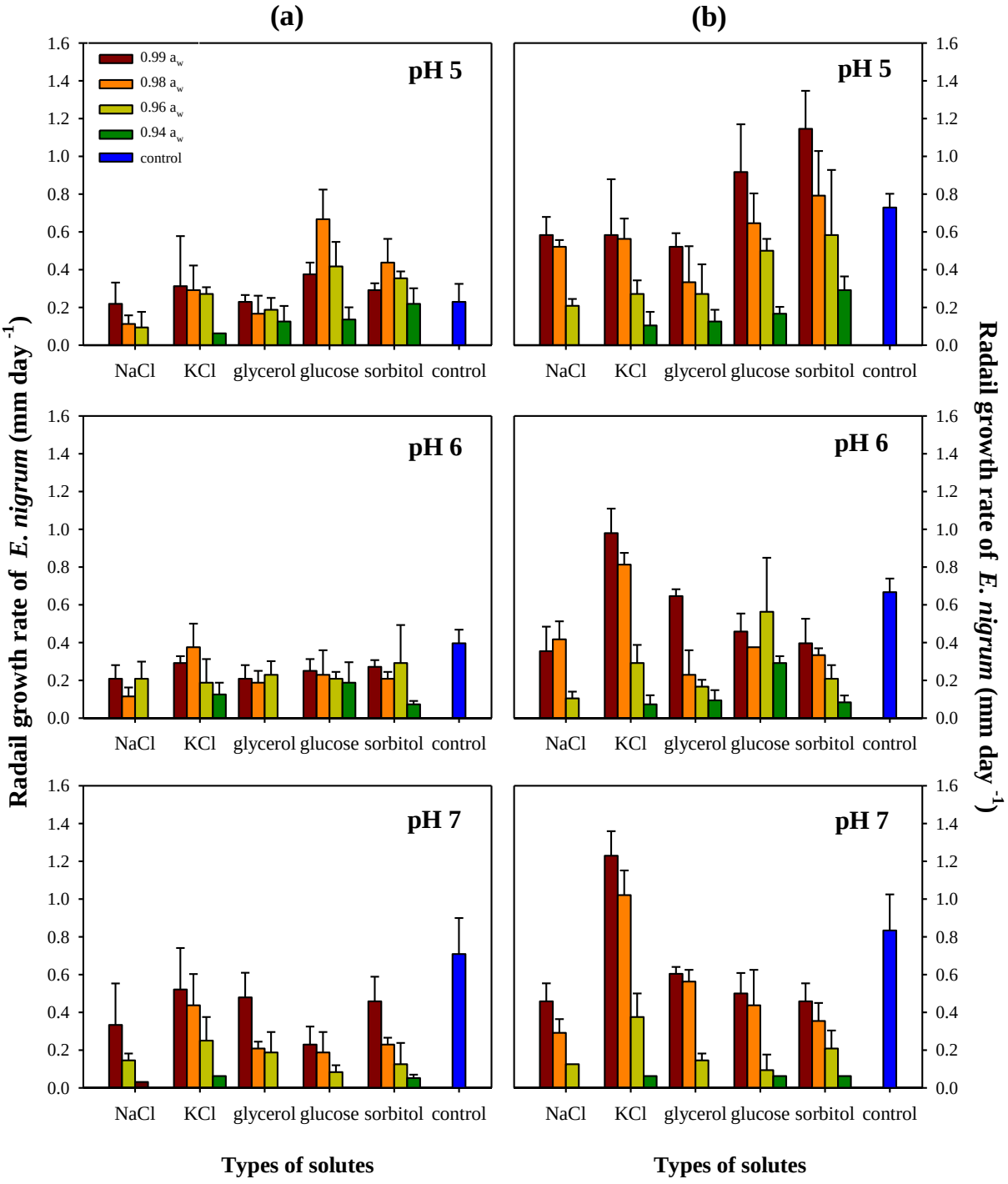
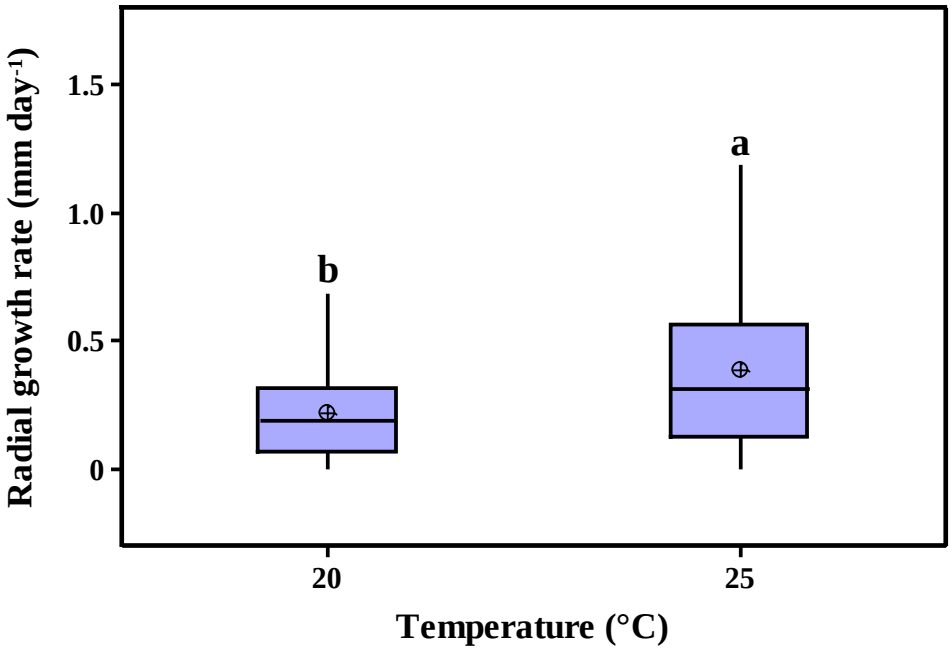



Figure 3.14 Effect of temperature, $a_w$, types of solutes and pH on the radial growth rate of *E. nigrum* at incubation temperature (a) 20°C and (b) 25°C.

(a)




(b)

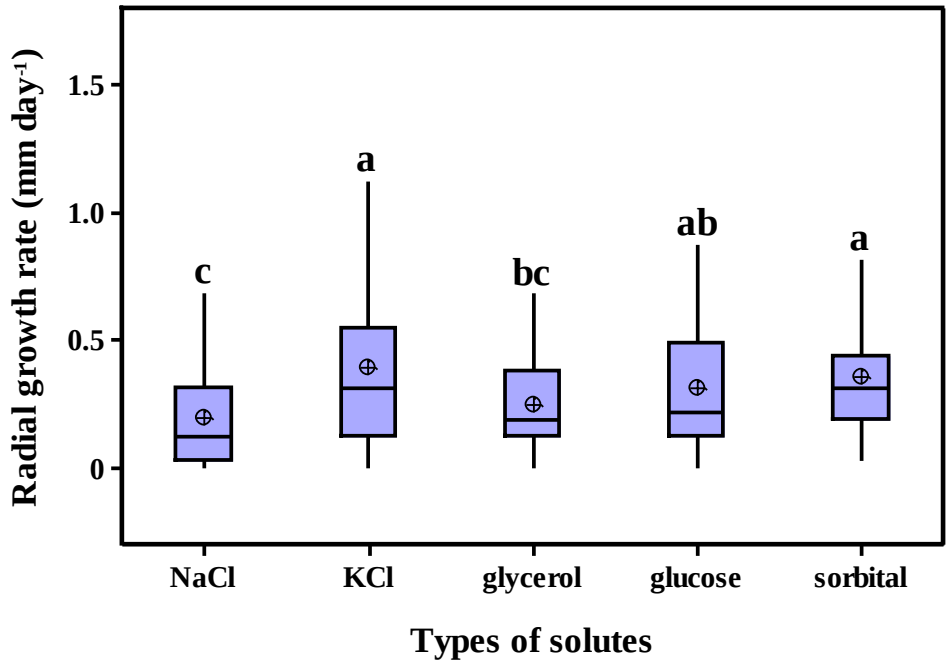
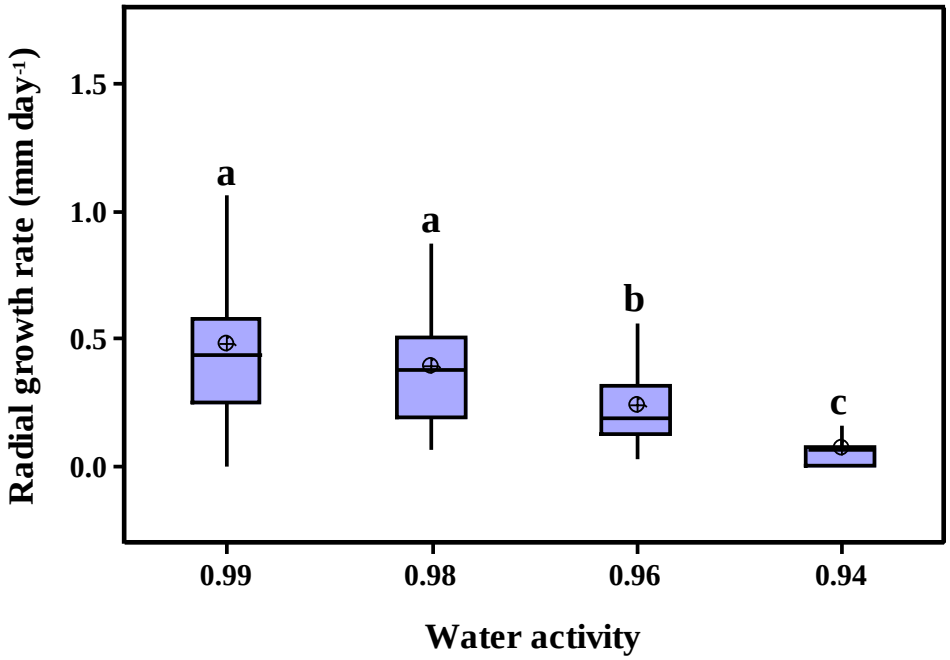



Figure 3.15 Box plot of change in radial growth rate of *E. nigrum* in response to (a) temperature and (b) types of solutes. The Bonferroni individual alpha for multiple comparisons is 0.05. Boxes labelled with the same letter shared the same group.

(a)




(b)

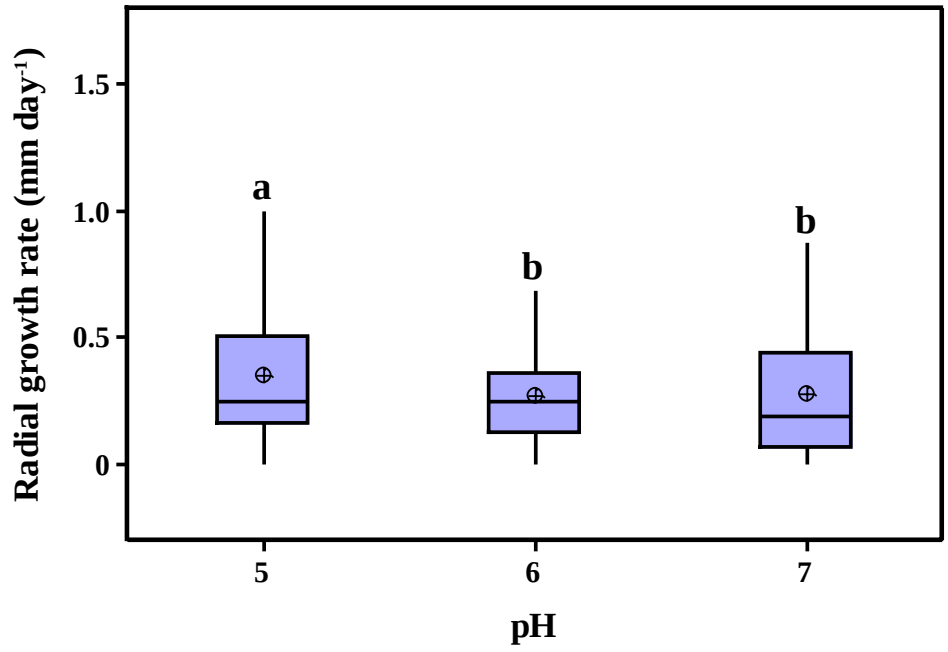



Figure 3.16 Box plot of change in radial growth rate of *E. nigrum* to (a) $a_w$ and (b) pH. Bonferroni individual alpha for multiple comparison is 0.05. Boxes labelled with the same letter shared the same group.

### 3.3.3 The application of response surface methodology to predict the radial growth rate of *P. variabile* and *E. nigrum*

The two independent variables including temperature and $a_w$ showed a statistically significant influence on growth rate of both taxol producing strains. Hence, these were the chosen variables in this study in order to develop the second order model. A two-factor face-centred central composite design was used for this purpose. The solute, sorbitol, was selected for this study due to the inhibitory effects on the growth rate lower than the other solutes used. After the data were collected and fitted, the second-order polynomial equations were developed for both strains based on the regression of statistically significant variables. The following model was obtained in the coded variables.

For *P. vaiabile*,

$$y = 1.42 - 0.049x_1 + 0.98x_2 - 0.46x_1^2 + 0.19x_2^2 + 0.14x_1x_2 \quad (3.5)$$

For *E. nigrum*,

$$y = 1.60 - 0.72x_1 + 1.20x_2 - 0.78x_1^2 + 0.40x_2^2 + 0.63x_1x_2 \quad (3.6)$$

where $y$ is the response (radial growth rate) and $x_1$ and $x_2$ are the factors studied including temperature and water activity, respectively.

The predicted values which were calculated from the Equation 3.5 for *P. variabile* and the Equation 3.6 for *E. nigrum*, together with their observed values are shown in Figure 3.17. Good agreement between the predicted and observed values in the range of operating variables was observed from the response surface model of *P. variabile*, whereas a less tendency in the linear relationship was obtained from the predictive model of *E. nigrum*.

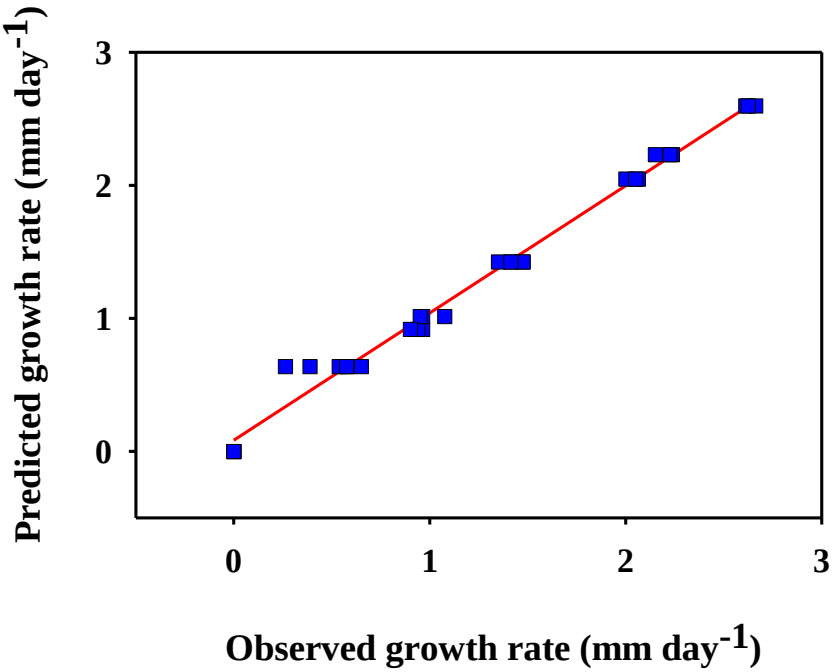
ANOVA was performed in order to test the significance of the regression model, coefficients and lack of fit for the models which is shown in able 3.6 for response surface quadratic model of *P. variabile*. The F-test of the model revealed that the model was significant ($P<0.05$). All model terms which included both main effects, the second-order effects and their interaction effects were also the significant terms ($P<0.05$). The $R^2$ for the models was approximately 0.99 which implies that the model explained 99% of the variability observed in the response in terms of radial growth rate.

Furthermore, the F-test for lack of fit is required to determine the appropriateness of the selected model to describe the experimental data. The statistical analysis showed that the lack of fit test was not significant but which indicated that the model did fit the observed data sets well.

Table 3.6 Analysis of variance for the response surface model for *P. variabile.*

| Source of variation | df | Mean square | F value | P value |
|---|---|---|---|---|
| Model | 5 | 3.82 | 1010.19 | 0.000 |
| Temperature | 1 | 0.043 | 11.24 | 0.003 |
| aw | 1 | 17.28 | 4568.69 | 0.000 |
| Temperature*aw | 1 | 0.24 | 62.73 | 0.000 |
| Temperature*Temperature | 1 | 1.41 | 373.95 | 0.000 |
| Aw*aw | 1 | 0.25 | 66.60 | 0.000 |
| Residual | 23 | 3.783E-003 | | |
| Lack-of-Fit | 3 | 4.973E-003 | 1.38 | 0.2779 |
| Pure Error | 20 | 3.604E-005 | | |
| $R^2 = 0.9955$ | | | | |

(a)




(b)

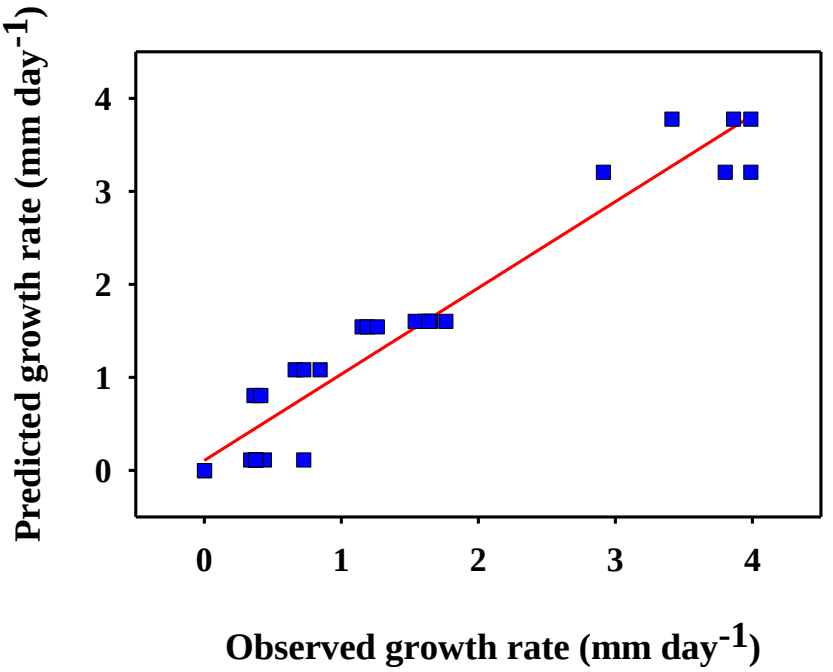



Figure 3.17 Comparison between observed and predicted growth rate (a) *P. variabile* and (b) *E. nigrum* for the model development set.

In addition, the analysis of the coefficient estimates revealed all of the coefficients were significant (P<0.05). Thus, all terms were kept in the model. This implies that all the factors directly affect the radial growth rate. The higher the value of linear coefficient ($\beta_1$ or $\beta_2$), the more influence of the corresponding factors (temperature or $a_w$). Therefore, the highest value for linear coefficient of $a_w$ illustrates the greater influence over the response when compared to temperature. When the $\beta_1$ value for temperature was -0.04833 and $\beta_2$ value for $a_w$ was 0.98056.

The same procedure was applied on the response surface quadratic model for *E. nigrum*. Based on the ANOVA analysis (Table 3.6), the F–test for all model terms indicated that the model terms were significant (P<0.05). Although the models also showed a high $R^2$ value of 93.19%, this response surface model exhibited significant lack of fit (P<0.05). Model improvement was required.

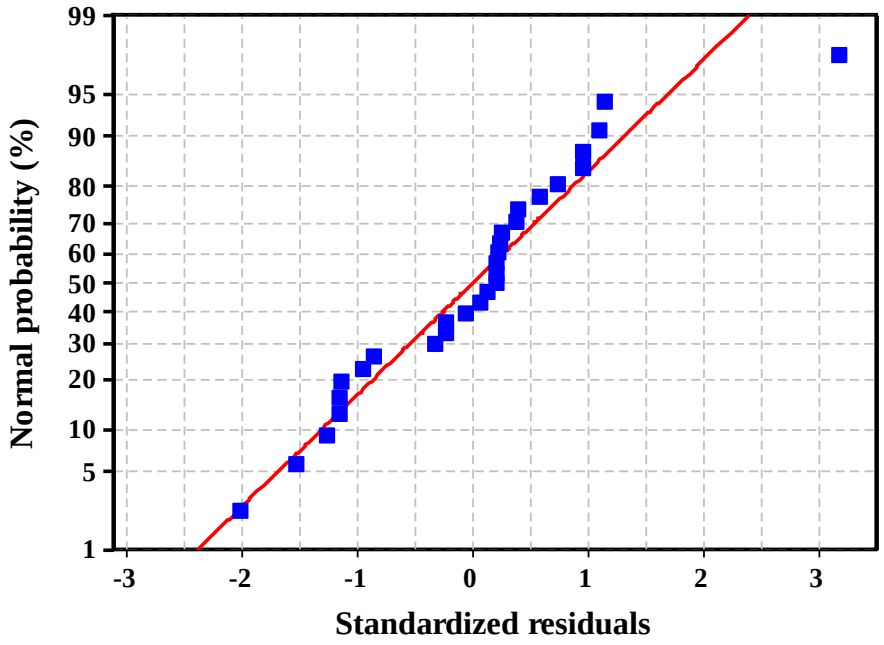
Table 3.7 Analysis of variance for the response surface quadratic model of *E. nigrum*.

| Source of variation | df | Mean square | F value | P value |
|---|---|---|---|---|
| Model | 5 | 8.9 | 63.00 | 0.000 |
| Temperature | 1 | 9.22 | 65.03 | 0.000 |
| $a_w$ | 1 | 25.91 | 182.81 | 0.000 |
| Temperature*$a_w$ | 1 | 4.80 | 33.90 | 0.000 |
| Temperature*Temperature | 1 | 4.06 | 28.66 | 0.000 |
| $a_w$*$a_w$ | 1 | 1.09 | 7.68 | 0.011 |
| Residual | 23 | 0.14 | | |
| Lack-of-Fit | 3 | 0.76 | 15.33 | 0.000 |
| Pure Error | 20 | 0.049 | | |
| $R^2$ = 0.9319 | | | | |

Graphical analysis is a useful method to examine the adequacy of the model. There are different types of plots. The normality probability plots of the residuals and the plots of the residuals versus the predicted response were selected for this objective. For the model of *P. variabile*, the distribution of residuals randomly lay along the straight line (Figure 3.18a). Only one residual (in the higher tail) departed from the straight line. This unexpected higher residual could be due to the outlier (unusual observed data). A plot of the residuals versus the corresponding predicted response in Figure 3.18b displayed the normal scattering pattern of the residuals which were contained in the horizontal band.

For *E. nigrum*, the residuals from the normal probability plot did not lie along a straight line and a positive skew (to the right) was found (Figure 3.19a). This pattern indicated that there was evidence of a problem with normality.Figure 3.19b displayed a scatterplot of residuals in which the points were not spread randomly and a few points in the plot were a long way from the rest of the points. The data appeared to violate the assumption of homoscedasticity (constant variance). In order to correct this problem, transformation on either the predictors or the response were required.

(a)




(b)

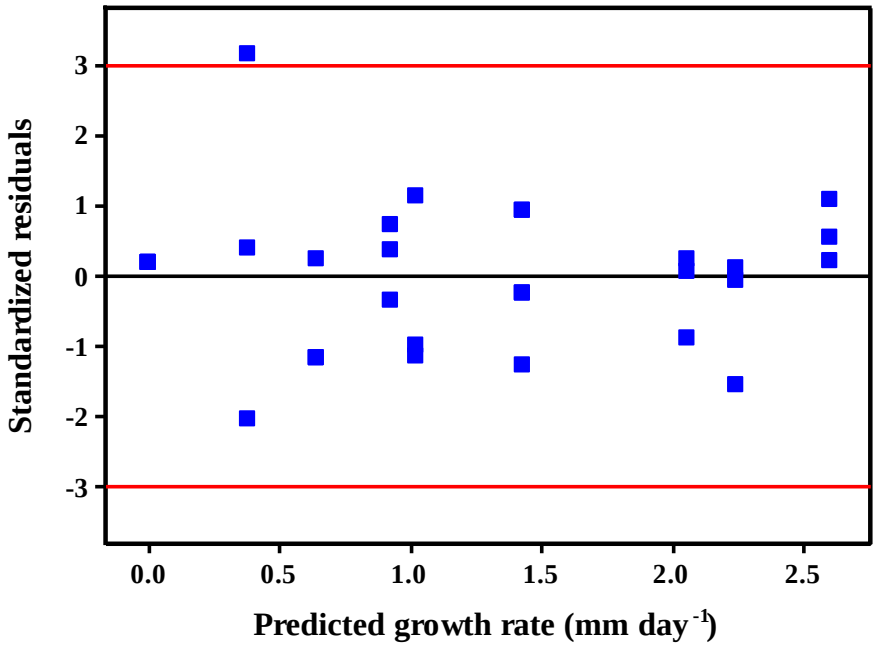
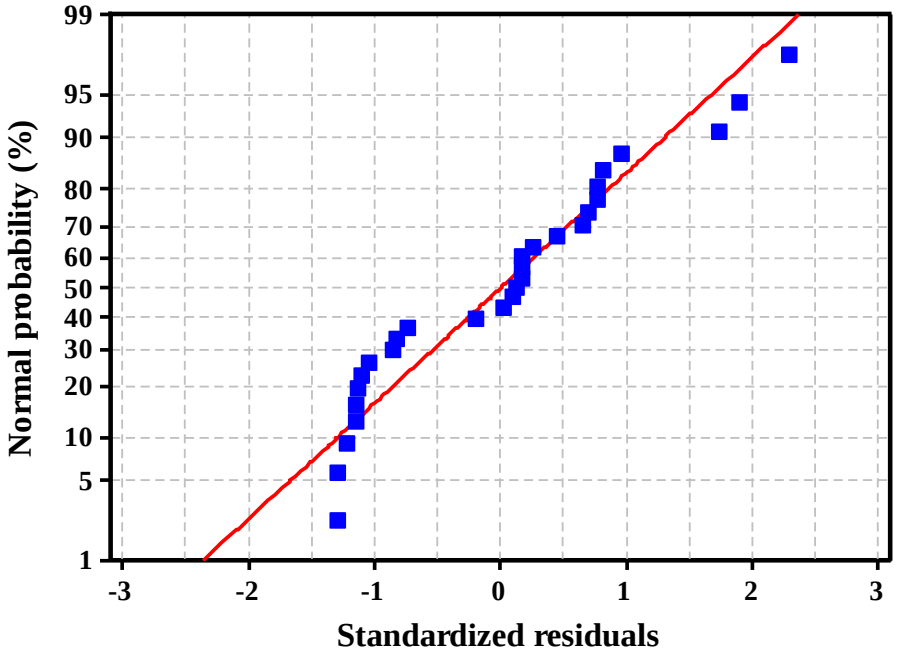



Figure 3.18 The Normal probability plot of standardized residuals (a) and the predicted growth rate (mm day-1) of *P. variabile* and standardized residuals plot.

(a)




(b)

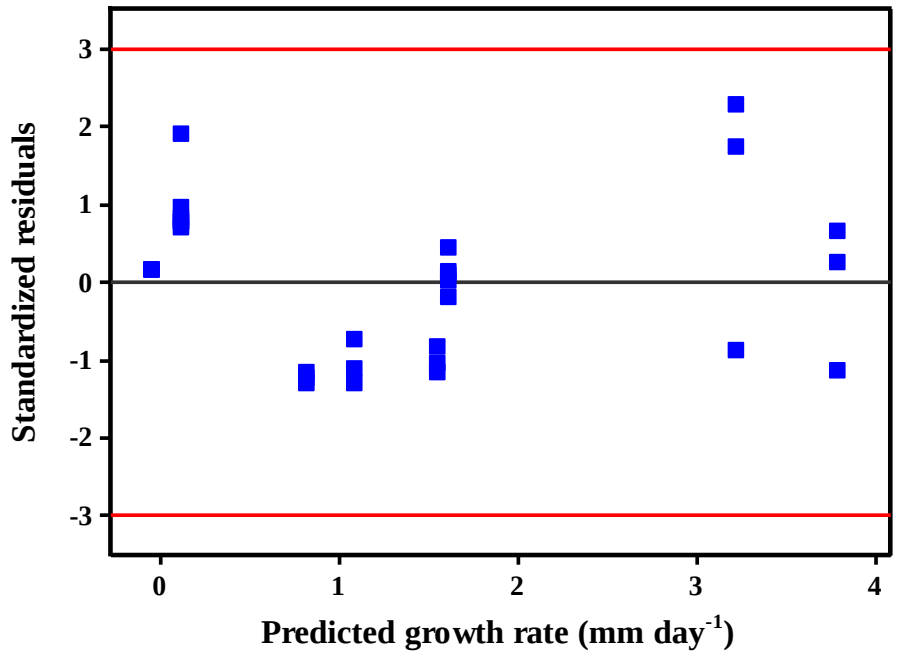



Figure 3.19 The Normal probability plot of standardized residuals (a) and the predicted growth rate (mm day-1) of *E. nigrum* and standardized residuals plot.

As the normality assumptions from the observed data of *E. nigrum* were not met, the normalization of the dataset was required. Better normalized data were generated from Johnson transformation (P>0.05, Kolmogorov–Smirnov test). The original dataset of radial growth rate of *E. nigrum* were transformed according to the following equation:

$$Y^* = -0.120616 + 0.868441 \times \ln(y + 0.245251) \quad (3.7)$$

In the equation, $Y^*$ represents the transformed radial growth rate and $y$ represents the original data.

The back-transformed data were calculated from the equation below:

$$y = -0.245251 + e^{\frac{Y^* + 0.120616}{0.868441}} \quad (3.8)$$

where $y$ represents back-transformed data, $Y^*$ represents the transformed radial growth rate of *E. nigrum*. After transformation, response surface reduced cubic model was then fitted in term of coded factors for radial growth rate of *E. nigrum* as below:

$$Y^* = 0.39 - 0.35x_1 + 0.71x_2 - 0.074x_1x_2 - 0.51x_1^2 - 0.099x_2^2 - 0.18x_1x_2^2 \quad (3.9)$$

where $Y^*$ is the transformed radial growth rate and $x_1$ and $x_2$ are the studied factors namely temperature and water activity, respectively.

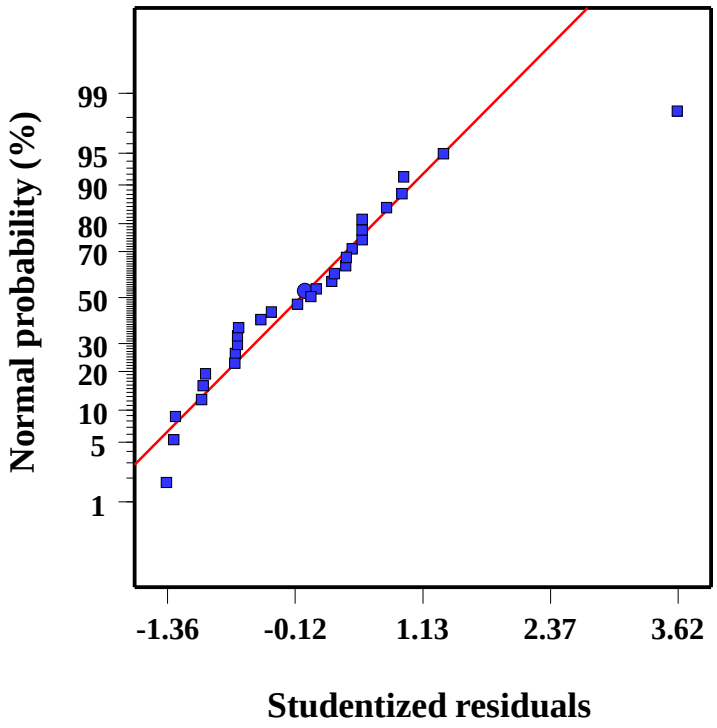
The significance of the model and all model terms were then analyzed with ANOVA (Table 3.8). After applying the transformation to the dataset of radial growth rate from *E. nigrum*, the previous established quadratic model was change to a reduced cubic model. All model terms were the significant terms and the model was also significant (P<0.05). A better $R^2$ was obtained (0.9837) compared with the previous model ($R^2$=0.9319). Moreover, when introducing the new model term in to the model, a non-significance of the lack of fit test was revealed. This statistical analysis indicated that the model did fit the observed transformed-data sets well.

Table 3.8 Analysis of variance for the response surface reduced cubic model of *E. nigrum* with transformed-dataset.

| Source of variation | df | Mean square | F value | P value |
|---|---|---|---|---|
| Model | 6 | 8.9 | 220.82 | 0.000 |
| Temperature | 1 | 9.22 | 64.98 | 0.000 |
| $a_w$ | 1 | 25.91 | 790.42 | 0.000 |
| Temperature*$a_w$ | 1 | 4.80 | 5.73 | 0.026 |
| Temperature*Temperature | 1 | 4.06 | 152.72 | 0.000 |
| $a_w$*$a_w$ | 1 | 1.09 | 5.72 | 0.003 |
| Temperature*$a_w$*$a_w$ | 1 | 0.13 | 11.20 | |
| Residual | 22 | 0.012 | | |
| Lack-of-Fit | 2 | 00031 | 3.27 | 0.0591 |
| Pure Error | 20 | 9.548E-0.003 | | |

To diagnose the adequacy of the established model, graphical analysis was performed. The residuals points on the normality probability plot seemed better linear with one residual deviated from the straight line (Figure 3.20a). It could be due to the outliner (unusual observed data). The scattering of residuals were fallen in a symmetrical pattern more than the model without transformation (Figure 3.20b). A plot of the observed values with the model predicted values showed better fit the linear regression line (Figure 3.21).

(a)




(b)

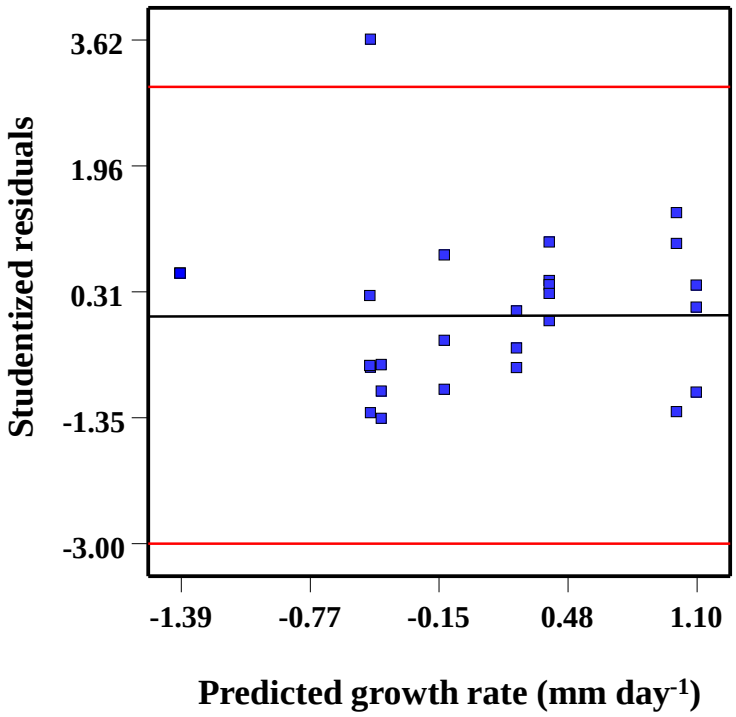



Figure 3.20 The Normal probability plot of standardized residuals (a) and the predicted growth rate (mm day$^{-1}$) of *E. nigrum* and standardized residuals plot (b).

(a)

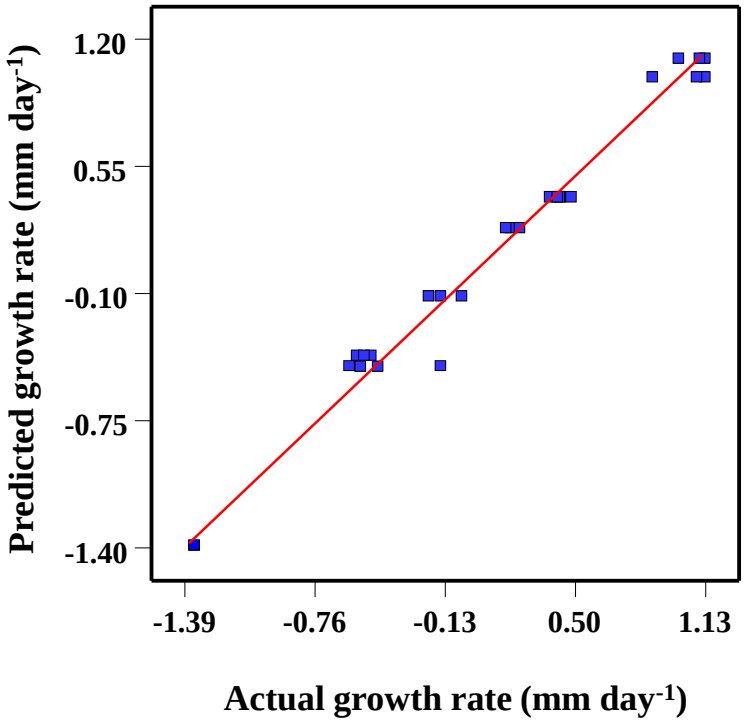
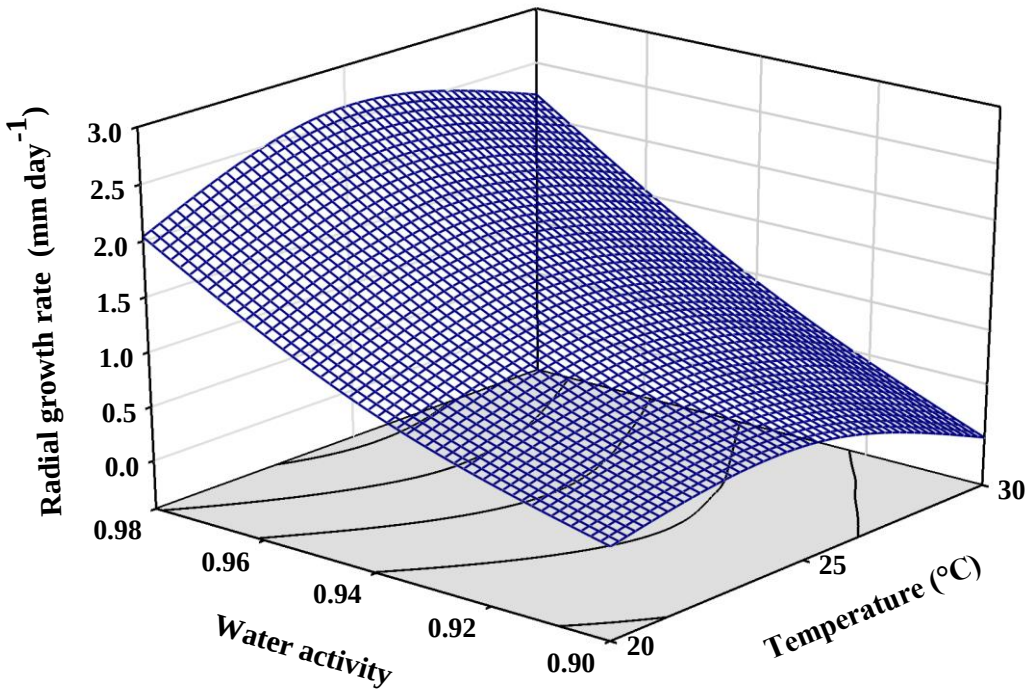



Figure 3.21 The observed values with the model predicted values plot for *E. nigrum* (transformed data).

Plots of the three-dimensional response surface and contour provided a better understanding of the interaction among the experimental variables on the response. From the model Equation 3.5 in this study, the response surface plot with the effects of temperature and $a_w$ on the growth rate of *P. valiabile* was generated (Figure 3.22a). Examining this plot, it can be seen that both variables had the desired effect on radial growth rate. The increasing trend in growth rate with increasing $a_w$ could be predicted. The incubation temperature of 25℃ favoured the highest growth rate at any given $a_w$. The contour plot from this response surface model was provided in Figure 3.22b. The significance of the quadratic term of temperature and $a_w$ indicated that a higher growth rate (more than 2.50 mm $day^{-1}$) was observed at above 0.98 $a_w$ with 25°C, whereas cultivation temperature higher than 26°C with 0.90 $a_w$ resulted in a meaningful reduction of the growth rate (less than 0.50 mm $day^{-1}$).

(a)




(b)

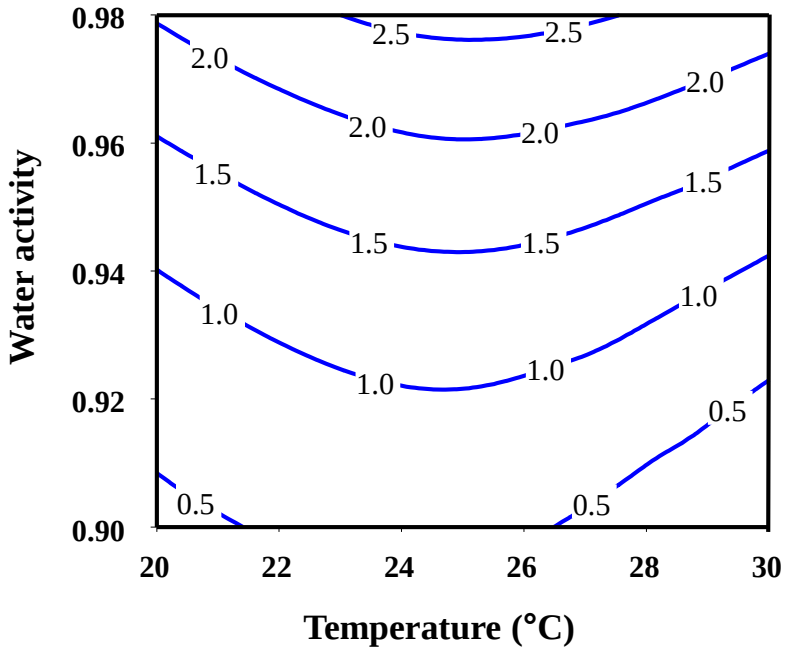



Figure 3.22 (a) The three dimension surface plot (showing the combined effect of $a_w$ and temperature on the radial growth rate. (b) The contour plot showing the combined effect of aw and temperature on the radial growth rate of *P. variable.*

For *E. nigrum*, the 3D surface graph and contour plot were analyzed from the response surface model (Figure 3.23). The surface graph exhibited a curvilinear between the experimental factors and the response. A similar response to both environmental stresses was observed. Higher growth rate was achieved when cultured with high media-water availability. An increasing trend in growth rate with lowering incubation temperature at <30°C was predicted. Growth at temperatures between 22-23.5°C at any $a_w$ can result in a higher growth rate (Figure 3.24). This temperature range was slightly reduced when the level of $a_w$ was ≥0.98. A higher growth rate which was more than 3.77 mm $day^{-1}$ was observed at above 0.98 $a_w$, whereas growth at the temperature above 28°C with high water stress (0.90 $a_w$) inhibited growth.

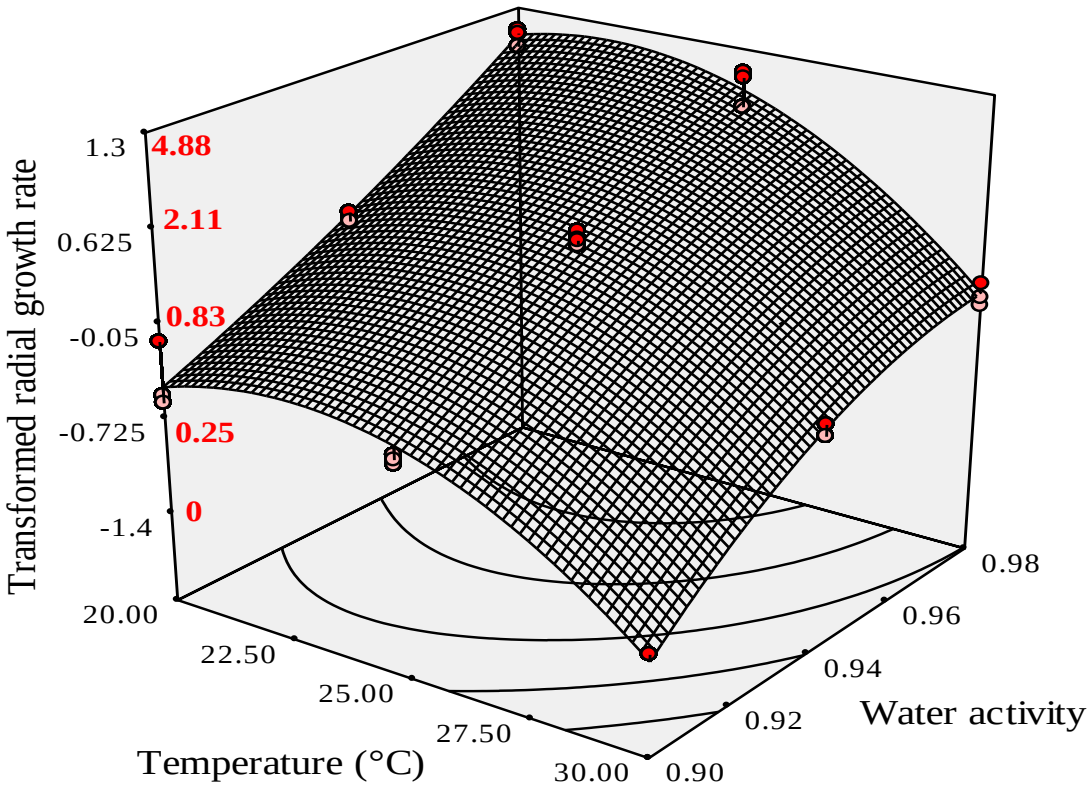



Figure 3.23 The response surface resulting from the reduced cubic model describing the effect of temperature and aw on radial growth rate of *E. nigrum* (the number in red at Z-axis indicated the back transformed scale).

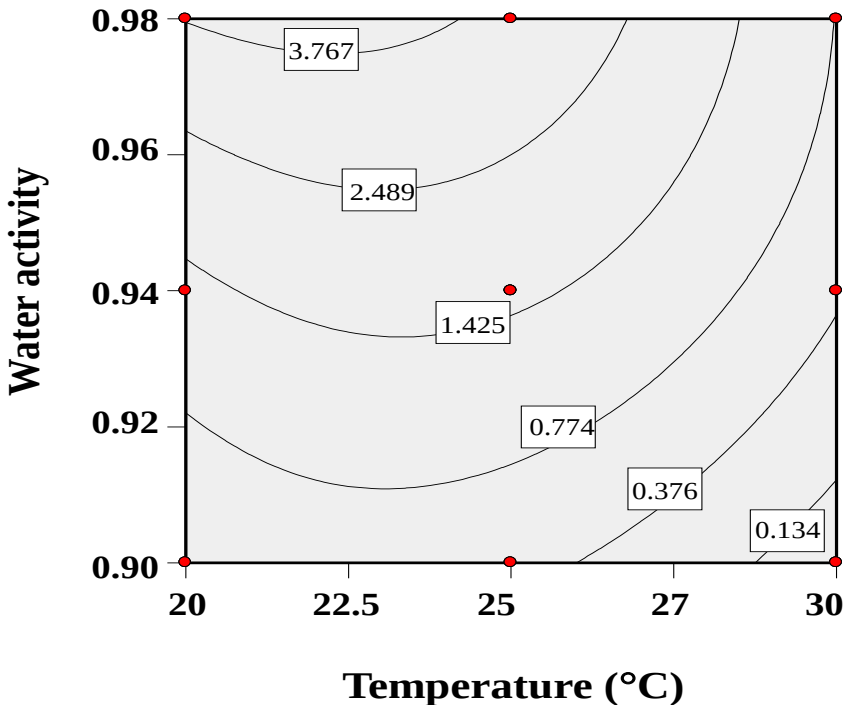



Figure 3.24 The contour plot showing the combined effect of $a_w$ and temperature on the radial growth rate of *E. nigrum* (back transformed data).

***Model validation***

To test the quality of the constructed model, the additional experiments in the experimental area were carried out. For *P. variabile* model, there is a good agreement between the results of experimental data and the predicted model data (Figure 3.25). A high correlation ($R^2$=0.92) between the predicted data and the experimental data was observed. The average error from the model is encapsulated in the RMSE. The smaller values of RMSE (0.117), the closer predicted data to the experimental data were obtained. RSEP value was low as 6.6%. Moreover, the nonparametric Mann-Whitney U-test was determined as P>0.05 which indicated that there was no statistically significant difference between the experimental data and the predicted data.

The $R^2$ value for *E. nigrum* model was 0.86 which the model can explain 86% of the variability observed in the response in terms of radial growth rate. The difference between the observed data and predicted data was not revealed significantly (P>0.05, Mann-Whitney U-test). The less accurate prediction was evidenced from the RMSE value (0.583) and RSEP (24.76%). Better fitted regression by both observed and predicted data was observed (Figure 3.26).

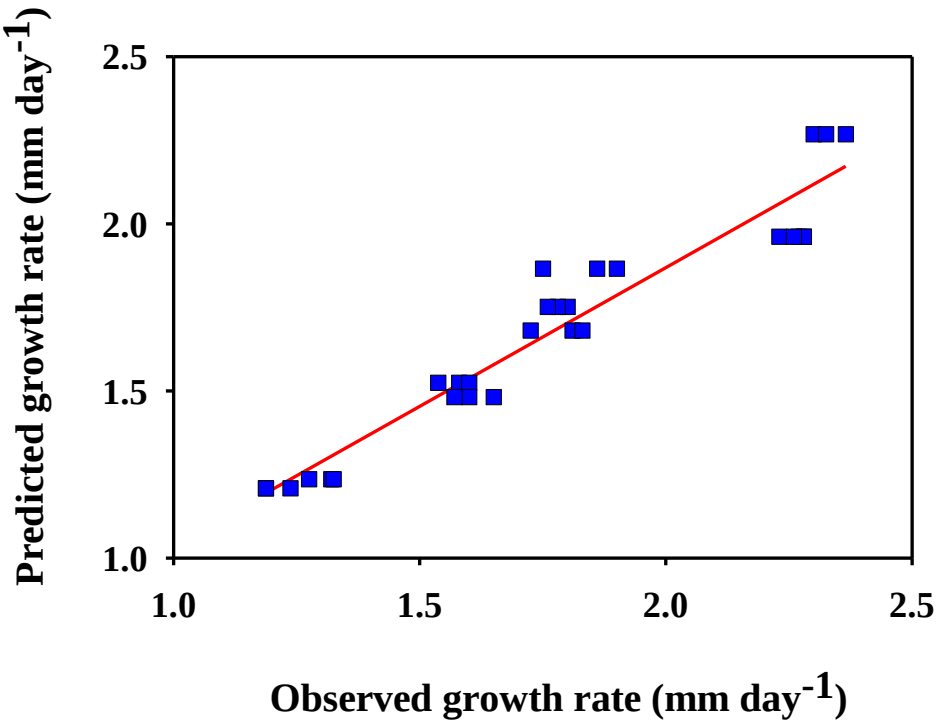



Figure 3.25 The observed values with the model predicted values plot for *P. variabile* (

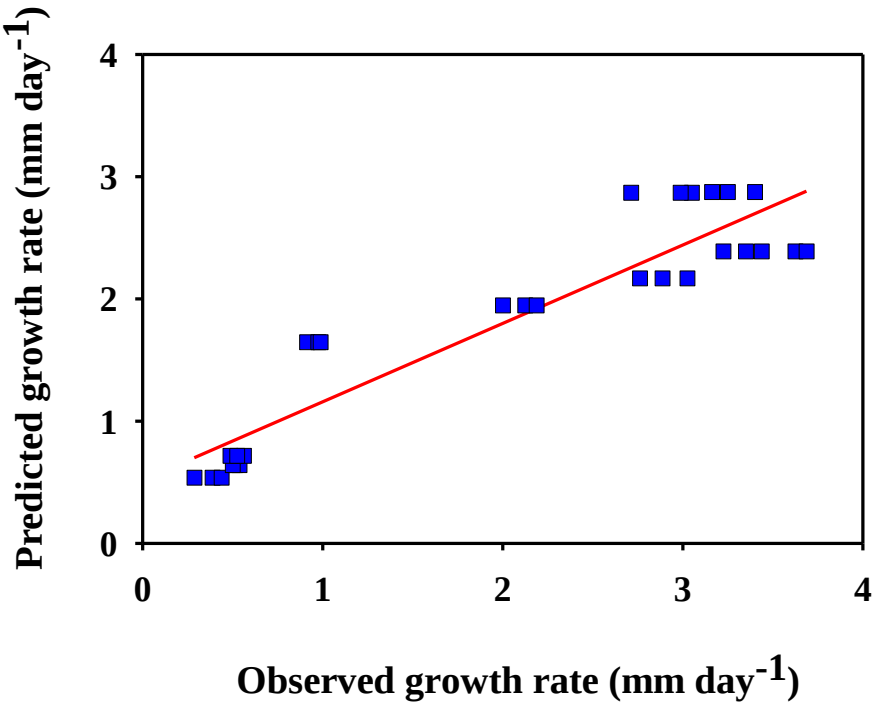



Figure 3.26 The observed values with the model predicted values plot for *E. nigrum* (backed transformed data).

## 3.4 Discussion

***Ecophysiological patterns of endophytic fungi under environmental stress***

Under particular micro-environmental niches within host plant tissues, fungal endophytes are likely to encounter different types of stress. Stressful conditions such as drought, salinity and temperature are stress factors that can result in an adverse effect on plant growth, which can in turn influence fungal symbionts. However, very little is known about the growth pattern of fungal endophytes capable of producing host plant compounds under abiotic stresses in vitro, especially taxol-producing strain. In this study, the possible combinations of environmental stress were used to mimic ecophysiological stress conditions to evaluate how these affect growth by such endophytic fungi.

A similar pattern of growth was made among three isolates when the maximum growth occurred with decreasing water stress (0.99-0.98$a_w$). Although two endophytes of *P. variabile* were isolated from the same species of host plant but different location, a *P. variabile* (taxol non-producing strain) exhibited less water stress tolerance. This indicated the different physical response may associate with single host plant. Similar observation was documented by Hutton et al., 1996. Two of the isolated endophytes with a high degree of homogeneity were isolated from the same species of host plant at different locations which demonstrated the same growth response when tested for water stress tolerance. While another isolated endophyte from the same plant species (different location) exhibited a different pattern of response.

The different responses of *P. variabile* and *E. nigrum* to $a_W$ stress were observed. *P. variabile* was found to be able to grow in the range 0.99 to 0.90 $a_w$, with the minima influenced by the solute types used. While *E. nigrum* showed less tolerant of lowered $a_w$ as described by Griffin, 1981. The lower limit for growth of this strain was found at 0.89 (Magan and Lacey, 1984) which was lower than in the present study of 0.92 $a_w$ at the same incubation temperature (25°C). However, in the Magan and Lacey (1984) study the *E. nigrum* was isolated from the phyllopshere of cereals and not as an endophyte. With ionic solutes, especially NaCl, inhibitory effects was found for both strains. These finding agree with the results of previous studies of *Penicillum expansum* which failed to produce substantial growth at high concentrations of NaCl (0.93 $a_w$, 25°C), whereas non-ionic solutes-amended media supported growth, even at the lowest tested water stress examined (0.89 $a_w$, 25°C) (Lahlali et al., 2005). Similar negative effects of salinity were observed by Lahlali et al. (2007) for *Botrytis cinerea* when the limit of growth was observed at 0.93 $a_w$ with the presence of NaCl or at 0.89 $a_w$ with non-ionic solute.

Beside the osmotic effect from salt stress, the cytosolic accumulation of toxic $Na^+$ and $Cl^-$ ions directly could be the additional explanation of such toxicity in living cell. Metal ion $Na^+$ can create a significant impact on cellular systems due to its physiochemical similarity with $K^+$ ion which then compete for binding sites interfering metabolic process (Griffin, 1994; Maathuis and Amtmann, 1999) In addition, the

similarity between $Na^+$ and $K^+$ ions leads the difficulty to remove the toxic $Na^+$ ion from the cell, this could be the basis of $Na^+$ toxicity. Excess salt also causes membrane disorganization, impairment of ionic homeostasis and produces the reactive oxygen species (Kramer, 1983; Greenway et al., 1986; Blomberg and Adler, 1993; Niu et al., 1995; Yeo, 1998; Hasegawa et al., 2000; Mahajan and Tuteja, 2005). Fungal growth and its viability therefore depends on adaptation to such high ionic concentrations. Thus salt toxicity at high concentrations could be the reason for limited growth observed in these conditions.

Within the same $a_w$ level but adjusted with non-ionic solutes clearly showed less inhibitory effect on fungal growth at relatively low $a_w$ (Marin et al., 1996; Begoude et al., 2007; Dagno et al., 2010). In the present study, the highest growth rate was mostly obtained with non-ionic solutes. At lower $a_w$, non-ionic solutes may affect the growth by osmotic stress which may interfere the movement of water. The strategy to overcome the changes in the external osmotic pressure is compatible solute accumulation which such osmolytes are produced and/or accumulated from the environment. However, the amount and types of osmolytes strongly depends on type of $a_w$ depressors (Redkar et al., 1995; Hallsworth and Magan, 1996; Ramos et al., 1999; Ramirez et al., 2004; Tarocco et al., 2005).

With regard to the influence of temperature stress, the observed higher growth rates of *P. variabile* at optimum temperature (25°C) at nearly all $a_w$ levels is supported by the view that the greatest tolerance to lowered $a_w$ occurs at the optimum growth temperature (Ayerst, 1969; Aldred et al., 1999; Magan, 2007). *E. nigrum* showed optimal growth at 20 to 25°C. This finding can indicate the tolerance to lowered $a_w$ was solute and fungal strain dependent.

Under non-modified $a_w$ conditions with well-buffered medium, *P. variabile* was more acidophilic with grow increasing with decreasing pH down to 5. While *E. nigrum* was more alkaliphilic strain, the highest average growth rate was obtained at pH up to7. The notable influence of pH with salt-amended media used in this study is exampled by *E. nigrum*. Growth on medium imposed with NaCl (pH 5-7) this isolate with

alkaliphilic pH optima (pH ≥7) was inhibited at 0.94 $a_w$. Inhibition of growth in NaCl-amended medium could be due to the salt toxicity or their interactions between stress factors but not due to sub-optimal pH alone. Good agreement was obtained between the observed and predicted results when testing the relationship between water stress and pH. Growth of filamentous fungi was considered to be more affected as a function of $a_w$ (salt stress) when ionic NaCl solute was used rather than pH (Panagou and Kodogiannis, 2009; Panagou et al., 2003; Valik et al., 1999).

***Application of response surface methodology to predict the growth***

Response surface methodology was used to generate the predictive model, which can adequately describe or predict the response of fungi to studied environmental stresses. The use of face-CCD facilitated more information in order to identify the factors and their interaction effects. The response surface model relating to growth rate of *P. variabile* exhibited good performance in terms of $R^2$, RMSE and RSEP. Whereas, lower performance was found from the response surface reduced cubic model of *E. nigrum.*

The different in quality of these constructed models are particular interest. Morphological studies of both strains revealed different colony characteristics which could be an additional explanation. *P. variabile* produces colonies of circular form with an entire margin, whereas an irregular form with undulate to lobate margin in *E. nigrum* colony morphology was observed. Radius measurement was used to obtain information computing radial growth rate. The irregular morphology provided more difficulty in measurement and created high difference between replicates which then generated unusual residuals of predicted data. In contrast, more accurate data was obtained as its symmetrical form of *P. variabile*. This finding is supported with high $R^2$ and lower RSME from the polynomial response surface model which was generated to predict the growth of the circular-colonies of fungi such as *Penicillium chrysogenum, Rhizopus oligosporus* NRRL2710, *Trichoderma asperellum, Botrytis cinerea* and *Moascus ruber* affecting by combined effect of temperature, pH and $a_w$ (Sautour et al.,

2001; Sparringa et al., 2002; Panagou et al., 2003; Begoude et al., 2007; Lahlali et al., 2007; Panagou and Kodogiannis, 2009).

According to the results obtained for the calculating RMSE, RSEP and $R^2$ from the validation of the model, this could be an empirical approach to predict the effects of $a_w$ and temperature conditions on the growth rate when cultured in MID using sorbitol as a $a_w$ depressor. Therefore, this can be applied to predict the growth under solid state fermentation for taxol production.

# 4 Effect of ecological interacting factors on taxol production by endophytic strains

## 4.1 Introduction

The amounts of relative yield of taxol from endophytic fungi have been reported in the range of ng to µg/l and still too low to reach industrial scale production. There has been a lot of interest in ways to improve the yields. As taxol is a secondary metabolite, efficient production of such a product is regulated by various factors including precursors, carbon:nitrogen ratio and source, phosphate, trace elements and environmental growth conditions (Griffin, 1994; Li et al., 1998a,b; Parra et al., 2005; Xu et al., 2006).

There have been very few detailed studies on the ecophysiology of taxol producing fungi and on optimization conditions for taxol production. This is surprising as this product is produced via fermentation in the same way as other secondary metabolite products from fungi, especially penicillin. Media design and environmental conditions are often examined. Taxol production in *Nigrospora* sp. increased with increasing concentration of the base medium M1D by 8 fold in solid state fermentation (Ruiz-Sanchez et al., 2010). Aldred et al. (1999) demonstrated that solid substrate fermentation and environmental manipulation could significantly enhance the production of cholesterol lowering drugs such as squalistatins.

Ecological niches in which fungal groups grow need to be considered in trying to optimize production systems using fungi. There are several abiotic factors affecting growth and yield of secondary metabolites such as temperature, pH, water activity and light (Carlile et al., 2001; Deacon, 2006; Magan and Aldred, 2007). Temperature and water are described as critical factors for fungal growth and metabolite production, especially secondary metabolites (Baxter et al., 1998; Sanchis et al., 2005; Magan, 2007). Moreover, cultures of *Taxus chinensis* plant

revealed the temperature shift from the optimum growth to another optimum temperature for taxol production. When cultured such plant cells under water stress with mannitol, sorbitol and polyethylene glycol modified media, resulted in a taxol yield enhancement (Choi et al., 2000; Kim et al., 2001). Therefore, the hypothesis was made that such environmental stress factors may stimulate fungal taxol yield.

Another effective method to enhance taxol yield by fungi was elicitation with biotic and abiotic elicitors. The use of abiotic elicitors such as sodium acetate had a significant effect on increasing taxol yield from *Fusarium maire* (Xu et al., 2006). Supplementation of benzoate was also shown to affect the accumulation of fungal taxol by *Pestalotiopsis microspore* when lowering inorganic phosphate in culture media (Li et al., 1998a). Furthermore, benzoate at 0.01 mM also activated taxol production by Periconia sp. which increased taxol production 8 fold from the control condition (Li et al, 1998b). Other studies have indicated the ability of other elicitors such as phenylalanine and salicylic acid improving the productivity of fungal taxol (Veeresham et al., 2003; Soliman and Raizada, 2013).

Interestingly, limited information exist on the ecology of taxol producing fungi and whether temperature and aw interactions may affect the yield of taxol. The objectives of this work were to (a) study the effect of ecophysiological factors on taxol production, (b) to study the potential of elicitors to stimulate taxol production and (c) to study the effect of interactions between $a_w$, pH and elicitors on taxol production by *P. variabile* and *E. nigrum*.

## 4.2 Material and methods

### 4.2.1 Preparation of mycelial inoculum

The inoculum was prepared from 7-days-old culture of both strains (*P. variabile* and *E. nigrum*) grown on PDA at 25°C. The mycelial agar plug (5 mm diameter) was cut using a sterilized cork borer. Three agar plugs were used as the inoculum in 250ml of culture broth in a 50 ml flask.

### 4.2.2 Effect of water activity, types of solutes and temperature on taxol production

An experiment was designed to determine how the studied stress factors affected taxol production by the isolated strains. A 6x5x3 factorial design was applied with the MINITAB version 16 (Minitab, Inc., USA). Table 4.1 shows the experimental variables and their levels. All treatments were carried out with two replicates per treatment. The basic media in this study was M1D. The $a_w$ of media was modified by adding non-ionic (glycerol, sorbitol, glucose) and ionic solutes (NaCl, KCl). The $a_w$ level was prepared by adding increasing amounts of solute to obtain the following $a_w$ levels of 0.99, 0.98, 0.96, 0.94 and 0.92. These were checked with the $a_w$ meter (AquaLab, Decagon Devices, Inc., USA). After inoculation, the cultured media were incubated at different tested temperatures for 21 days under static conditions. The cultured media were extracted and analysed as detailed later.

Table 4.1 Experimental factors and their levels used in 6x5x3 factorial design

| Factor | Levels | | | | |
|---|---|---|---|---|---|
| $a_w$ | 0.99 | 0.98 | 0.96 | 0.94 | 0.92 |
| Types of solutes | NaCl | KCl | glycerol | glucose | Sorbitol |
| Temperature (°C) | 20 | 25 | 30 | | |

### 4.2.3 Use of elicitors to stimulate taxol production

Acetic ammonium, jasmonic acid, phenyl alanine, salicylic acid, serine, silver nitrate and sodium acetate were used as elicitors in this study. Stock solutions were prepared by dissolving each elicitor in appropriated solvent and then were sterilized by filtration through 0.20 µm filter. Each inducer was added to the M1D broth (250 ml) at the different concentration which was showed in Table 4.2. Taxol amounts were measured

Table 4.2 The levels of elicitors used the experiment.

| Types of elicitors | Levels (mg/l) | | |
|---|---|---|---|
| Acetic ammonium | 0.5 | 1 | 5 |
| Jasmonic acid | 210 | 1051 | 3154 |
| Phenylalanine | 1 | 5 | 10 |
| Salicylic acid | 50 | 100 | 150 |
| Serine | 85 | 100 | 150 |
| Silver nitrate | 1 | 2 | 3 |
| Sodium acetate | 4 | 123 | 410 |

### 4.2.4 Effect of inducer, water activity and pH on taxol production

The effect of inducer, $a_w$ and pH on taxol production were studied for *P. variabile* and *E. nigrum* at two $a_w$ conditions (non-modified, 0.98 $a_w$) and two levels of pH (5 and 6). The best inducers were selected from the previous experiment where salicylic acid (20 and 50 mg/l) for *P. variabile* and serine (30 and 85 mg/l) for *E. nigrum*. M1D was a basic medium used in this study. The $a_w$ of the medium was adjusted by adding the selected solutes to obtain the target 0.98 $a_w$ which was checked with the $a_w$ meter (AquaLab, Decagon Devices, Inc., USA). The initial pH of the medium was adjusted by using 1 N HCl or 1N NaOH to give the desired pH. Stock solutions of inducer were prepared by dissolving each inducer in appropriated solvent and were then sterilized by filtration through 0.22 µm filter. Each inducer was added to the M1D broth (250 ml) at the tested concentration. After inoculation for 21 days at 25°C, the cultured media were extracted for taxol quantification by HPLC.

### 4.2.5 Fungal taxol extraction

After inoculation, the cultured media were incubated at the treatment temperatures for 21 days as static cultures. The fungal mycelia were separated from broth by filtration. This filtered culture was subsequently extracted by adding dichloromethane in two equal volume of culture broth (Strobel, 1996). The extracted solvent was evaporated by using the rotary evaporator to dryness at 35°C (Eyela, Tokyo, Japan). The dry residual was re-dissolved in 5 ml of 100% dichloromethane and then was subjected to a SPE column (silica gel) 15 ml bed weight 2 g (Thermo Scientific, UK) which was eluted in a stepwise elution. The elution was carried out starting with 15 ml of 100% dichloromethane and then continued with 15 ml of dichloromethane:ethyl acetate at 1:1 v/v and 100% ethyl acetate. The last four of eluents were collected, combined and then evaporated to dryness under a gentle stream of nitrogen. The dry residual were dissolved in 1 ml of 100 % methanol. All samples were

filtered through 0.22 µm before further analyses with HPLC. The standard taxol (Paclitaxel) was purchased from SIGMA.

### 4.2.6 HPLC analysis

Taxol in samples were analyzed by HPLC (Algilent 1200, Agilent Technologies, USA) with a C18 analysis column (Agilent Zorbax Eclipse, Part No. 990967-902). Fifty microlitres of sample was injected each time and detected at 230 nm. The mobile phase was methanol:water (80:20 v/v) at flow rate of 1.0 ml/min.

### 4.2.7 Statistical analysis

After conducting the experiments, the effect of the experimental factors on the amount of taxol produced was analysed statistically by using MINITAB version 16.0 (Minitab Inc., USA). When assumptions of normality and equal variance were met, ANOVA was adopted to test for significant differences in response among the studied treatments. Post-hoc inter factor differences were calculated with Tukey multiple comparison tests. If these assumptions of a parametric test were violated, the significant differences were assessed using Kruskal-Wallis nonparametric ANOVA at statistical significant level of $P<0.05$. Post-hoc inter factor differences were calculated with Dunn's multiple comparison nonparametric tests. Bonferroni correction was used to keep the Bonferroni individual alpha=0.05.

## 4.3 Results

### 4.3.1 Effect of water activity, types of solutes and temperature on taxol production

All three main factors affected taxol production by *P. variabile* significantly ($P<0.05$), however, no interaction effect was found between the studied factors ($P>0.05$). The maximum amount of taxol was 7.11 μg/l when cultured with KCl modified media at 0.98 $a_w$ and 25°C. While cultivated under control conditions with unmodified $a_w$ medium at 25°C yielded about 1.15 μg/l of taxol. The effect as a function of individual factors from the main effect plots of the average amount of taxol are shown in Figure 4.1. When NaCl and sorbitol were used to impose water stress, this strain showed no taxol production regardless of $a_w$ levels and cultivation temperature used. Under severe $a_w$ stress (0.92-0.96) conditions, strong inhibitory effects on taxol production were revealed. A sharp decline in average productivity was observed as a function of high cultivation temperature (30°C). Better yield was achieved by using the ionic solute KCl as the $a_w$ depressor to modify this to 0.98 with a temperature of 25°C. This study also examined effects of these treatments on *E. nigrum* and no taxol was production, regardless of the experimental conditions used.

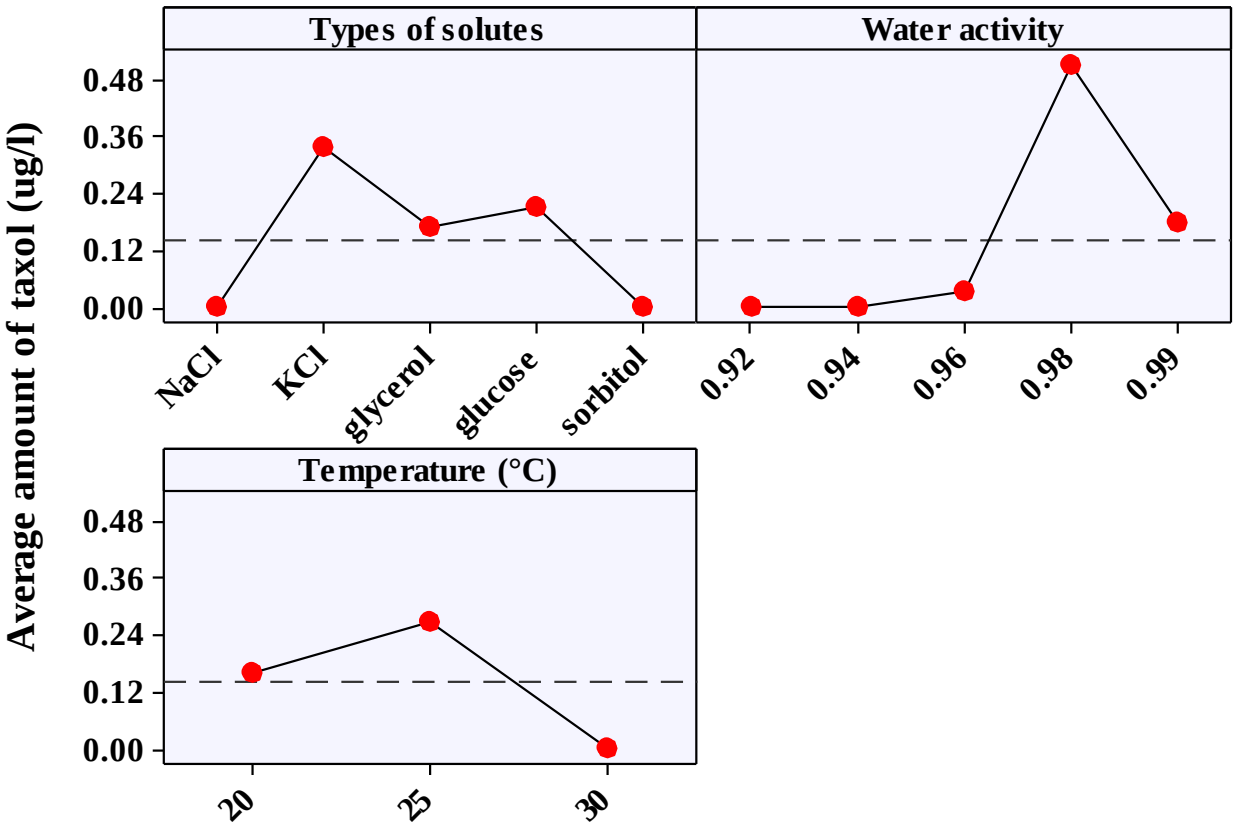



Figure 4.1 Main effect plots showing the mean value of the amount of taxol produced by *P. variabile* as a function of the experimental factors. Dashed lines represent grand mean.

### 4.3.2 Stimulation of taxol production by elicitors

As both strains exhibited an unstable productivity of taxol during cultivation, seven inducers were examined to evaluate whether they might stimulate taxol production. There was a significant increase in taxol production under all treatments when compared to that of the control conditions (Figure 4.2). When supplementing the media with salicylic acid, the most pronounced effect on increasing yield of taxol by *P. variabile* was observed. The highest taxol production (14.74±4.80) was obtained after induction with salicylic acid at 50 mg/l, whereas no taxol production was observed under control conditions.

Phenyl alanine-supplemented media had less effect on taxol yield. For *E. nigrum*, the maximum induction of taxol yield was obtained with serine (39.80±18.60) at 85 mg/l, while acetic ammonium had less of an influence on yield (Figure 4.3).

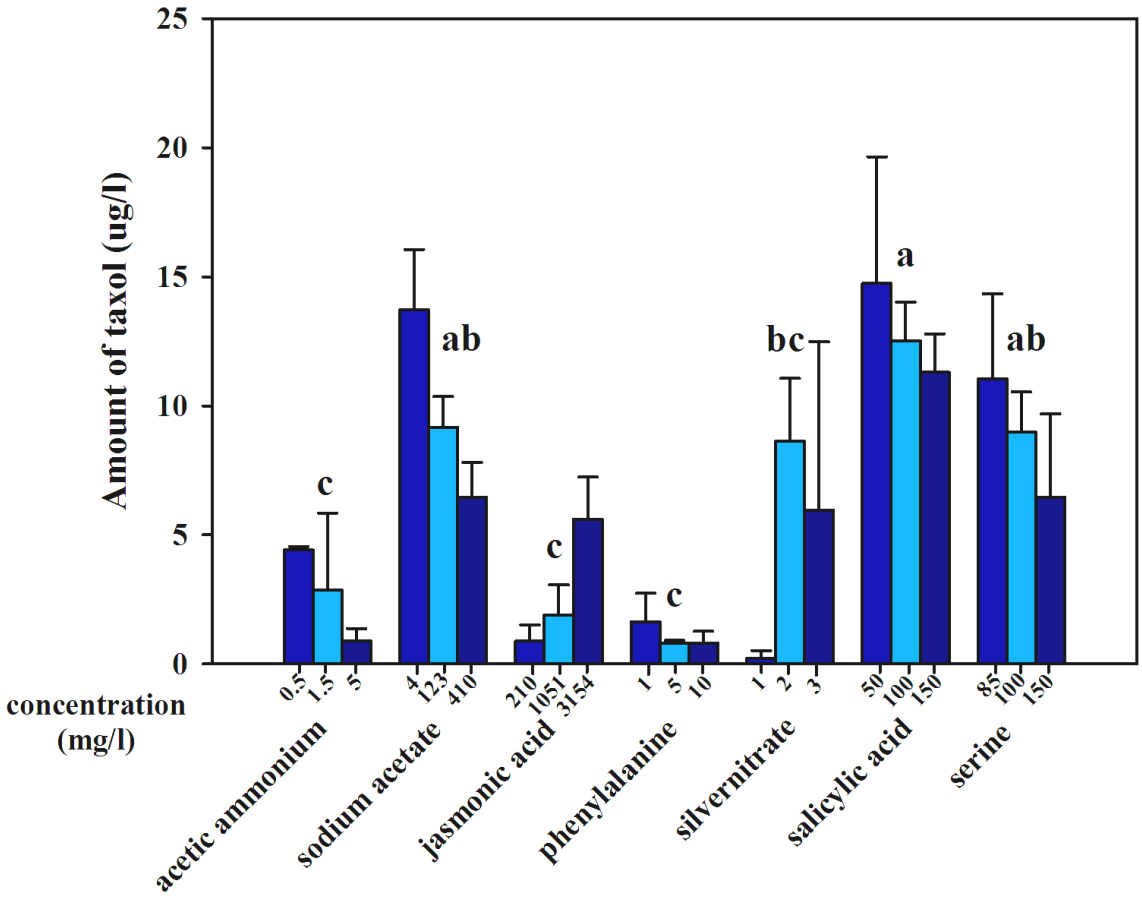



Figure 4.2 The effect of elicitors at different concentrations on taxol production by *P. variabile*. Data are presented as the mean ± SD from three replications. Bars with the same letters are not significantly different (data for three combined concentrations) using the post-hoc multiple comparison Tukey test ($P<0.05$).

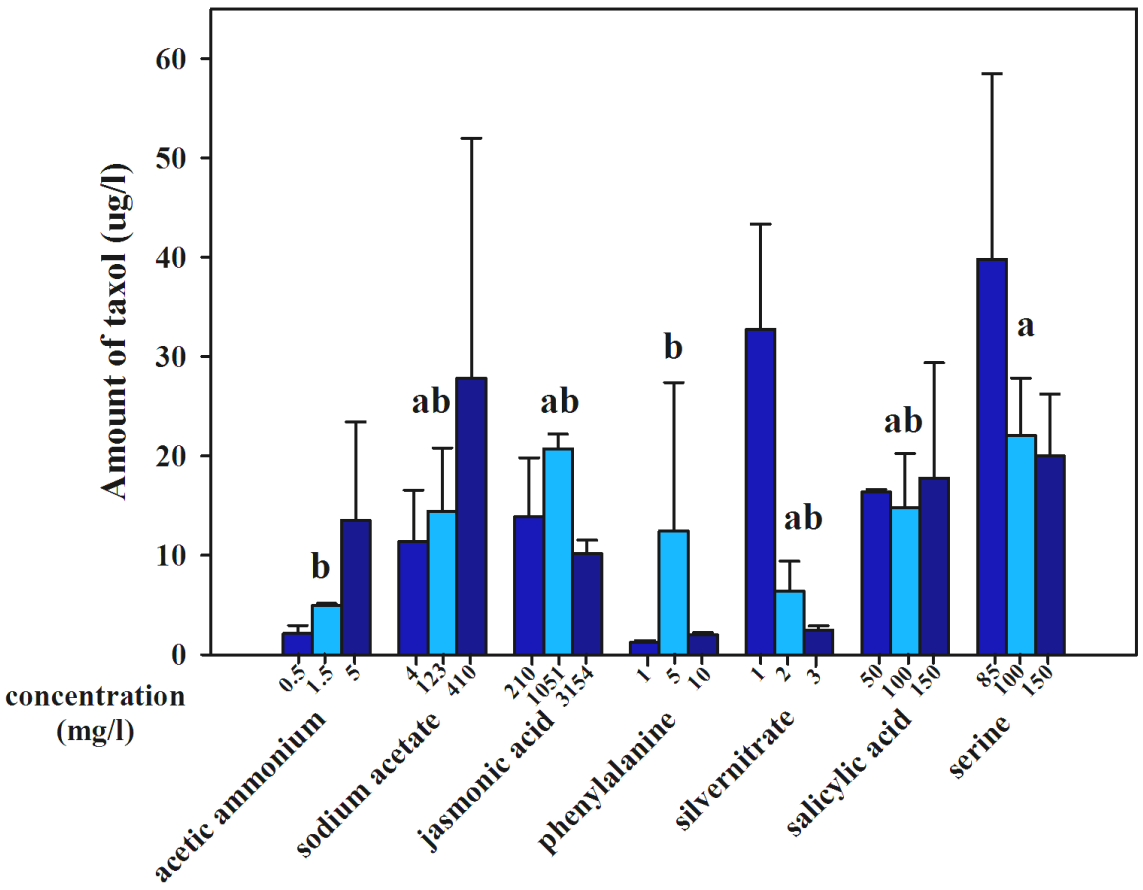


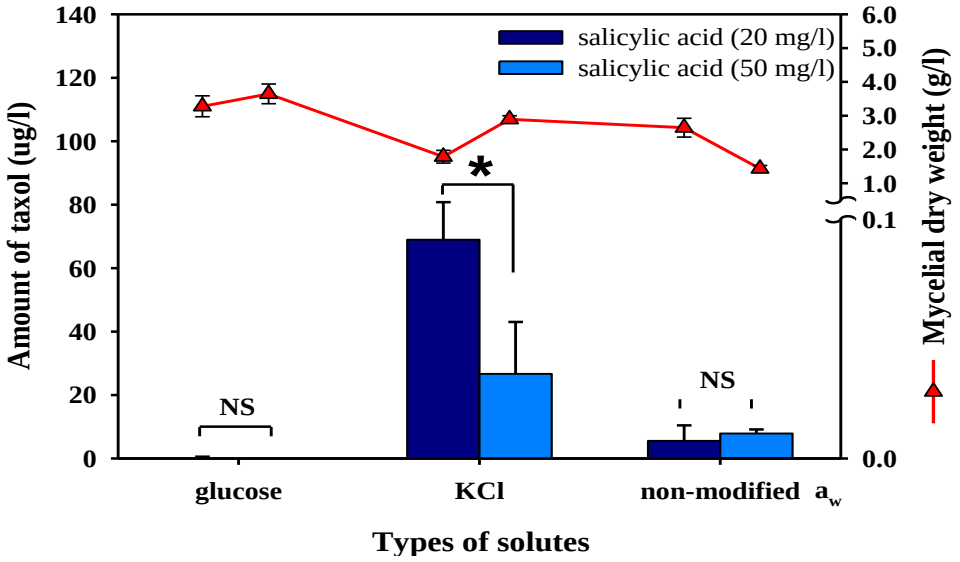

Figure 4.3 The effect of elicitors at different concentrations on taxol production by *E. nigrum*. Data are presented as the mean ± SD from three replications. Bars with the same letters are not significantly different (data for three combined concentrations) using the post-hoc multiple comparison Tukey test ($P<0.05$).

### 4.3.3 Effect of elicitor, water activity and pH on taxol production

Studies were extended to determine the impact of elicitor, pH and $a_w$ on taxol yield. A significant improvement in taxol yield by *P. variabile* was obtained (Figure 4.4). Using the ionic solute KCl as the $a_w$ depressor combined with salicylic acid at 20 mg/l resulted in the highest taxol production regardless of the cultivation pH used. However, culturing at pH 5 was a more favourable condition where the maximum yield was obtained (68.92±11.86 µg/l) (Figure 4.4a). This was a 10.27 fold increase when compared with non-modified $a_w$ conditions. The difference in taxol yield was significant with different concentrations of salicylic acid under acidic condition at pH 5, whereas had no significant effect occurred at pH 6 (Figure 4.4b). When the non-ionic solute glucose was used as the $a_w$ depressor, practically no taxol production was observed. The dry weights of mycelium were also examined at the same time for its ability to grow in the presence of the inducer. There was a noticeable reduction in the mycelial biomass when taxol yield increased.

For *E. nigrum*, the highest taxol yield was obtained (57.13±11.84 µg/l) after cultivated at pH 5 in a medium with a modified $a_w$ and an ionic KCl solute + 30 mg/l of serine as an inducer (Figure 4.5a). The difference in the taxol production between this condition and under non-modified $a_w$ conditions was approx. 8.5 times different. Taxol yields were again not significantly improved with the use of non-ionic solutes such as glucose and sorbitol to modify the $a_w$ of the media. Within the different concentrations of serine used at pH 6 there was no significant effects on taxol yield (Figure 4.5b). The different concentration of serine had no influence on the change in mycelial dry weight of *E. nigrum*. However, cultures with sorbitol-modified media resulted in higher dry weight of mycelia.

(a)




(b)

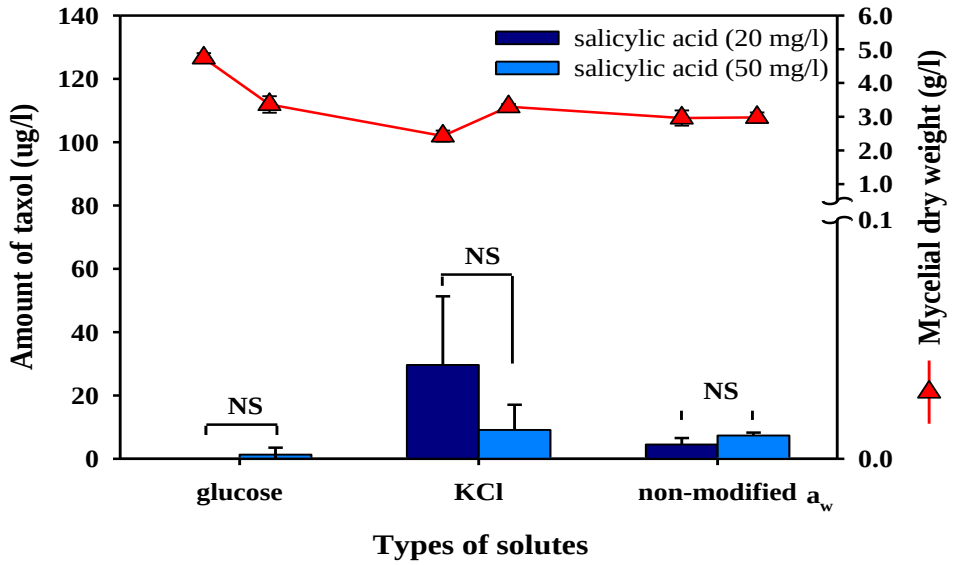
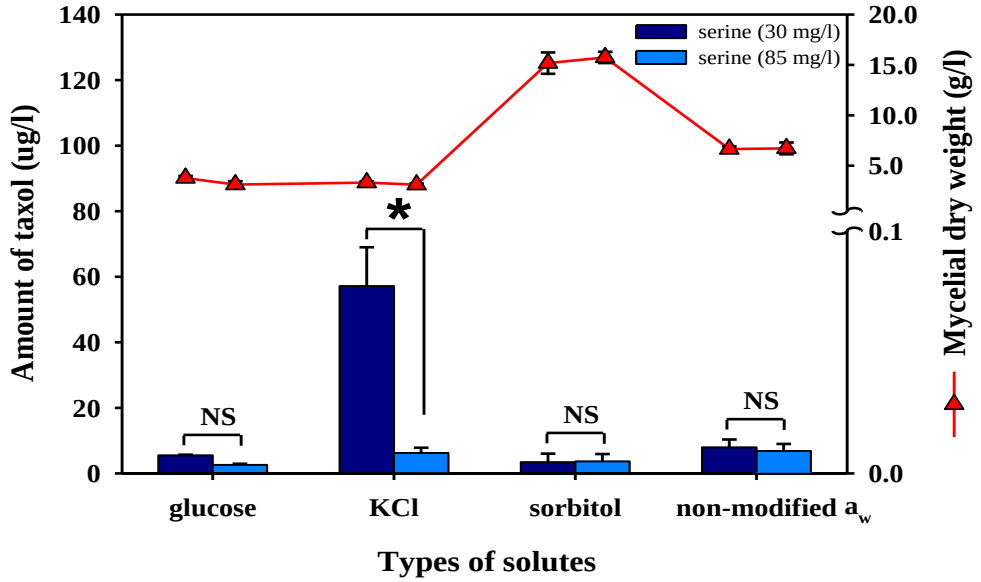



Figure 4.4 The effect of the different concentrations of salicylic acid, aw, solutes and pH on taxol production and mycelial dry weight of *P. variabile* in M1D broth. Two different pH levels: pH 5 (a) and pH 6 (b). The $a_w$ of media were modified with glucose and KCl at 0.98 $a_w$. Data are presented as the mean ± S.D. from three replications. Asterisk indicates significant differences ($P<0.05$, Student's *t*-test) while NS represents not significant.

(a)




(b)

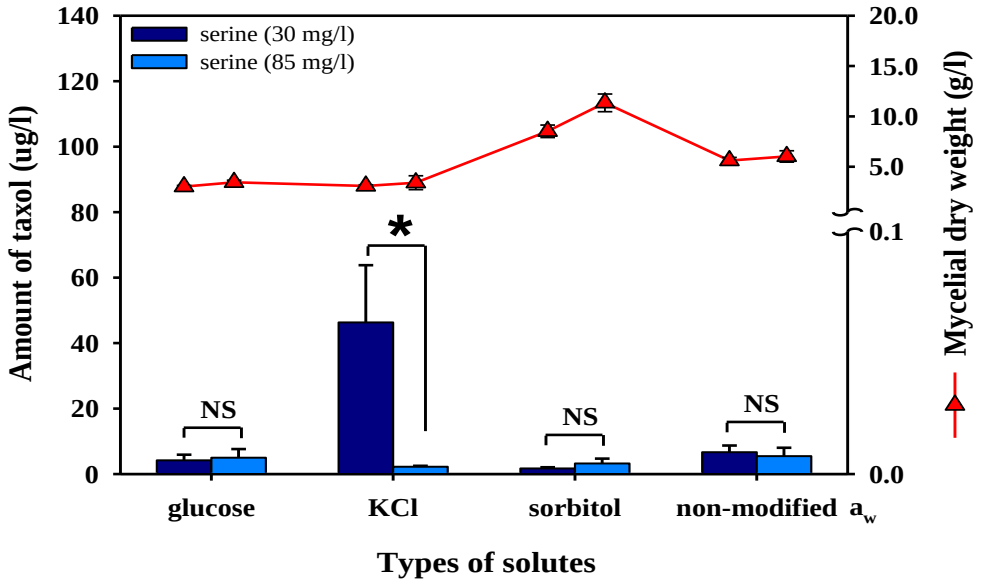



Figure 4.5. The effect of the different concentrations of salicylic acid, $a_w$, solutes and pH on taxol production and mycelial dry weight of *E. nigrum*. Two different pH levels: pH 5 (a) and pH 6 (b). The $a_w$ of media were modified with glucose, KCl and sorbitol at 0.98 $a_w$. Data are presented as the mean ± SD from three replications. Asterisk indicates significant differences ($P<0.05$, Student's *t*-test) while NS represents not significant.

### 4.3.3.1 Evaluation of the effect of salicylic acid, pH and $a_w$ on taxol production in liquid cultures of *P. variabile*

Statistical analysis was extended to evaluate the effect of salicylic acid, pH and $a_w$ on taxol production in liquid M1D cultures of *P. variabile* when KCl was used to modify $a_w$. An ANOVA analysis was carried out on the experimental data (Table 4.3). Among the studied quantitative variables, all three of them and their interactions had a significant effect on taxol yield within the range used in this study ($P<0.05$).

Table 4.3 Analysis of variance for experimental data.

| Source | DF | Mean square | F value | P value |
|---|---|---|---|---|
| **Main effects** | 3 | 2328.9 | 18.24 | 0.000 |
| pH | 1 | 1281.2 | 10.04 | 0.005 |
| Salicylic acid | 1 | 1244.3 | 9.75 | 0.006 |
| $a_w$ | 1 | 4461.0 | 34.94 | 0.000 |
| **2-Way interactions** | 2 | 1438.1 | 11.26 | 0.001 |
| pH*aw | 1 | 1145.0 | 8.97 | 0.001 |
| Salicylic acid*$a_w$ | 1 | 1731.2 | 13.56 | 0.008 |
| Lack of fit | 2 | 177.6 | 1.46 | 0.261 |

After the collected data were fitted, a first-order model with interaction in terms of the actual factors for taxol yield was developed as shown below:

$$Taxol\ amount = 43122.4 - 5443.54pH - 222.997 salicylic\ acid - 43771.2a_w + 5525.63pH * a_w + 226.48 salicylic\ acid * a_w \quad (4.1)$$

The main effects plot is useful in the practical interpretation of the results. In terms of the screening experiment, these plots can be used to compare the changes in the mean level to examine the direction of the response in relation to the tested level of factors providing the information for the subsequent experiment. Figure 4.6 represents the main effect plots of pH, $a_w$ and salicylic acid concentration in term of experimental data. A recommended setting of

factor levels to achieve a high response was obtained. Better yield could be achieved by choosing a relative more acidic pH level, setting the salicylic acid concentration at a lower level and modifying the $a_w$ of the culture broth.

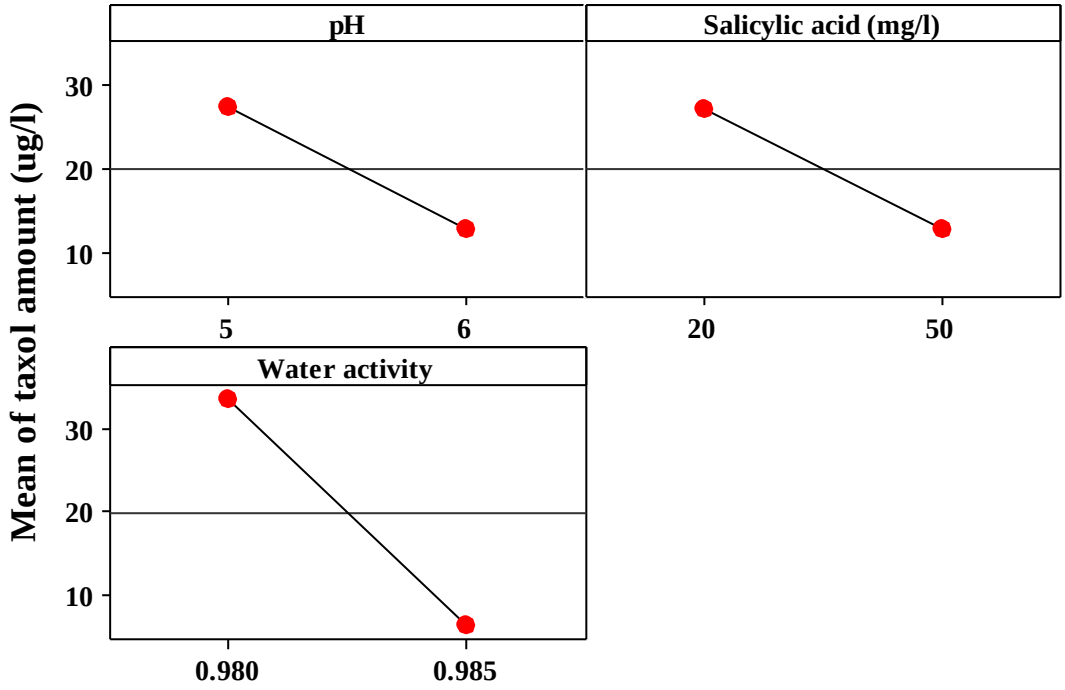



Figure 4.6 Main effects plot for the mean taxol production quantities (µg/l).

***Interaction effect of pH and $a_w$***

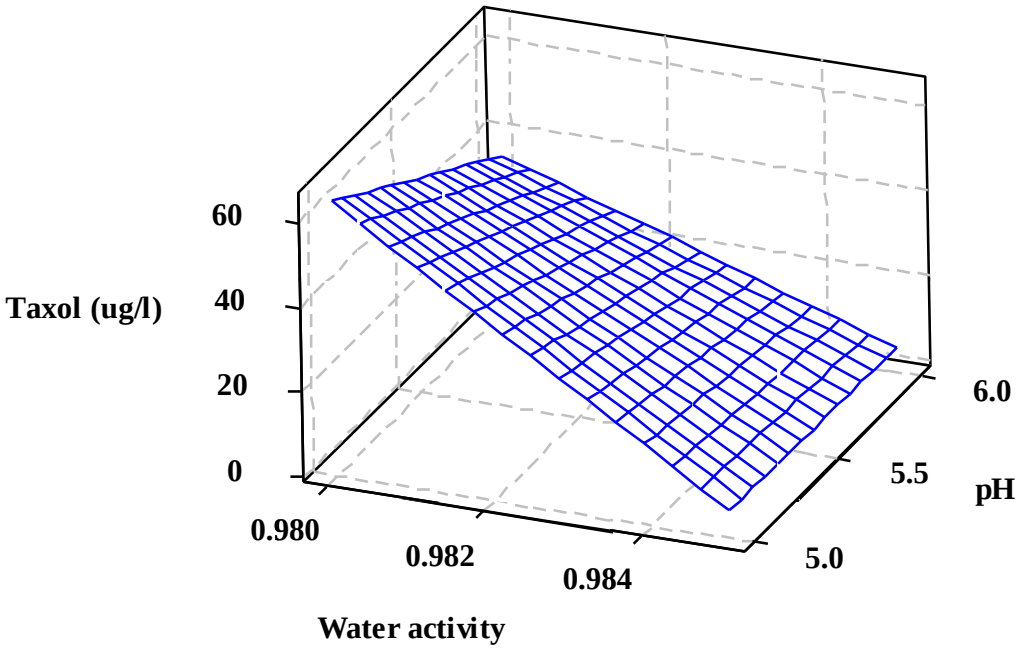
Figure 4.7a presents the 3D plot of the effect of pH x $a_w$ on taxol production when the level of salicylic concentration was held at a fixed level (20 mg/l). Examining this plot, it can be seen that $a_w$ had the desirable effect on taxol yield. An increasing trend in yield with a slight increase in water stress was observed. Moreover, when the water stress is increased, the impact of pH on responses became more significant**.** It seemed that the response depended almost entirely on $a_w$. This finding is in good agreement with the high F value of $a_w$ (F value=34.94). The higher the value of F, the more impact the particular parameter. Figure 4.7b provides the corresponding 2D contour plot. It is notable

that by adding an interaction term into the model, the curved shape of the surface was observed. Changing pH from 5.0 to 6.0 produced more change in taxol yield when $a_w$=0.980 than when $a_w$=0.985.

***Interaction effect of $a_w$ and salicylic acid***

Taxol production showed an increasing trend with increasing water stress at lower concentrations of salicylic acid (Figure 4.8a). It appeared that the effect of $a_w$ as a factor was clearly predominant and impacted on the relative taxol production response. Under water stress, the concentration of salicylic acid had a more important impact on the response, however, the response depended mostly on $a_w$. This was confirmed with the higher F value of $a_w$. Figure 4.8b provides a graphical interpretation of this interaction. Changing the concentration of salicylic acid from 20 to 50 mg/l resulted in a much smaller change in taxol yield when $a_w$ was 0.984 than when $a_w$ was 0.98.

(a)




(b)

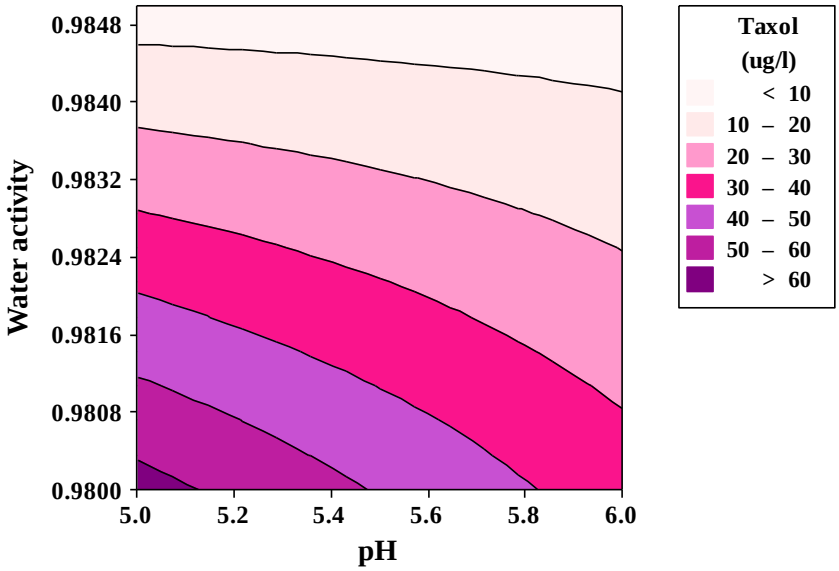
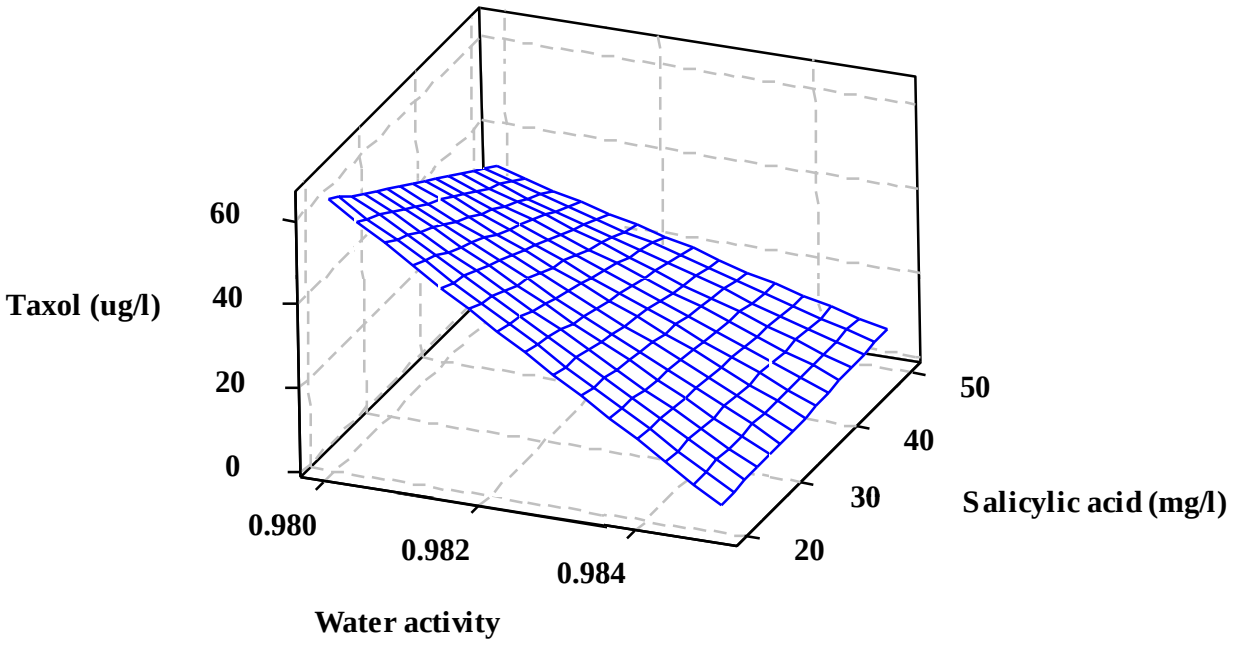



Figure 4.7 Three dimension plot of the response surface (a) and the contour plot (b) for taxol yield as a function of pH and $a_w$ with a fixed factor salicylic acid concentration at 20 mg/l.

(a)




(b)

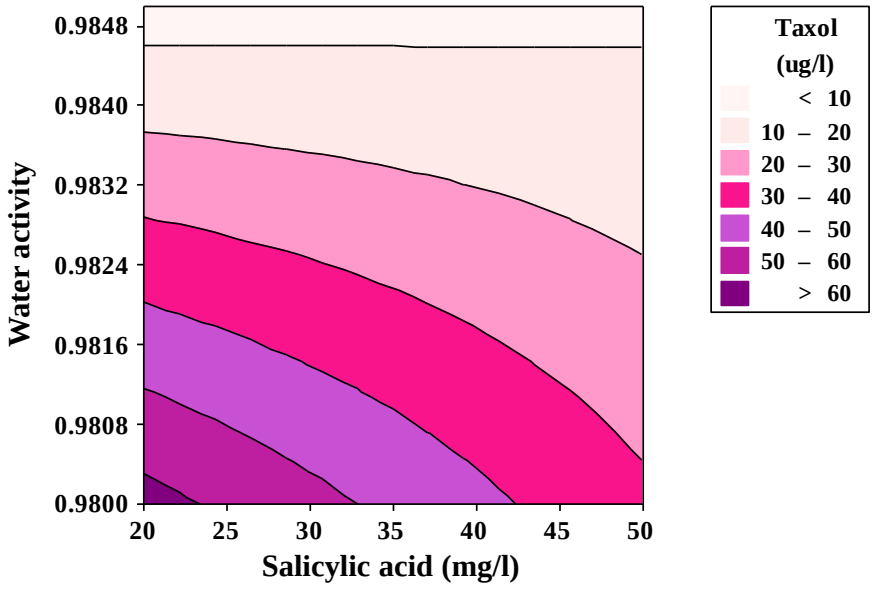



Figure 4.8 Three dimension plot of the response surface (a) and the contour plot (b) for taxol yield as a function of $a_w$ and salicylic acid concentration with a fixed factor pH at 5.0.

### 4.3.3.2 Evaluation of the effect of serine, pH and $a_w$ on taxol production in liquid cultures of *E. nigrum*.

Evaluation of the effect of serine, pH and $a_w$ on taxol production in M1D liquid cultures of *E. nigrum* was also investigated when KCl was used to modify the $a_w$. Normality of the distribution of the population was tested using the Kolmogorov–Smirnov test. The test revealed that the response variable was not normally distributed and needed to be normalized by transformation prior to analyses. The data sets were transformed by the Johnson transformations (P>0.05; Figure 4.9). The original data sets of taxol amounts were thus transformed according to the following equation:

$$Y^* = -0.786458 + 0.502649 \times Asinh\left(\frac{(x-4.74745)}{0.690764}\right) \quad (4.2)$$

In the equation, $Y^*$ represents the transformed taxol amount and $x$ represents the original data.

The back-transformed data were calculated from the equation below:

$$y = 4.74745 + \left(0.345382\, e^{\left(\frac{(Y^*+0.786458)}{0.502649}\right)}\right) - \left(0.345382 e^{\left(-\frac{(Y^*+0.786458)}{0.502649}\right)}\right) \quad (4.3)$$

where $y$ represents back-transformed data, $Y^*$ represents the transformed taxol amount.

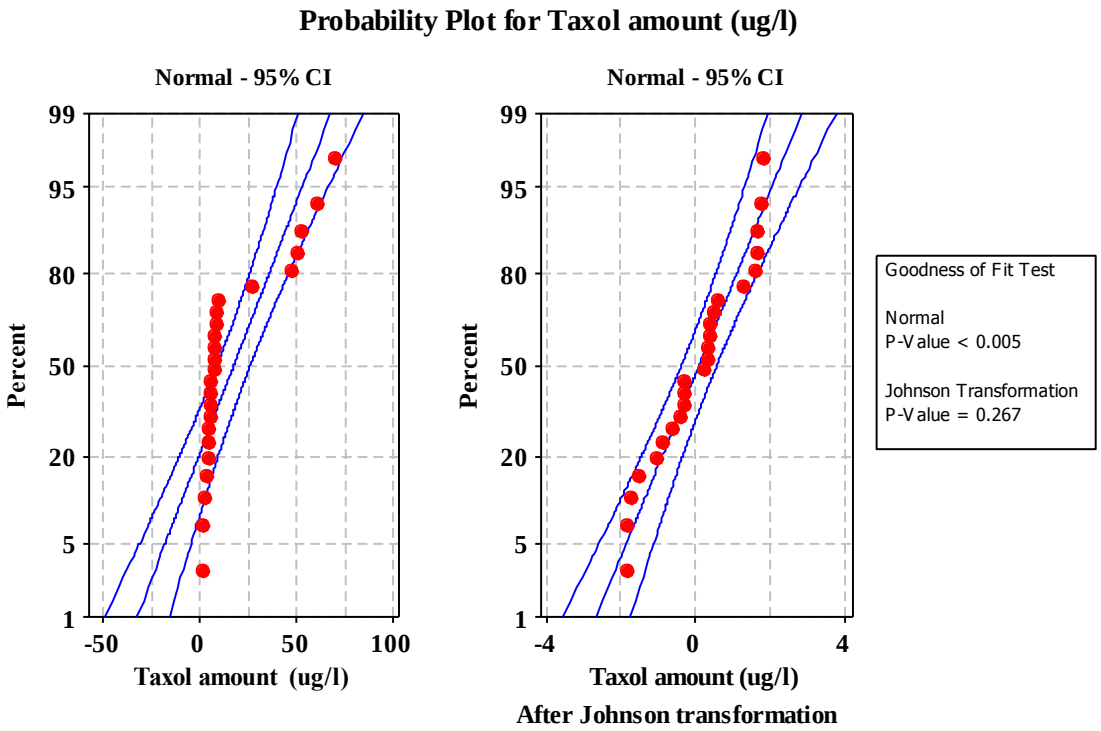



Figure 4.9 Normal probability plots for original data of taxol amount and transformed data using the Johnson transformation.

An ANOVA analysis was carried out on the transformed experimental data (Table 4.4). All three of studied factors including pH, serine concentration and $a_w$ had a significant effect on taxol yield within the range used in this study ($P<0.05$). Moreover, there was a significant effect on the interaction of serine concentration and $a_w$ ($P<0.05$).

Table 4.4 Analysis of variance for transformed data.

| Source | DF | Mean square | F value | P value |
|---|---|---|---|---|
| **Main effects** | 3 | 6.1660 | 17.53 | 0.000 |
| pH | 1 | 2.5256 | 7.18 | 0.015 |
| Serine | 1 | 14.5249 | 41.28 | 0.000 |
| $a_w$ | 1 | 1.4474 | 4.11 | 0.055 |
| **2-Way interactions** | 2 | 7.0862 | 20.14 | 0.000 |
| Serine*$a_w$ | 1 | 7.0862 | 201.4 | 0.000 |
| Lack of fit | 2 | 0.6727 | 2.31 | 0.116 |

The first-order model with interaction in terms of the actual factors for taxol yield was developed as shown below:

$$Y^* = 548.304 - 0.648795pH - 7.79362serine + 7.903652a_w + 7.90365serine * a_w \quad (4.4)$$

In the equation, $Y^*$ represents the transformed taxol amount.

The main effects plot of pH, $a_w$ and serine concentration in term of back-transformed data is shown in Figure 4.10. The results showed that with the decrease in pH level, a slight increase in water stress and the lower serine concentration provided the increasing trend in taxol yield.

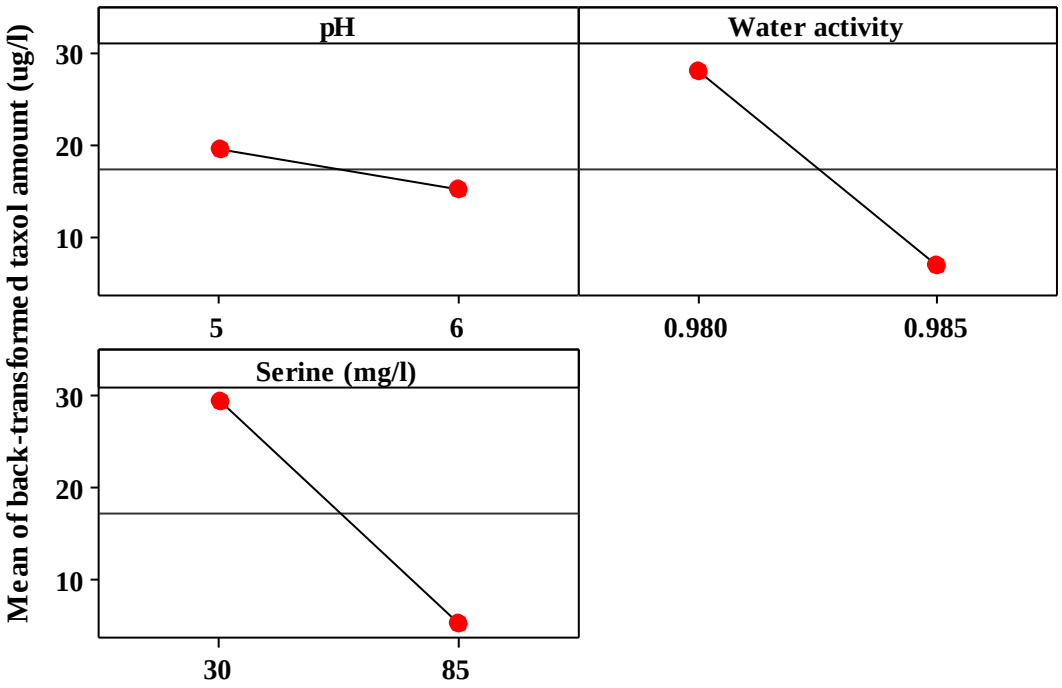
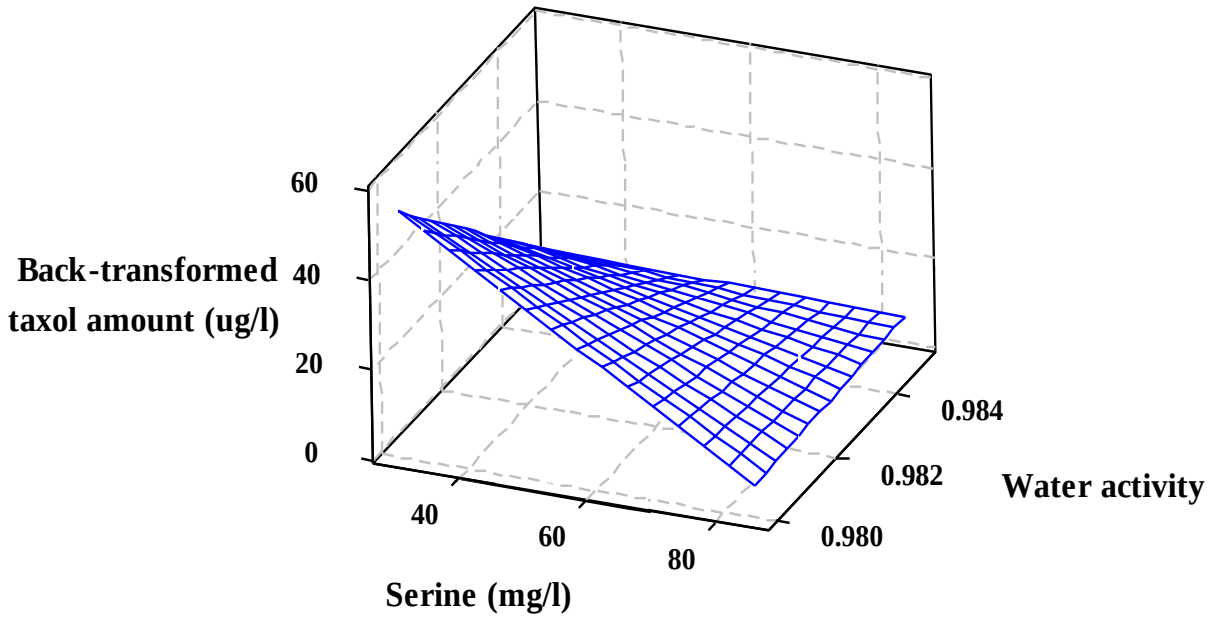



Figure 4.10 Main effects plot for the mean of back-transformed taxol amounts (µg/l).

***Interaction effect of serine concentration and water stress (water activity, $a_w$)***

Figure 4.11a presents the 3D plot of the effect of serine concentration x $a_w$ on taxol production when pH was held at 5.0. An increasing trend in yield with a decrease in serine concentration stress was observed. At lower levels of serine, the impact of water stress on the responses became more significant**.** However, it was noticeable that the concentration of serine had a stronger effect with this fungal species. This was confirmed with the high F value of serine concentration (F value=41.25). The corresponding 2D contour plot is shown in Figure 4.11b. The smaller change in taxol yields were obtained when cultured under more freely available water conditions (>0.984 $a_w$) than 0.98 $a_w$, although the concentration of serine was changed from 85 to 30 mg/l.

(a)




(b)

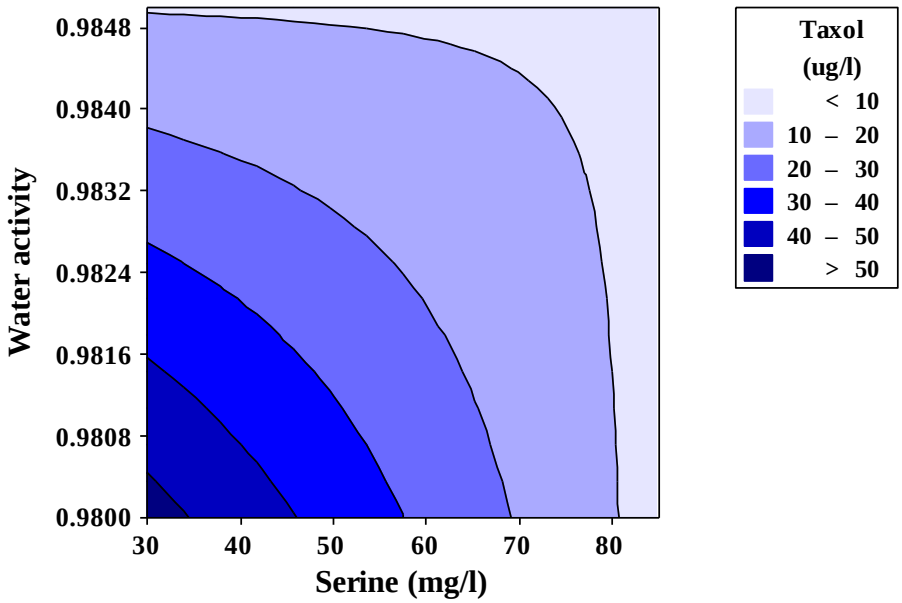



Figure 4.11 The 3D response surface plot (a) and contour plot for the effect of the interaction between $a_w$ and serine concentration on back-transformed taxol yield (µg/l). The pH level was held at 5.0. Original statistical analysis was performed on transformed data sets.

## 4.4 Discussion

The interaction effect between the environmental factors is one of the critical considerations to enhance the production of secondary metabolites from fungi (Deacon, 2006; Carlile et al., 2001; Magan and Alderd, 2007). This study has provided the detail on taxol production in relation to temperature, $a_w$, types of solutes and elicitors.

Similarity, the ionic solute NaCl and also the sugar sorbitol had a negative effect on taxol production. With the former ionic solute there was practically no taxol produced at lowered $a_w$ levels tested. Generally, the $a_w$ range for production appeared to be from 0.96 to 0.995 $a_w$. However, there was greater production of taxol under slight water stress (0.98 $a_w$) when compared with almost freely available water (0.99 $a_w$). The higher yield of this secondary metabolite at lowered $a_w$ levels were different from the effects on growth where optimum was at 0.99 $a_w$. This suggests that some slight ecophysiological manipulation of growth conditions may stimulate taxol production. Similarly, Baxter et al. (1998) found that the optimum $a_w$ for growth of a *Phoma sp*. was at 0.995 $a_w$, whereas 0.995-0.980 $a_w$ was optimum for the production of the cholesterol lowering metabolite squalestatin S1 production. In addition, Aldred et al. (1999) used solid substrate systems and obtained similar results with the *Phoma* species. However, they found best S1 production at 0.96 $a_w$, in contrast, the maximum growth rate was found at 0.998-0.99 $a_w$. These were natural heterogenous cereal-based substrates and not defined media.

With regard to the influence of temperature stress, ecophysiological patterns of the taxol producing strains in terms of growth and metabolite production – these were similar with both being optimum at 25°C. In addition, the results for growth rate with statistical analysis from studies showed that $a_w$ had a greater effect than temperature not only on growth rate, but also on taxol production.

The problems of using endophytic fungi to produce taxol under optimized fermentation processes are the low and unstable productivity. This was also observed for the strains isolated from the Cranfield Campus yew trees which produced taxol. *E. nigrum* had lost its ability to produce taxol when examining the ecophysiological growth patterns, although this strain was previously confirmed to produce taxol. The reduction and unstable productivity of the endophytic secondary metabolite production in optimized vitro conditions may be independent from the chemical communication from their host plants which were influenced by the environmental conditions (Kusari et al., 21012; Selim et al., 2012). It has been suggested that the high variation in quantities produced suggested caution for larger scale production systems.

Some of the elicitors were able to increase taxol yield by *P. variabile* when compared with the untreated control cultures. Salicylic acid affected the production of taxol most significantly. Salicylic acid has been previously shown to elicit the accumulation of taxol in suspension plant cell culture of *Taxus* sp. (Miao et al., 2000; Khosroushahi et al., 2006; Wang et al., 2007; Rezaei et al., 2011). Recently, Soliman and Raizada (2013) have been reported the significant enhancement in the taxol yield (two times) from *Praconiothyrium* SSM001 when supplemented with salicylic acid. The biosynthesis of secondary metabolites in plants, as well as taxol, is widely accepted as the chemical defence responses to counter infection by plant pathogens. The infection by plant pathogen has reported the change in signalling molecules including salicylic acid (Bari and Jones, 2009). This then activates the plant defence system by accumulating the anti-plant pathogen agents. This could be another explanation for the elicitation properties of salicylic acid increasing taxol yield from endophytic fungi.

For *E. nigrum*, the use of elicitors resulted in a restoration in the biosynthesis of taxol production when this strain showed attenuation of taxol production on the M1D broth medium. The serine-induced conditions stimulated the most taxol biosynthesis and yield. In the presence of serine, previous reports have shown the significance of the increase in taxol accumulation from plant cell cultures of

*Taxus cuspidate* (Fett-Neto et al., 1994). However, limited information on the influence of such amino acids on taxol production by endophytic fungi is available.

Under modified $a_w$ conditions with KCl (0.98 $a_w$) at pH 5, the production of taxol yield by *P. variabile* and *E. nigrum* was significantly increased when decreasing the concentration of salicylic acid to 20 mg/l and serine at 30 mg/l, respectively. The induction effect of this elicitor was likely as the maximum production was obtained when the optimum concentration of the elicitor was applied. The induction under non-modified $a_w$ conditions resulted in unexpectedly low production, although the elicitor was used. This finding is similar to some previous findings that taxol production by endophytic fungi was significantly reduced by repeated sub-culturing on defined artificial media in vitro.

Similar optimum pH conditions for taxol production and growth of *P. variabile* and *E. nigrum* was found in the acidic pH of 5. Interestingly, with water stress effects on mycelial growth, there was a slight shift in taxol production in the presence of the elicitor. Cell growth was optimum at 0.99-0.98 $a_w$ while taxol production was at 0.98 $a_w$. This finding may suggest that there may be an interaction effect of some slight of growth conditions and elicitor which may stimulate taxol production. Good agreement was obtained between the observed and predicted results when evaluating the effect of elicitor, pH and $a_w$ on taxol yield from both strains. This study showed that the elicitors combined with mild water stress conditions (0.98 $a_w$), enhanced taxol yield significantly. Previously, Sanchis et al. (2005) found that when examining optimum and marginal conditions of $a_w$ and temperature for growth, secondary metabolite production optima were very different. The optimum conditions for fumonisin production by *Fusarium verticillioides* were found at 0.97 $a_w$, while the most favourable conditions for growth of this strain was at 0.98-0.96 $a_w$.

In the present study, the reduction of fungal biomass of *P. variabile* was observed with taxol yield increased when salicylic acid (20 mg/l) was applied regardless of the treatments used. In contrast, supplementation with 50 mg/l of salicylic acid, decreased taxol amounts while a higher fungal biomass was observed. The reduction of biomass may be explained by the accumulated-taxol effect rather than salicylic effect, although salicylic acid elicitor has been shown to inhibit hyphal growth (Sun et al., 2013).

# 5 Screening factor for lysozyme production by *Pichia pastoris*

## 5.1 Introduction and objectives

Lysozyme constitutes a family of enzymes which break down bacterial cell walls by cleaving 1-4 glycosidic linkage between N- acetylmuramic acid (NAM) and N- acetylglucosamine NAG) of peptidoglycan in the cell wall (Hughey and Johnson, 1987; Salazar and Asenjo, 2007; Huang and Demirci, 2009; Maidment *et al*., 2009). This enzyme occurs naturally in tears, human mucus, saliva and hen egg white. Its antibacterial properties have been beneficial for applications in medical sciences and in the food industry. In the medical area, lysozyme has efficacy as an antiviral, immune modulatory, anti-inflammatory and antitumor agent (Goda et al., 2000). Although hen egg-white lysozyme is available commercially and used in various applications, several studies have revealed that hen egg-white lysozyme is allergenic. In addition, human lysozyme shows higher antimicrobial activity (Fremont et al., 1997; Sampson, 2004; Sicherer and Sampson, 2006; Mine and Yang, 2008).

In recent years, there has been considerable interested in recombinant pharmaceutical products from recombinant yeasts. Due to several positive points overcoming the problems with *Saccaromyces cerevisae, Pichia pastoris* has been used as a yeast expression system to produce recombinant human products. Today's understanding of different stress condition of host cells has played a major role in the development of cell factories for the overexpression of foreign genes. There are two categories of stress: metabolic stress and environmental stress. Metabolic stress concerns the gene copy number and transcription process. On the other hand, environmental stress examines the cultivation condition such as temperature, pH, osmolarity and oxygenation. However, few studies have examined the effect of interacting environmental conditions and how they may affect the physiology of the cell factory and how this would affect growth of *P. pastoris* and production of human lysozyme. Previous studies by Parra et al. (2004, 2005) showed that such physiological manipulation of a xerotolerant strain of *Aspergillus niger* resulted in a significant enhancement of lysozyme production.

While several studies have examined the effect of individual environmental factors on lysozyme production by *P. pastoris*, few if any, have examined interacting environmental factors on (a) growth and (b) lysozyme production. The objective of this study was to examine the effect of water activity x temperature x pH effects interactions to identify optimum and marginal conditions for both growth and lysozyme production by a recombinant *P. pastoris* strain.

## 5.2 Material and methods

### 5.2.1 Strain used in this study

Recombinant *P. pastoris* strain GS115/Mut$^+$ expressing human lysozyme under the control of alcohol oxidase promoter was used in this study. Stock cultures were maintained on YPD (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) medium and suspended with 15% glycerol (v/v) then kept at – 80 $^oC$ (Invitrogen, Carlsbad, USA). The strain was kindly provided by Prof. D. Archer, Nottingham University, UK.

### 5.2.2 Effect of water activity, temperature and types of solutes on growth of *P. pastoris*

The inoculum culture was prepared from a 3-day-old colony of *P. pastoris* grown on YPD agar at 30°C which was transferred into 20 ml YPD broth. The culture was incubated at 30°C in a shaker at 200 rpm for 24 hrs. The culture medium used for this study was YPD. The $a_w$ was modified by adding the non-ionic solutes (glycerol, sorbitol) and an ionic solute (NaCl). The accuracy of the $a_w$ treatments was checked with a $a_w$ meter (AquaLab, Decagon Devices, Inc., USA). Media were spread plated with 100 µl of inoculum and kept in plastic polyethylene bags at the tested temperatures. Colonies were counted manually. In order to identify which parameters significantly affected *P. pastoris* growth, a 4x5x3 factorial design was developed with

the MINITAB version 16 (Minitab, Inc., USA). Table 5.1 shows the experimental variables and their levels used. All treatments were carried out in triplicate.

Table 5.1 Experimental factors and their levels used in 4x5x3 factorial design to evaluate effects of interacting factors on growth of *P. pastoris*.

| Factor | Levels | | | | |
|---|---|---|---|---|---|
| $a_w$ | 0.99(0.98)[a] | 0.97(0.96) | 0.95(0.94) | 0.93(0.92) | |
| Temperature (°C) | 15 | 20 | 25 | 30 | 37 |
| Types of solutes | NaCl | Glycerol | Sorbitol | | |

[a] The level in blanket was used for sorbitol.

***Statistical analysis***

The impact of $a_w$, temperature and type of solute on the colony forming units (CFUs) per millilitre of *P. pastoris* was compared with MINITAB version 16 (Minitab, Inc., USA). Since the data was not normally distributed, a Kruskal-Wallis nonparametric ANOVA was conducted. A P-value of <0.05 was considered significant. A nonparametric multiple comparison, Dunn's test with the Bonferroni correction was used to keep the Bonferroni individual alpha = 0.05.

## 5.2.3 Effect of interaction between water activity, pH and type of solute on growth of *P. pastoris*.

In preliminary experiments $a_w$, temperature and type of solute were evaluated for their suitability to sustain good cell viability and determine their lag phase. These factors and their levels were selected from the result of this experiment. The effect of environmental stress on growth was examined by cultivating *P. pastoris* on YPD agar. A 2x2x5 factorial design was performed (Table 5.2). The pH of the YPD medium was modified by using a phosphate citrate buffer solution and was modified to the required $a_w$ levels. The $a_w$ was modified by adding a non-ionic (glycerol) or ionic solute (NaCl). The media treatments were spread plated with 100 µl of inoculum which was prepared as described in section 5.2.2. The inoculated plates were kept in plastic polyethylene

bags at the treatment temperature. The total viable CFUs were enumerated. All treatments were carried out in triplicate and incubated at 30°C.

Table 5.2 Experimental factors and their levels used in 2x2x5 factorial design to examine effect on growth of *P. pastoris*.

| | Level | | | | |
|---|---|---|---|---|---|
| $a_w$ | 0.97 | 0.95 | | | |
| Types of solutes | NaCl | Glycerol | | | |
| pH | 4.6 | 5.0 | 6.0 | 7.0 | 7.6 |

***Statistical analysis***

When assumptions of normality and equal variance were not met, significant differences were assessed using the Kruskal-Wallis nonparametric ANOVA at a statistical significant level of $P<0.05$. Post-hoc inter factor differences were calculated with Dunn's multiple comparison nonparametric tests with MINITAB version 16 (Minitab, Inc., USA). Bonferroni correction was used to keep the Bonferroni individual alpha = 0.05. Mann-Whitney U test was adopted for statistical analyses between two samples with the significant level alpha = 0.05.

## 5.2.4 Screening factors for lysozyme production using a factorial design approach

The factorial design of $2^5$ with repeated center points was firstly used to identify which variables have a significant influence on specific activity of lysozyme. The selected factors and their levels were chosen based on previous results. The variables were tested at two levels, a high (+1) level and a low (-1) level and tested at the center point (0) level for quantitative variables. The range of the coded and actual levels which were used in this study listed in Table 5.3.

Table 5.3 Coded and actual values of independent variable used to identify optimum conditions for lysozyme production by the strain of *P. pastoris.*

| Variable code | Variable | Low level (-1) | Center point (0) | High level (+1) |
|---|---|---|---|---|
| $X_1$ | $a_w$ | No modification | - | 0.97 |
| $X_2$ | pH | 5.80 | 6.0 | 7.0 |
| $X_3$ | Ttemperature (°C) | 20 | 25 | 30 |
| $X_4$ | Methanol (%) | 0.5 | 1 | 1.5 |
| $X_5$ | Expression method | Type 1 | | Type 2 |

For the expression of lysozyme, two methods were used to examine lysozyme production in this experiment:

(a) Type 1: the inoculum media were prepared from phosphate citrate buffer at the different pH levels (pH 5.8-7.0) with modified $a_w$ by adding glycerol at the $a_w$ level studied (0.97, unmodified medium). Then, it was used as solvent to prepare YPD medium. This 5 ml of YPD media were placed in a 50 ml flask. One single colony (3 days old) from YPD agar was transferred to the inoculum flask. Cultures were incubated for 18 h at 20-30°C and agitated at 250 rpm. The inoculum culture was transferred to 45 ml BMGY (2% peptone, 1% yeast extract, 1% glycerol in 0.1 M phosphate buffer pH 5.8-7.0) at the target $a_w$ (0.97, unmodified medium) in a 250 ml flask. Then, the inoculated expression media were cultivated at the target temperatures (20-30 °C) for 100 h in a shaking incubator at 250 rpm. The methanol (100%) was added at a final concentration of 0.5 to 1.5%) every 24 h to maintain the secretion.

(b) Type 2: the inoculum medium in this method was BMGY which was prepared from phosphate citrate buffer at the different pH levels (pH 5.8 to 7.0) with the $a_w$ modified by adding glycerol at 0.97 $a_w$. Then, the medium components of BMGY were dissolved in these prepared solutions. 50 ml of this BMGY was placed in a 100 ml flask and inoculated with a single colony (3 days old) taken from YPD agar. This inoculum was incubated over the temperature treatment range of 20 to 30°C in a shaking incubator at 250 rpm for18 hrs. After 18 h, this inoculum was centrifuged at 2500 g for 10 min at 4°C (Labofuge 400R, Thermo scientific) and the supernatant discarded. The cell pellet

was re-suspended in 50 ml BMMY (2% peptone, 1% yeast extract, methanol) in 0.1 M phosphate buffer. The 0.1M phosphate buffer was prepared for the target pH values of between 5.8 to 7.0 and 0.97 $a_w$ as detailed in Table 5.3. These inoculated expression media were incubated at 20 to 30°C for 100 h on a shaker at 250 rpm. The methanol (100%) was added to a final concentration of 0.5 to 1.5% every 24 h to maintain the secretion. The overall flowchart of the method used is shown in Table 5.1.

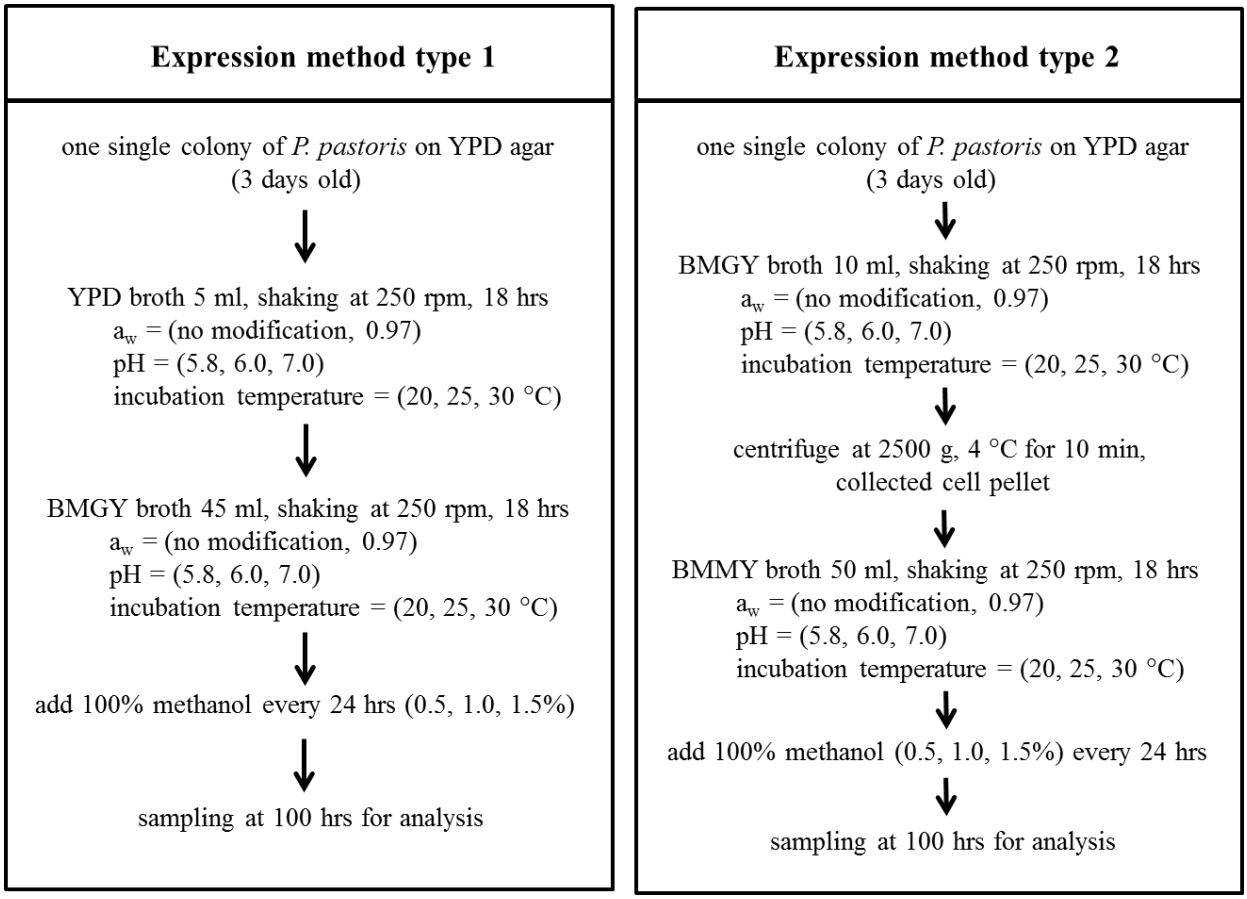



Figure 5.1 Flowchart showing the experimental procedure of the expression methods

***Measurement of lysozyme activity***

After expression, the samples of fermentation medium at 100 hrs were centrifuged for 5 min at 9000 g (Labofuge 400R, Thermo scientific). The supernatant was analyzed for lysozyme activity. This activity was determined by the turbidimetric method of Mackie *et al.* (1976) with slight modifications. The solution of the free-cell supernatant (100µl) was added to 900 µl of a suspension of lyophilized *Micrococcus lysodeikticus* cells (Sigma-Aldrich, Inc., USA) in 0.1 M potassium phosphate buffer pH 7 ($OD_{450}$= 0.5-0.7). The change in absorbance at 450 nm was measured for 2 min using a spectrophotometer. A change in absorbance of 0.001 absorbance unit per minute was defined as 1 U enzyme activity. Thus lysozyme activity was calculated by the following equation

$$Y^* = Lysozyme\ activity\ \left(\frac{U}{ml}\right) = (\Delta abs/min) \times 10^4 \quad (5.1)$$

In the equation, $\Delta abs$ is the change in absorbance.

The total protein of the sample was quantified using the bicinchoninic acid (BCA) method using the Pierce® BCA protein assay kit applying bovine serum albumin as standard (Thermo scientific, USA). The specific activity of lysozyme can be calculated from the following equation:

$$Specific\ activity\ (U/mg) = \frac{Unit\ of\ lysozyme\ per\ ml}{mg\ total\ protein\ per\ ml} \quad (5.2)$$

***Statistical analysis***

The experimental design and statistical analyses were analyzed using Design Expert version 8 (Stat-Ease, Inc., USA). All non-normal distributed datasets of specific activity of lysozyme were transformed to fit normal distribution prior to performing statistical analysis. Johnson transformation (Johnson, 1978) in MINITAB software version 16.0 (Minitab Inc., USA) was applied. The transformed datasets were normally distributed and analysed using Kolmogorov–Smirnov test with 95% confidence intervals.

Significant differences were assessed with the analysis of variance (ANOVA) at the statistical significant level of $P<0.05$.

### 5.2.5 Influence of buffer systems on lysozyme production

Three buffer systems which included MES buffer, phosphate citrate buffer (McIlvaine's buffer) and potassium phosphate buffer were selected to examine the impact of buffer systems together with selected quantitative variables. The factorial experimental design for 4 variables at 2 levels with the center point level was used (Table 5.4).

Table 5.4 Experimental factors and their levels used to examine the effect of different pH buffer modifications to obtain the targeted values at different temperatures and $a_w$ levels.

| Variable | Low level | Center point | High level |
|---|---|---|---|
| $a_w$ | 0.97 | 0.98 | 0.99 |
| pH | 5.6 | 6.2 | 6.8 |
| Temperature (°C) | 15 | 20 | 25 |
| Methanol (%) | 0.6 | 1.1 | 1.6 |

As in the previous screening experiment, no significant differences were observed in expression method used. Thus, expression method type 1 was selected for this experiment. The inoculum media were prepared from different buffer systems (MES buffer, phosphate citrate buffer and potassium phosphate buffer) at the different pH levels (pH 5.6-6.8) with $a_w$ modified by adding glycerol to reach the target treatment levels listed in Table 5.4. Then, the components of medium (YPD) were dissolved in these prepared solutions.

The inoculum media were prepared in a 50 ml flasks with 5 ml of prepared media as described previously. A single colony (3 days old) from YPD agar was transferred to the inoculum flask. Cultures were incubated for 18 h at 15, 20 and 25 °C at 250 rpm. The inoculum culture was transfer directly to 45 ml BMGY in 250 ml flask. BMGY

media were prepared as for YPD in terms of the buffer system at the different pH and $a_w$ levels in Table 5.4. Inoculated expression media were cultivated at 15 to 25 °C with shaking at 250 rpm for 100 h. The necessary methanol treatments were added every 24 h to maintain the expression. After expression, the samples of the fermentation medium were quantified for lysozyme activity as described in section 5.2.4.

***Statistical analysis***

The experimental design and statistical analyses were analyzed using MINITAB software version 16.0 (Minitab Inc., USA). When assumptions of normality and equal variance were not met, significant differences were assessed using the Kruskal-Wallis nonparametric ANOVA at a significance level of $P<0.05$. Post-hoc inter-factor differences were calculated with the Dunn's multiple comparisons nonparametric test. Bonferroni correction was used at 0.99 to keep the Bonferroni individual alpha = 0.028, when the number of comparisons were 36 pairs.

## 5.3 Results

### 5.3.1 Effect of water activity, temperature and types of solutes on *P. pastoris* growth

The influence of $a_w$, temperature and types of solutes on the lag phases prior to growth and relative growth of *P. pastoris* are shown in Figure 5.2. This showed that the two significant factors were aw and temperature ($P<0.05$). *P. pastoris* exhibited the most sensitive response when cultured at 37°C ($P<0.05$). The growth was inhibited at 0.95 $a_w$ with the ionic NaCl and non-ionic solute glycerol, but only at 0.94 $a_w$ with sorbitol. Adding glycerol in YPD medium supported growth, especially at the lowest temperature (15°C) examined with the maximum number of yeast cell populations (3.06 x103 cfu/ml). While the maximum yield of the control was 2.75 x $10^3$ cfu/ml. In contrast, this $a_w$ depressor resulted in lower populations at elevated temperatures. However, the main effect of types of solutes on the *P. pastoris* populations was not statistically significant (Figure 5.3a). Growth at 15°C seemed to be better than at any other temperature. However, this involved a longer initial lag phase duration (6 – 13 days) prior to growth (Figure 5.3b).

The highest number of *P. pastoris* population was found in the $a_w$ range of 0.96 - 0.97 but was not significantly greater than cultured in the range 0.98 - 0.99 $a_w$ ($P<0.05$) (Figure 5.4). The reduction of $a_w$ not only influenced the yeast populations, but also affected the lag phase. However, the result depended on the types of solute and incubation temperature. For example, at 0.97 $a_w$ with NaCl, the shortest lag phase (2 days) was at 30°C**,** while this was 6 days at 25°C. Using glycerol to modiufy YPD medium resulted in a short lag phase. However, the populations of *P. pastoris* were lower than when using NaCl as a solute, especially at higher temperatures.

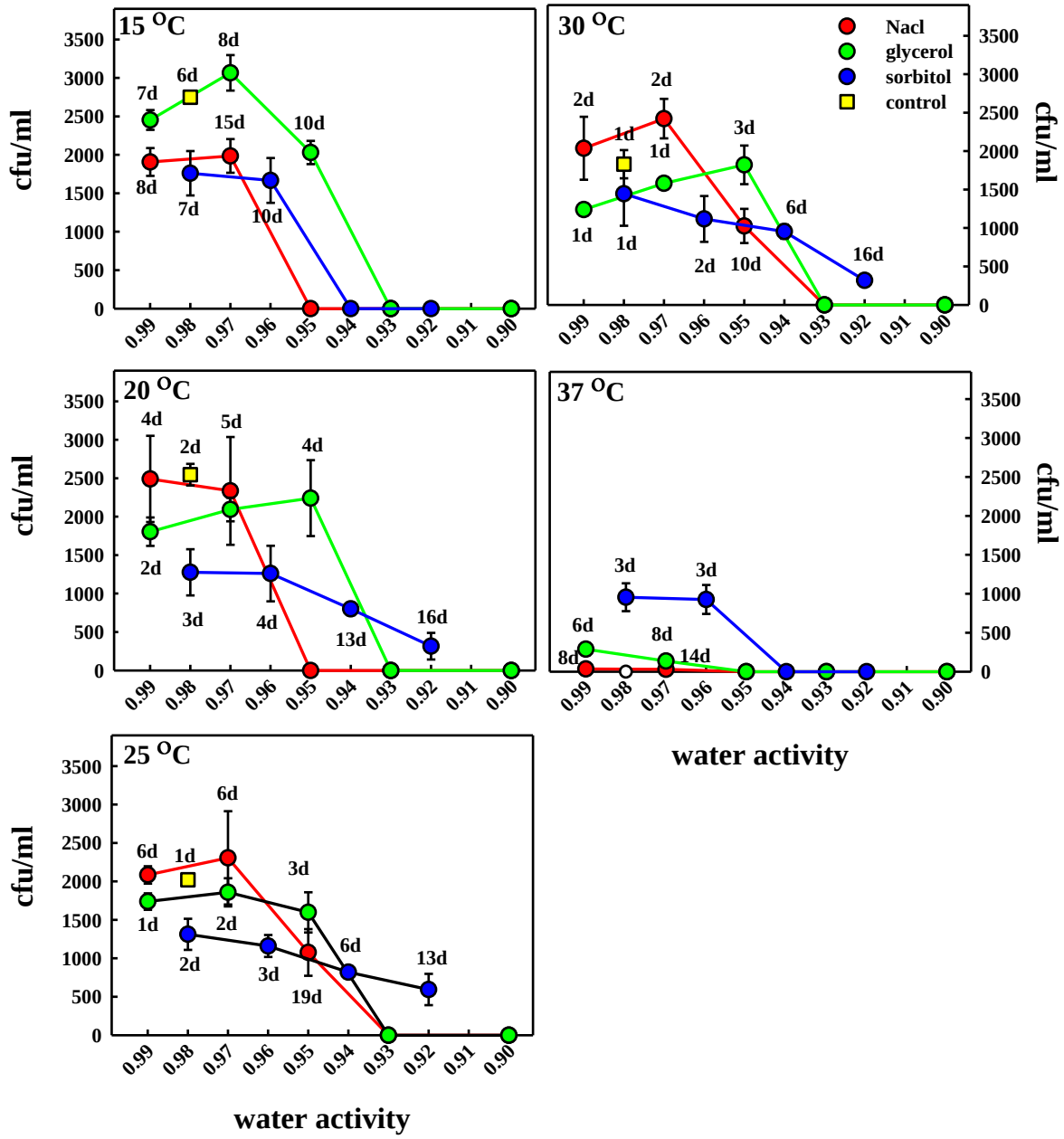
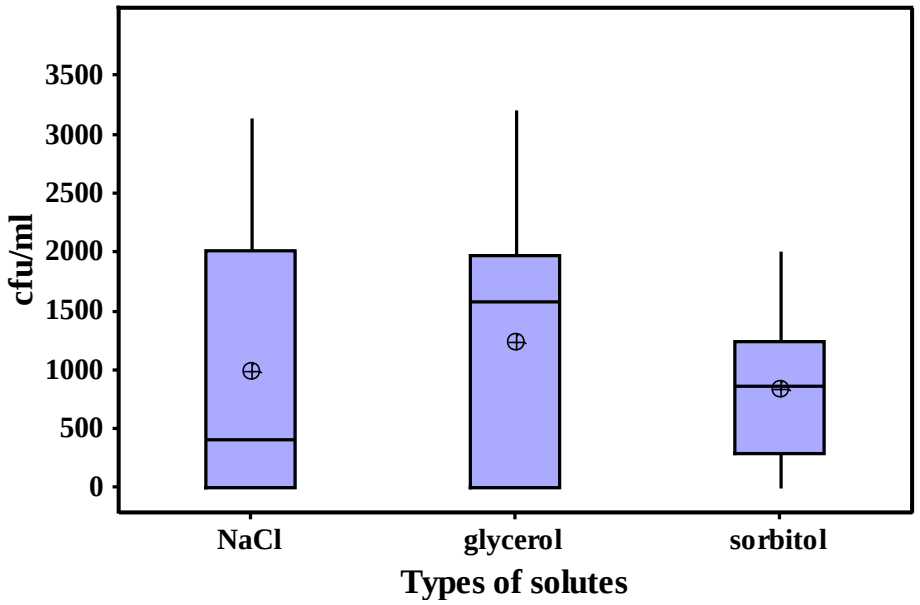



Figure 5.2 Effect of $a_w$, temperature and solute type on lag phase and growth of *P. pastoris* in media modified with NaCl, glycerol and sorbitol at 15, 20, 25, 30 and 37 °C. The numbers of days for initiation of growth are indicated.

(a)




**(b)**

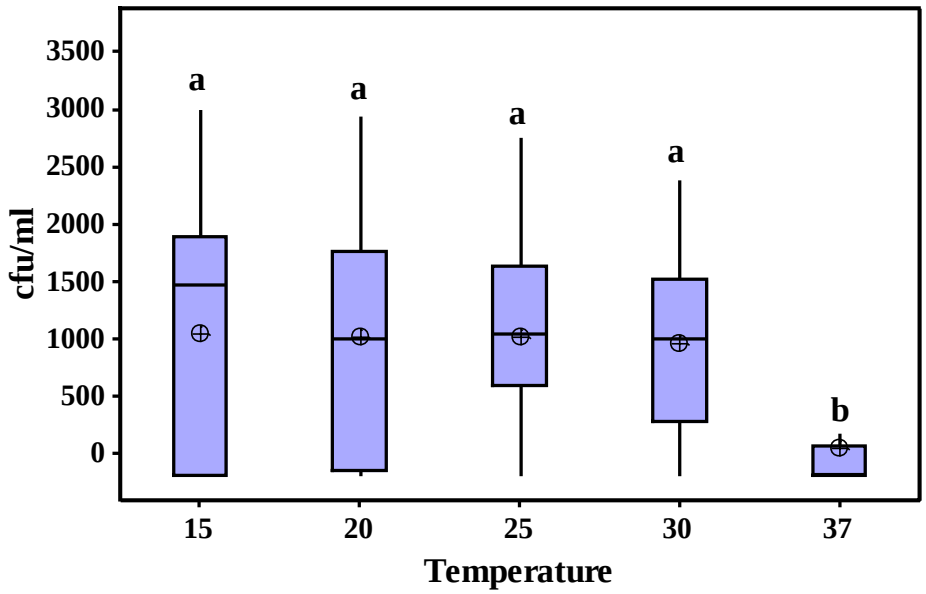



Figure 5.3 Differences in cell number of *P. pastoris* in response to types of solutes (a) and temperature stress conditions (b). Box plots show the median (horizontal lines within each box), upper and lower quartiles (boxes). The circle inside the box is the mean. Boxes labelled with the different letter indicate significant median difference following Dunn's multiple comparisons test. The Bonferroni individual alpha for multiple comparisons is 0.05.

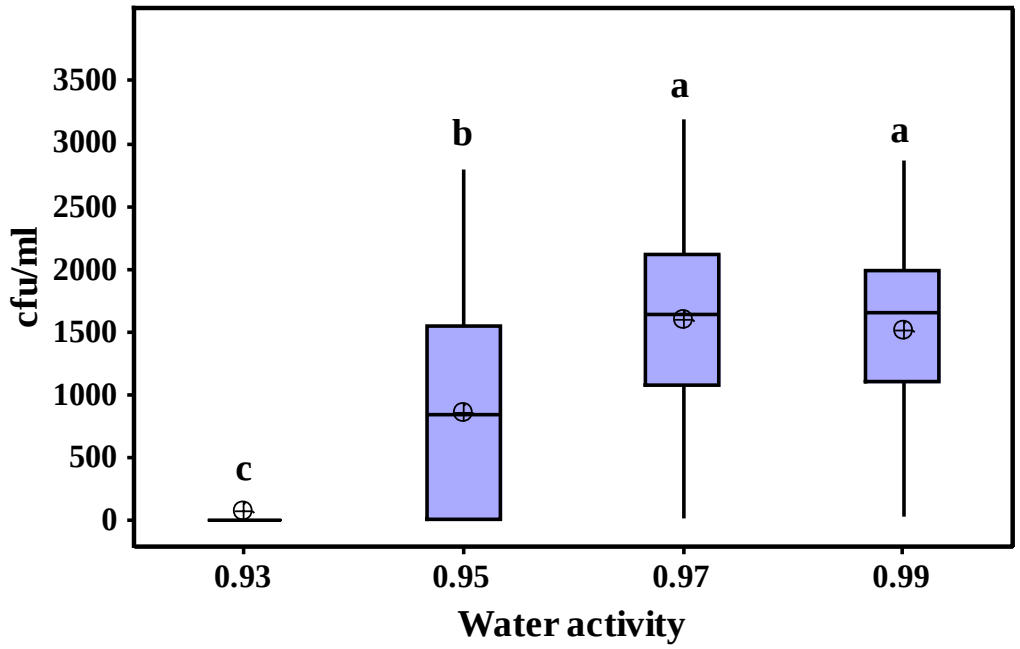



Figure 5.4 Differences in cell number of *P. pastoris* in response to $a_w$ stress conditions. Box plots show the median (horizontal lines within each box), upper and lower quartiles (boxes). The circle inside the box is the mean. Boxes labelled with the different letter indicate significant median difference following Dunn's multiple comparisons test. The Bonferroni individual alpha for multiple comparisons is 0.05.

### 5.3.2 Effect of interaction between $a_w$, pH and solutes on growth of *P. pastoris*

To evaluate the effect of several factors on growth by using a factorial design, NaCl and glycerol were selected as the solutes to test at 0.95 and 0.97 $a_w$ based on previous results. Figure 5.5 summarizes the lag phases and growth rates. The highest cell number when using glycerol as the solute was 1.97 x $10^3$ cfu/ml (pH 7, 0.97 $a_w$) and the lowest was 1.57 x $10^2$ cfu/ml (pH 7, 0.95 $a_w$). For NaCl, the highest and lowest cell populations obtained were 1.49 x $10^3$ cfu/ml (pH 7, 0.95 $a_w$) and no growth in the most stressed conditions (pH 4.6-7.6, 0.95 $a_w$) respectively. It was clear that glycerol-modified media allowed the best range of $a_w$ conditions for growth over the pH range tested with the shortest lag phases.

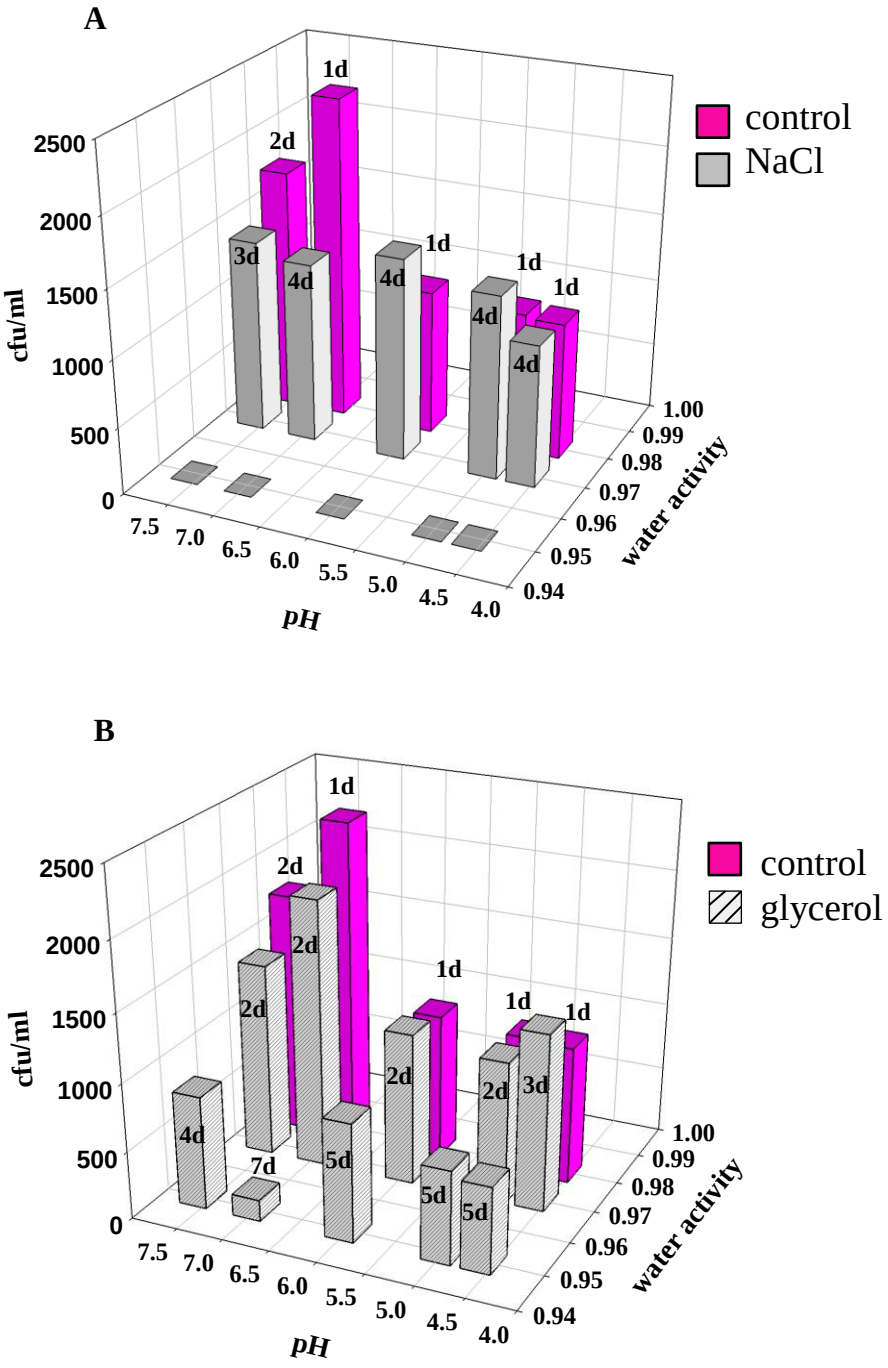



Figure 5.5 Comparison of growth of *P. pastoris* in YPD medium with pH x $a_w$ modified medium with the ionic solute NaCl (A), non-ionic solute glycerol (B) and that in unmodified YPD. The number of days (d) for initiation of growth at 30°C under the treatment conditions are shown above each histogram bar.

To determine which effects were significant, a statistical analysis was conducted. This revealed that $a_w$ was the most significant factor influencing cell growth ($P<0.05$). *P. pastoris*, when cultivated on YPD agar at 0.97 $a_w$ had a significantly higher cell yield ($P< 0.05$) than at 0.95 $a_w$ (Figure 5.6). Furthermore, using the non-ionic solute glycerol as the $a_w$ depressor resulted in a higher viable cell yield than the ionic solute NaCl (Figure 5.7). However, the main effect of solute type on yeast cell populations was not statistically significant. There were differences in the populations of *P. pastoris* in the pH range of 4.6 to 7.6.

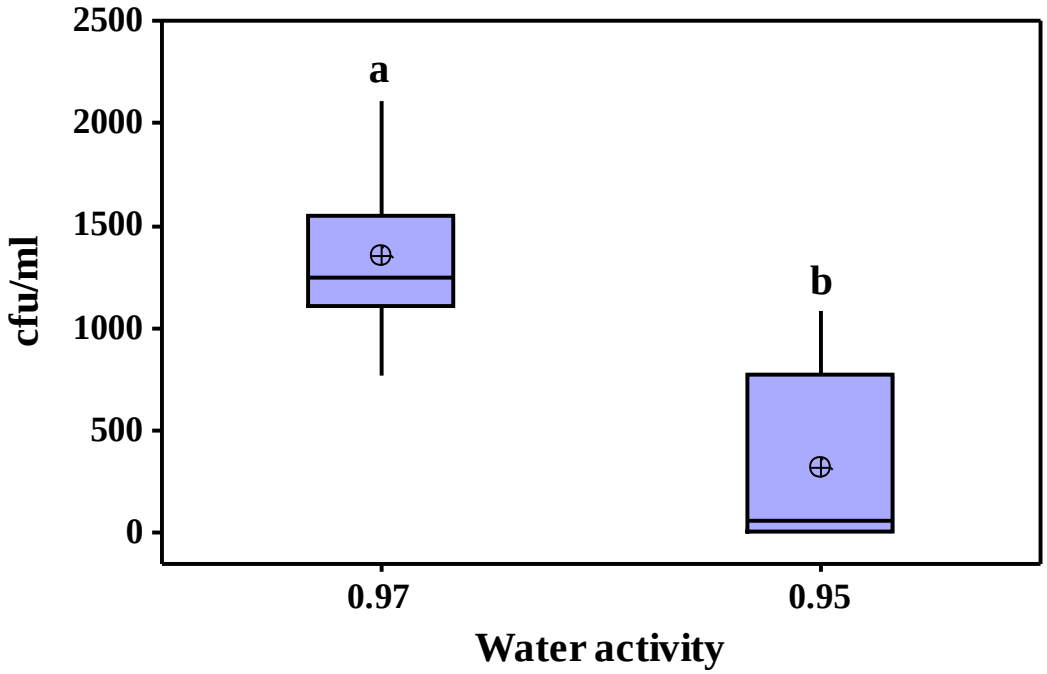
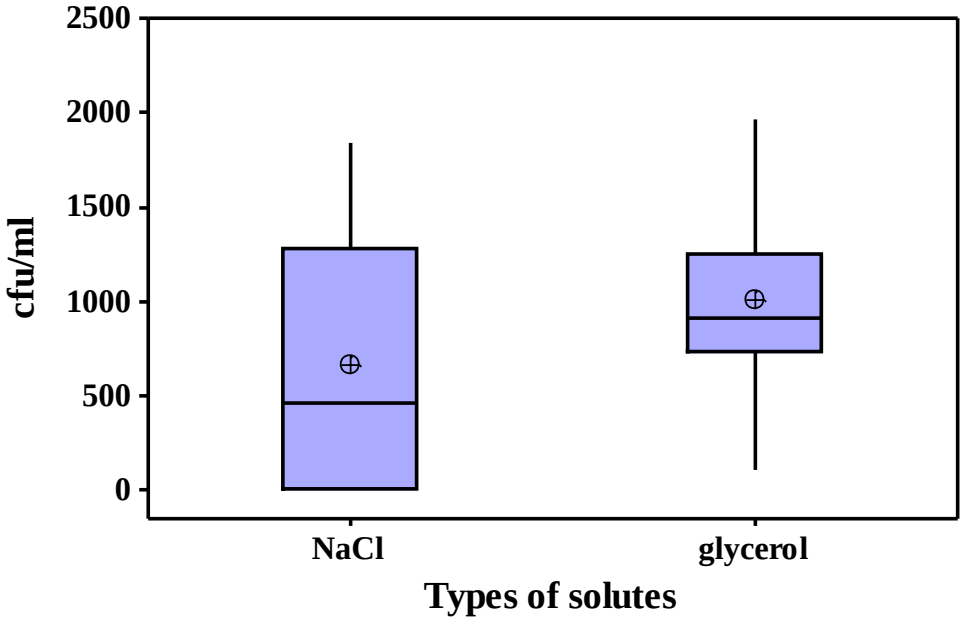



Figure 5.6 Box plots display of the populations of *P. pastoris* in response to $a_w$ stress. The line within the box indicates the median cell number of *P. pastoris*, the 25th and 75th percentile (lower and upper margin of the box) together with the minimum and maximum values (whiskers). The circle inside the box is the mean .Statistical differences between groups were determined using the non-parametric Man-Whitney test is indicated by the letter above columns. The different letter indicate statistic significant differences ($P<0.05$).

(a)




(b)

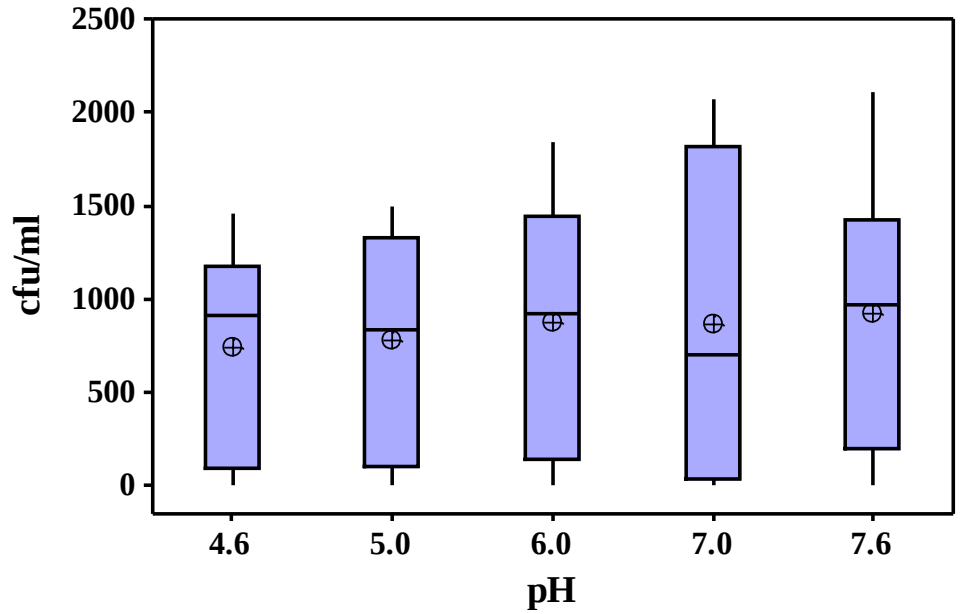



Figure 5.7. Box plots show the cell number populations of *P. pastoris* in the response to (a) type of solute and (b) pH. The line within the box indicates the median cell number of *P. pastoris*, the 25th and 75th percentile (lower and upper margin of the box) together with the minimum and maximum values (whiskers). The circle inside the box is the mean.

### 5.3.3 Screening factors for lysozyme production by factorial design

Based on the results obtained using the factorial experiments for the effect on *P. pastoris* growth, some of the environmental stress factors were selected for examining effects on enhancing lysozyme production. Because a factorial design was used for the screening experiment, the impact of the experimental factors and their interactions on specific activity of lysozyme could be evaluated.

The highest yield (75.06 U/mg) was at a pH of 5.8, 20°C, and with methanol 1.5%, using the type 1 expression method and without any $a_w$ modification. This maximum yield increased approximately by 3.58 times compared with those cells cultured under the control conditions. In contrast, the lowest yield (25.06 U/mg) was obtained at pH 5.8, 20°C and methanol 0.5% addition in the modified $a_w$ treatment.

Normality of the distribution of the population was tested using the Kolmogorov–Smirnov test. The null hypothesis ($H_0$) was that the data followed a normal distribution and the alternative hypothesis ($H_a$) was that data they were non-normally distributed. For the responses variable, the $H_0$ was rejected in favor of the $H_a$. This was done with 95% confidence level (Figure 5.8). Moreover, the inequality of variance was revealed from the Levene's test.

One approach to address non-normal distributed datasets is to normalize the dataset using transformation prior to analyses. Johnson transformations was considered as more normalized data sets were generated (P>0.05, Kolmogorov–Smirnov test). Clearly, the Johnson transformation method gave the new transformed data set which was closer to a normal distribution than the original one (Figure 5.8).The original data sets of specific activity of lysozyme were thus transformed according to the following equation:

$$Y^* = -0.137633 + 1.05657 \times Asinh\left(\frac{(x-44.3134)}{7.70277}\right) \quad (5.3)$$

In the equation, $Y^*$ represents the transformed specific activity of lysozyme and $x$ represents the original data.

The back-transformed data were calculated from the equation below:

$$y = 44.3134 + \left(3.851385\, e^{\left(\frac{(Y^*+0.137633)}{1.05657}\right)}\right) - \left(45.4094\, e^{\left(-\frac{(Y^*+0.137633)}{1.05657}\right)}\right) \qquad (5.4)$$

where $y$ represents back-transformed data, $Y^*$ represents the transformed specific activity of lysozyme.

An ANOVA analysis was carried out on the transformed data (Table 5.5). Both qualitative variables of this experiment including $a_w$ modification and expression method were observed as non-significant changes in specific activity of lysozyme ($P>0.05$). Among the studied quantitative variables, temperature had a significant effect on specific activity ($P<0.05$). The remaining variables, namely, methanol concentration and pH were considered non-significant within the range used in this study.

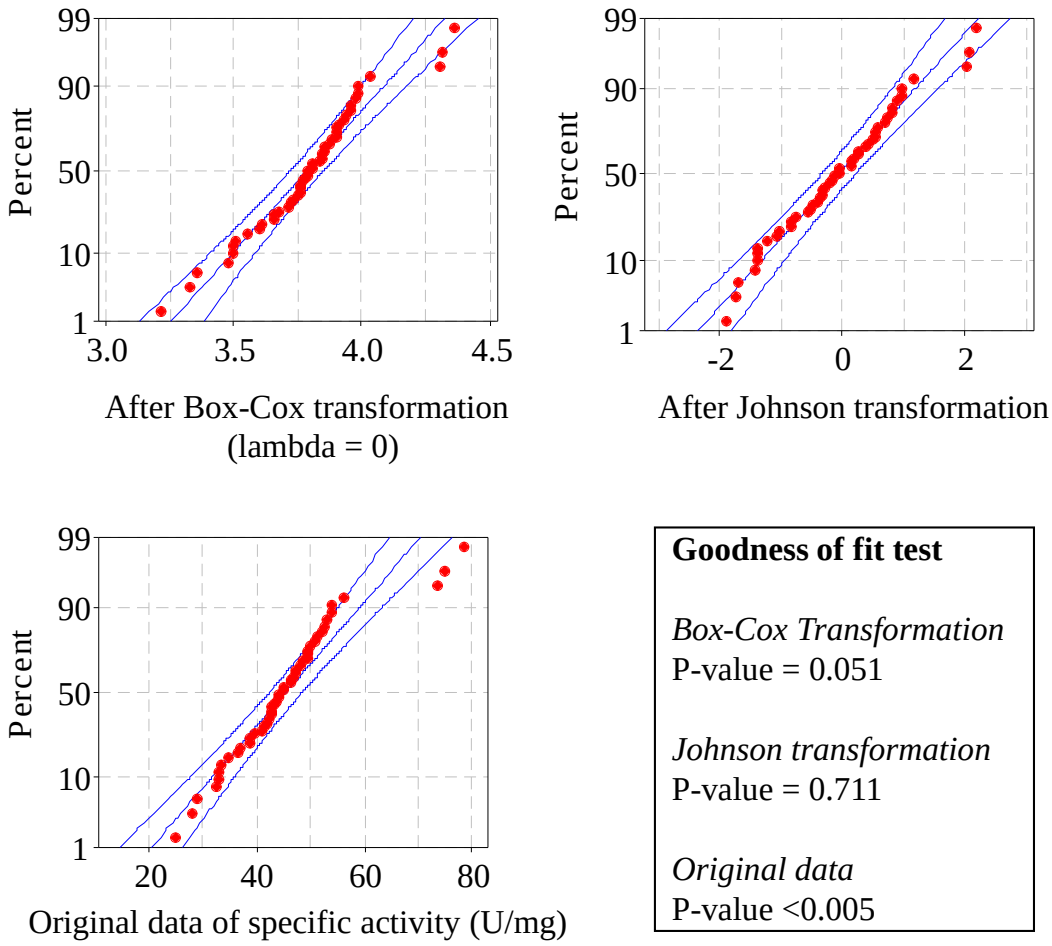



Figure 5.8 Normal probability plots of specific activity of original lysozyme data, transformed data with Box-Cox and Johnson transformations.

Table 5.5 Analysis of variance for transformed data

| Source | df | Mean square | F value | P value |
|---|---|---|---|---|
| pH | 1 | 2.44 | 3.59 | 0.0649 |
| Temperature | 1 | 14.31 | 21.05 | <0.0001 |
| Methanol concentration | 1 | 0.014 | 0.02 | 0.8884 |
| $a_w$ modification | 1 | 4.923E-003 | 7.243E-003 | 0.9326 |
| Expression method | 1 | 0.33 | 0.049 | 0.8264 |

After the collected data were transformed and fitted, a linear first-order model in terms of the actual factors for specific activity was developed as shown below:

$$Y^* = 5.94182 - 0.42972\, pH - 0.13372\, temperature + 0.041146\, methanol\ concentration \quad (5.5)$$

In the equation, $Y^*$ represents the transformed specific activity of lysozyme.

The main effects plot is useful in the practical interpretation of the results. In term of the screening experiment, these plots can be used to compare the changes in the mean level to examine the tendency of the response on the tested level of factors providing the information for the subsequent experiment. Figure 5.9 represents the main effects plots of pH, temperature and methanol concentration in term of back-transformed data. A recommended setting factor levels to achieve a high response and the temperature which should be used. Although the pH and methanol concentration factors were not significant (P>0.05), there was some useful information from these results. Better yield could be achieved by choosing a relative more acidic pH level and setting the methanol concentration at a much higher level.

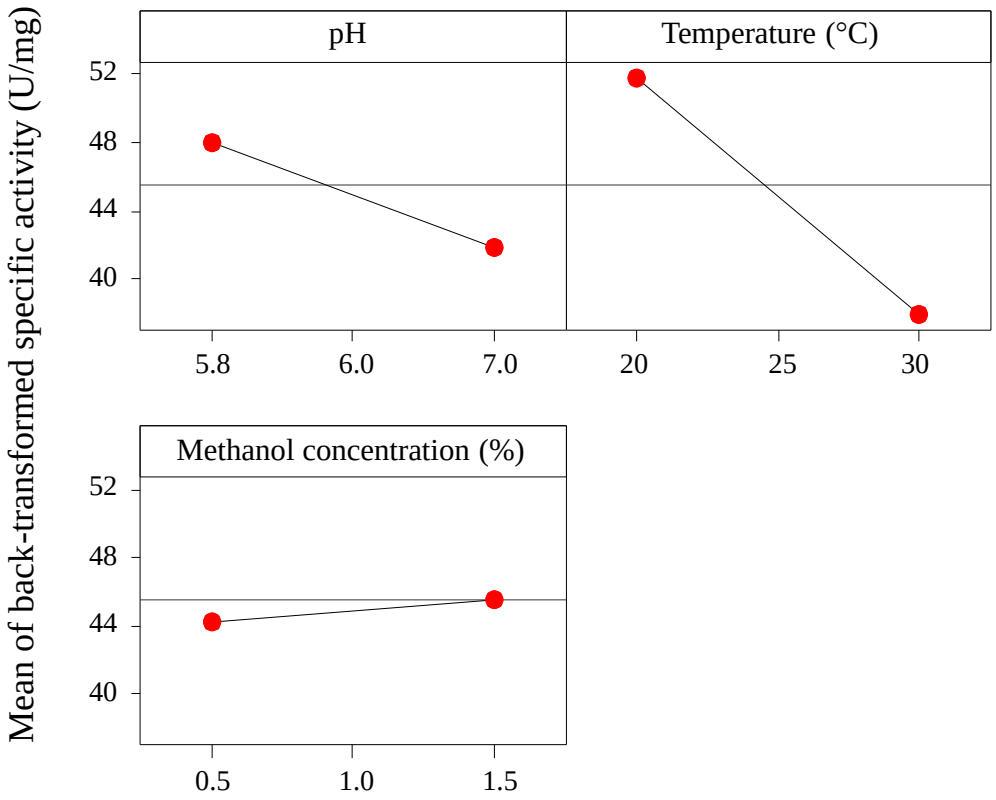



Figure 5.9 Main effects plot for the mean of back-transformed specific activity of lysozyme (U/mg).

***Interaction effect of pH and temperature***

Plots of the three-dimensional (3D) response surface provide a better understanding of the interaction among the experimental variables on the lysozyme production responses observed. When the level of another factor was held at a fixed level for each plot, the response surface for lysozyme production can be observed as a function of two factors. Figure 5.10 presents the 3D plot of the effect of pH x temperature effects on specific activity of lysozyme. Examining this plot, it can be seen that temperature had the desirable effect on lysozyme production. An increasing trend in yield with decreasing temperature at the lower level in the experimental pH can be observed. However, when the temperature is increased, the impact of pH on responses became less significant. It

seems that the response depended almost entirely on temperature regardless of pH level used.

This observation from 3D surface plot can also be confirmed by using statistical analysis. Interaction detection is an important approach providing useful information among experimental variables. When building the linear model from Equation 5.5 which included both of the interactions by forward stepwise selection, the interaction between two factors on relative lysozyme production can be identified. The effect of both factors were found to be additive on lysozyme production ($P>0.05$). When combined factors show no statistical interaction, it can imply that the effect of one factor on the response does not depend on the level of the other factor.

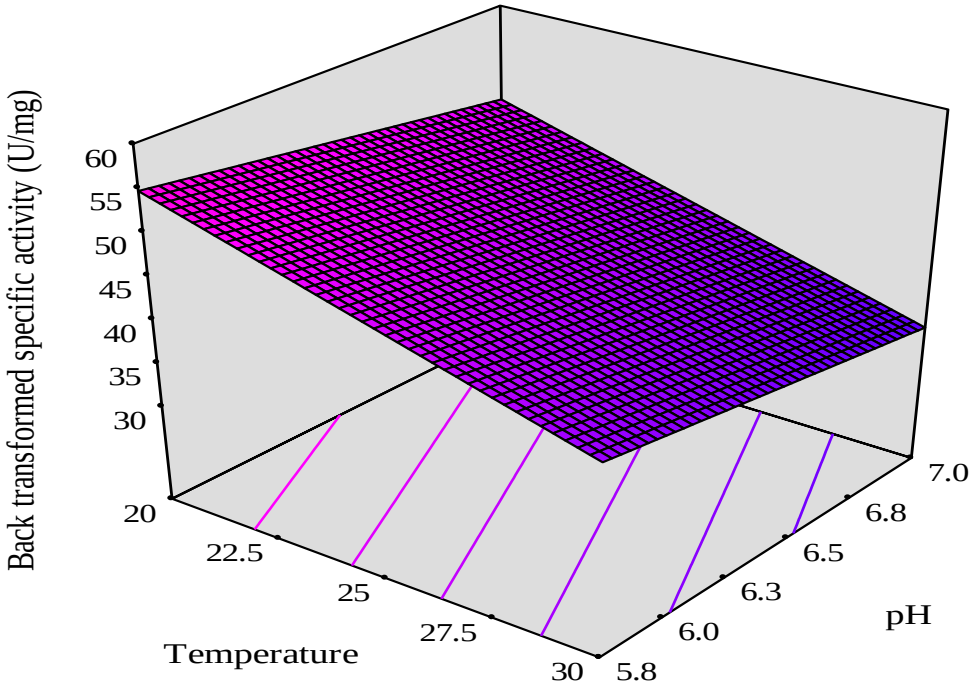



Figure 5.10 Three dimensional plot of the response surface for back-transformed specific activity of lysozyme as a function of pH and temperature with a fixed factor of 1% methanol concentration. Original statistical analysis was performed on transformed data sets.

***Interaction effect of pH and temperature***

Specific activity of lysozyme showed an increasing trend with decreasing methanol concentration at lower temperatures (Figure 5.11). It appeared that the effect of temperature was predominant on the relative lysozyme production response regardless of the methanol concentration used. However, at high concentrations of methanol with lower incubation temperature, the effect of methanol showed a slight tendency toward higher lysozyme production at a lower concentration of methanol addition. Statistical analysis also confirmed that there was no evidence for a significant effect of this interaction on lysozyme production (P>0.05).

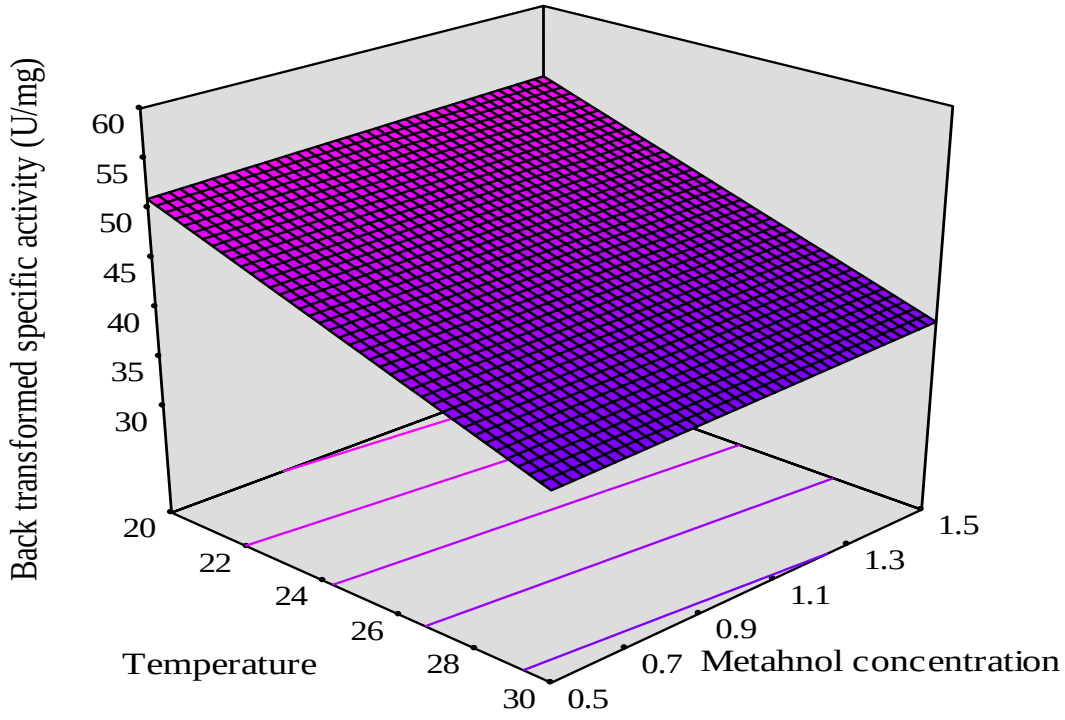



Figure 5.11 The 3D response surface plot for the effect of the interaction between temperature and methanol concentration on back-transformed specific activity of lysozyme. The pH level was held at 6.0. Original statistical analysis was performed on transformed data sets.

***Interaction effect of pH and methanol concentration***

The influence of pH and methanol concentration factor on specific activity were elucidated using the response surface plot shown in Figure 5.12. There was little increase in lysozyme production at the lower pH level with high methanol concentration. Both factors seemed to be less related to the response and did not interact to affect lysozyme production. Indeed, this was confirmed by no significant effect of interactions of these two factors on production.

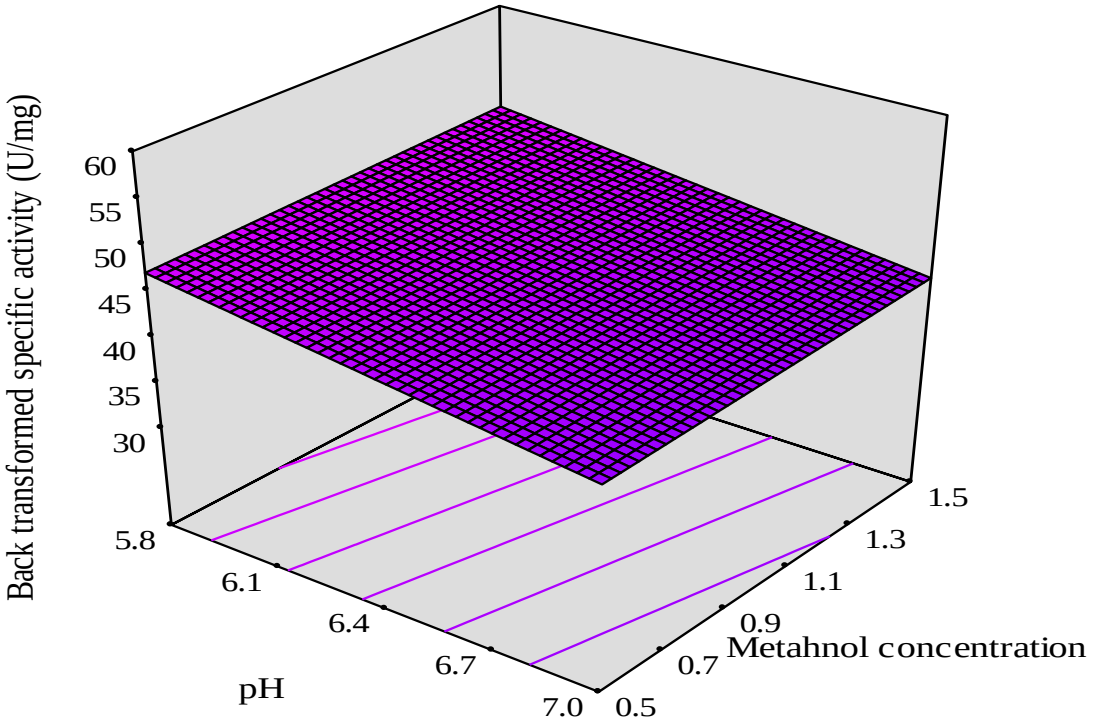



Figure 5.12 The 3D response surface plot showing the combined effects of pH and methanol concentration on back-transformed specific activity. The temperature level was held at 25 °C. Original statistical analysis was performed on transformed data sets.

### 5.3.4 Influence of buffer systems on lysozyme production

For the expression of recombinant protein in *P. pastoris*, a low pH setting of between 5.0-6.0 has been commonly used. A potassium phosphate buffer (0.1M) is recommended when preparing buffered medium to maintain a stable pH condition. From the previous study, only such buffer system was evaluated. In order to examine the effects of different buffer systems on lysozyme production, liquid culture experiments were studied with three different buffer systems, i.e., MES, potassium phosphate and phosphate citrate buffer. The highest enzyme production (53.79 U/mg) was obtained after incubation at pH 6.8, 25°C, a methanol concentration of 0.6%, and 0.97 $a_w$ with a MES buffer system. When the potassium phosphate buffered medium was used, the highest of enzyme production (44.29 U/mg) was observed at pH 6.2, 20°C, methanol concentration of 1.1% and 0.98 $a_w$. For phosphate citrate buffered medium, the maximum enzyme activity (28.92 U/mg) was observed when cultured at pH 6.8, 25°C, 0.6% methanol concentration and 0.99 $a_w$.

To illustrate the difference of the responses between groups, the non-parametric Dunn's multiple comparisons test was performed. An examination of Figure 5.13 showed that the highest median yield was in the MES buffered medium at pH 6.2 ($P<0.028$), whereas the maximum lysozyme production was obtained from pH 6.8. Using the phosphate citrate-buffered medium at pH 6.8, the production of lysozyme was significantly lower when compared to other pH levels and buffer systems used ($P<0.028$). The difference in the median lysozyme production between this condition and under the conditions favouring production (the highest median value) was approx. 12.54 times different. Buffering the media at pH 6.2, the higher median values were observed when comparisons were made within the same buffer system used.

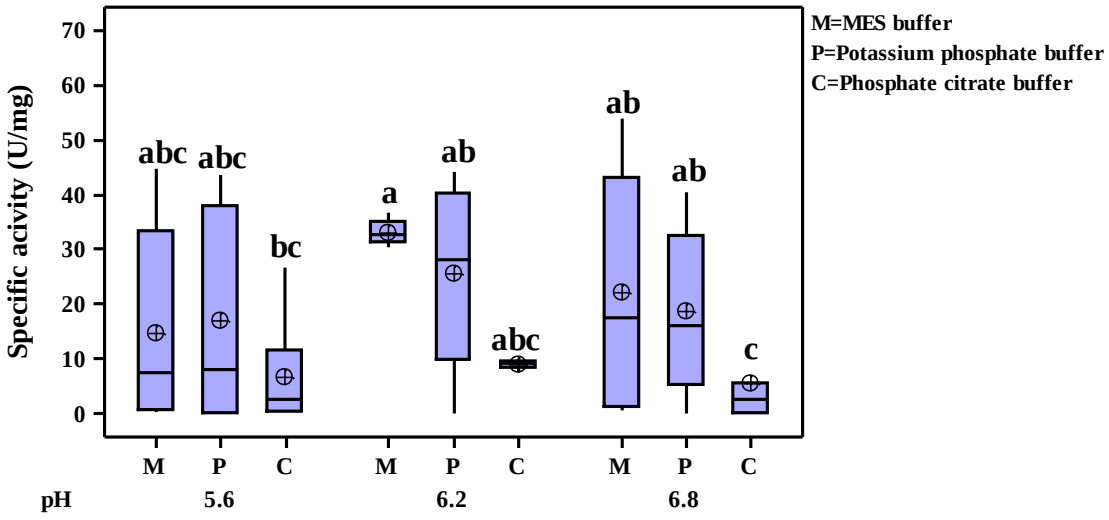



Figure 5.13 The box plot illustrates specific activity of lysozyme (U/mg) in the response to pH and buffer systems. Boxes contain the median (horizontal line within the box and the minimum and maximum values (whiskers). The circle inside the box is the mean. The non-parametric Dunn's multiple comparisons test was used with Bonferroni individual alpha for multiple comparisons is 0.028. The median of groups that share a letter are not significantly different.

In this study, buffered medium with MES and cultivation at 25°C was found to be favoured although no statistically difference in the median between MES buffered medium was found at 20°C (Figure 5.14).Within MES buffered pH media used, there was a significant difference between the decline in the median values (21.55–26.58 fold decline) when cultured at lower temperatures (15°C) ($P<0.028$). The buffering conditions with phosphate citrate buffer system showed lower median values and also lower amounts of enzyme production regardless of the temperature used.

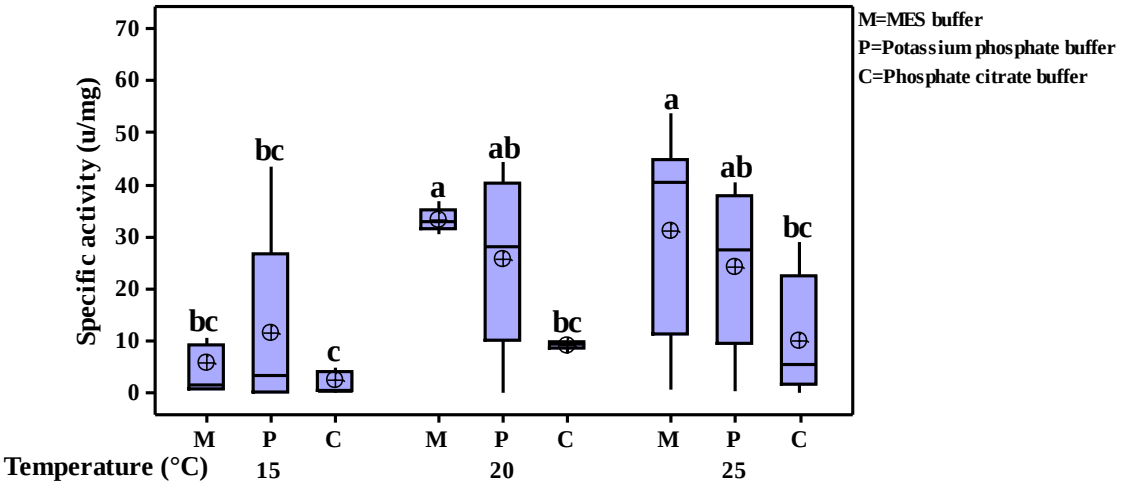
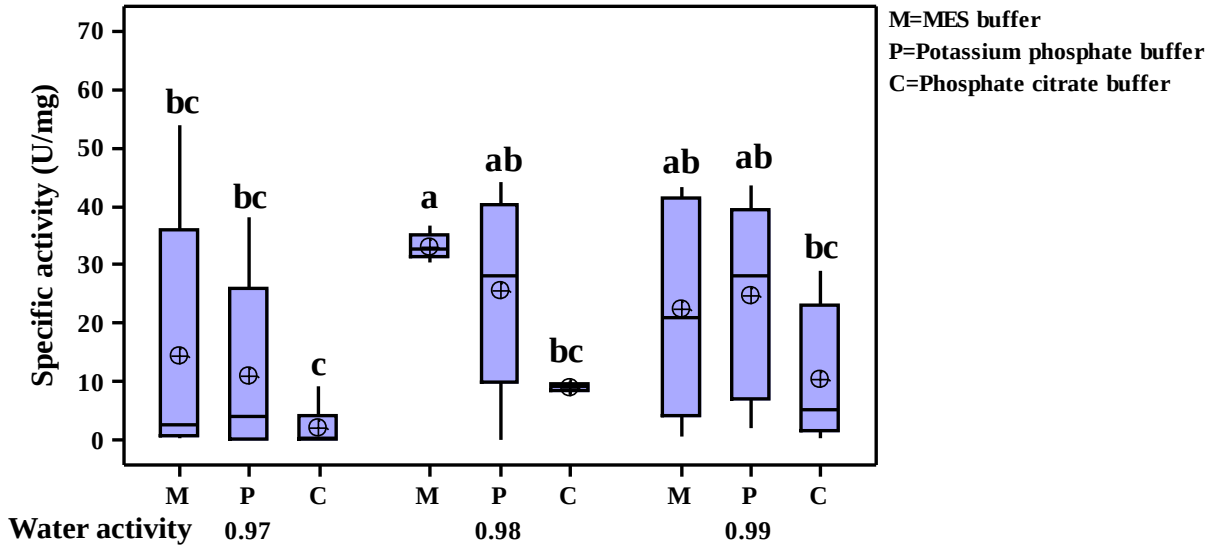



Figure 5.14 The box plot illustrates specific activity of lysozyme (U/mg) in the response to temperature and buffer systems. Boxes contain the median (horizontal line within the box) and the minimum and maximum values (whiskers). The circle inside the box is the mean. Bonferroni individual alpha for multiple comparisons is 0.028. The median of groups that share a letter are not significantly different.

Using the buffered MES medium at 0.98 $a_w$ gave the best yields of lysozyme (Figure 5.15a). However, the maximum value of lysozyme production was obtained in the potassium phosphate buffer at the same $a_w$ level. Phosphate citrate buffered media again supported the lowest median value and amount of enzyme production when cultured at 15°C. The influence of methanol concentration in the different buffer systems on lysozyme activity is shown in Figure 5.15b. Potassium phosphate buffered medium with methanol at 0.6% had the highest median production but the difference was not significant from MES buffered medium using 1.1% methanol (P>0.028). At methanol concentration of >1.1 %, a dramatic decline in the median lysozyme values were observed within all buffered medium treatments used. The sharpest decline in production levels was observed at the highest methanol concentration (1.7%) with the MES buffer system. There was a 26.16 fold decline when compared with the highest median production (Potassium phosphate buffered medium, 0.6 % methanol concentration).

(a)




(b)

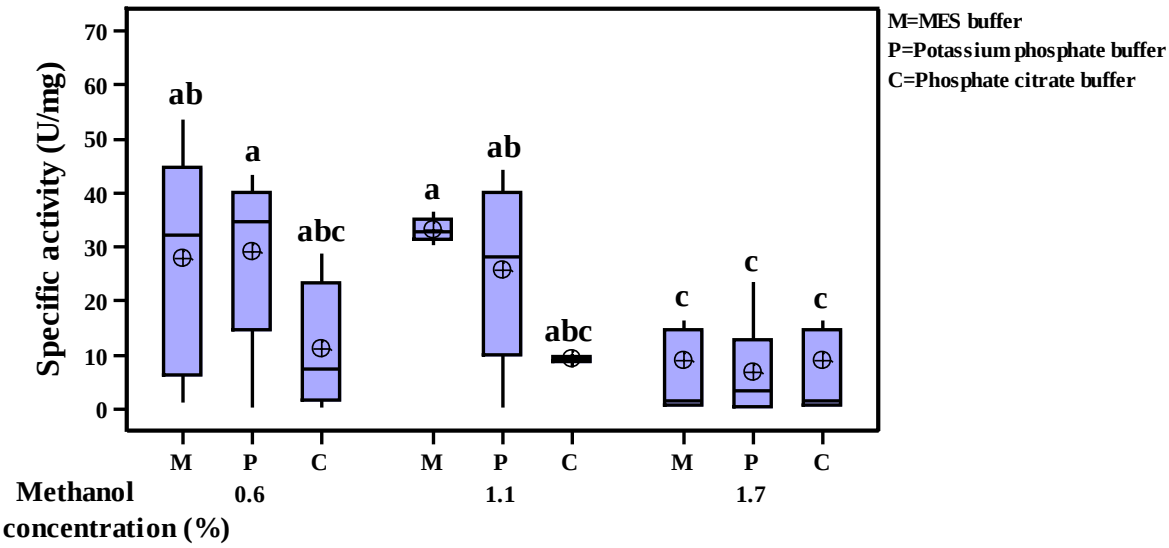



Figure 5.15 The box plot illustrates specific activity of lysozyme (U/mg) in the response to (a) $a_w$ and (b) methanol and buffer systems. Boxes contain the median (horizontal line within the box). The circle inside the box is the mean. The non-parametric Dunn's multiple comparisons test was used with Bonferroni individual alpha for multiple comparisons is 0.028. The median of groups that share a letter are not significantly different.

## 5.4 Discussion

*P. pastoris* was found to be able to grow in the range 0.99 to 0.95 $a_w$. The solute type affected the total cell numbers produced. The inhibitory effect of salt-induced stress with NaCl (low $a_w$) on growth was found. This finding agrees with the result of previous studies by Li et al (2007). The reduced specific growth rate of *P. pastoris* was obtained when toxic $Cl^-$ ions were increased. Two different effects from salt stress impact on cell include ion toxicity and osmotic stress. High accumulation of such toxic ions can cause a significant impact on cellular systems (Kramer, 1983; Greenway et al., 1986; Blomberg and Adler, 1993; Niu et al., 1995; Yeo, 1998; Hasegawa et al., 2000; Yale and Bohnert, 2001; Mahajan and Tuteja, 2005; Logothetis et al., 2007). The present study found that its toxicity of NaCl being more pronounced with pH stress. Without pH stress conditions, *P. pastoris* was able to grow on medium imposed with NaCl at 0.95 $a_w$, however, there was no growth on such medium at 0.95 $a_w$ when combined with pH stressed conditions (pH 4.6-7.6). The reduction of cell number under the influence of pH with salt-amended media was approximately 2-fold comparing to salt stressed conditions alone. When *P. pastoris* exposed to salt stress at different pH levels (pH 4.6-7.6), the average cell number of *P. pastoris* at pH 7.0-7.6 and at pH 4.6-5.0 were lowered than at pH 6.0. Inhibition on growth in NaCl-amended medium could be due to the salt toxicity or their interactions between stress factors but not due to sub-optimal pH alone. Good agreement was obtained between the observed and predicted results when testing the relationship between salt stress and pH.

Within the same $a_w$ level but adjusted with non-ionic solutes, glycerol, clearly showed less inhibitory effect on yeast growth at relatively low $a_w$ and pH. This could be due to its ability to transport glycerol across the cell membrane (Mattanovich et al., 2009). Transported glycerol serves not only as a carbon source for cell multiplication, but also as a compatible solute accumulating inside the cells directly, compensating for leakage. However, there was the inhibition on growth at high glycerol levels when glycerol was used to modify aw at 0.95. This was also observed by Li et al (2007). Changing the concentration of glycerol to 10% (v/v) produced the lower specific growth rate of *P. pastoris* than glycerol was at 5% (v/v).

Shake-flask culture is widely used as the first stage in the investigation of optimizing conditions. However, the levels of protein products are usually lower than when performed in a fermentor. The highest specific activity at 100 h (75.06 U/mg) obtained in the present study, was lower than some published reports. For example, Masuda *et al*. (2005) reported a high specific activity of hen egg lysozyme of 1,500 U/mg when using a 3 L fermenter. Limited aeration is a critical factor for $Mut^+$ strains in shake-flasks, since there is no constant supply of air and methanol (Romanos, 1995). Thus, the potentially toxic molecule formaldehyde from methanol metabolism can be accumulated in the cells with a detrimental effect when the cells are exposed to oxygen limitation (Sibirny et al., 1988; Sibirny et al., 1990; Cereghino and Cregg, 2000). However, the screening study was designed as a first step of an optimization process and the statistical test revealed that the optimal conditions were not located within the experimental parameters used and required a further more detail experimental design.

It was interesting to note that the recommended cultivation temperature (28 – 30°C) by Invitrogen has a negative effect on lysozyme production in this study. This meant that at lower cultivation temperatures, a higher average yield was obtained when compared with the higher recommended temperatures. Similarly, a reduction in temperature yielded a positive effect on growth, and lysozyme productivity could be significantly linked to the proteolytic activity. It has been suggested that this activity is decreased at lower temperatures. In addition, the stability of cell membranes can be improved and the rate of protease release into the culture supernatant is reduced (Jahic et al., 2003b; Gasser et al., 2007; Lin et al., 2007).

To express the recombinant protein from *P. pastoris* under standard conditions, pH 5-6 has been recommended (Cregg et al, 1993; Invitrogen). However, the present study has shown that the highest average lysozyme production was achieved at lowered pH values. This could be due to the reduction of proteolytic activity (Brierley et al, 1994; Koganesawa et al, 2002). To minimize proteolytic activity, lowering pH to 3.0 in the induction phase has been previously suggested (Scorer et al., 1993; Jahic et al., 2003a). However, other studies have indicated that expression levels were increased at

pH 7.0 – 8.0 (Shi et al., 2003). The lowest levels of protease activity were also detected at higher pH levels (pH 6.0, 8.0). Thus, cultivation pH is probably product dependent.

From this study, the best methanol concentration was found to be >1 to 1.5% for optimsing expression. However, a slight decrease in lysozyme production was observed when methanol concentration was added at 0.5% or >1.6%. This can be explained by the toxic effect of methanol. While *P. pastoris* is able to assimilate methanol, excess methanol is toxic to the yeast cells due to the accumulation of intracellular toxic compounds. Formaldehyde and hydrogen peroxide are the oxidized products from methanol which involve alcohol oxidase (AOX) and catalase in peroxisome using oxygen molecules as an electron acceptor (Cereghino and Cregg, 2000). For *P. pastoris* which has a strongly inducible AOX1 promoter for protein expression, better control the methanol level with a sufficient level of dissolved oxygen in cultured medium is highly required. Increasing the concentration of methanol from 0.15% to 1.0% was optimal for yeast growth and increased the expression level (Katakura et al., 1998). Moreover, the toxicity of methanol is one of the main reasons for cell lysis and consequently host cell proteins leakage into the culture supernatant; especially protease, which leads to lower yields and an increase in protein product impurities (Brierley et al., 1990; Chiruvolu et al., 1997; Minning et al, 2001; Jahic et al., 2003).

As the minimum inhibitory $a_w$ for growth of *P. pastoris* is 0.95–0.90 (Grant, 2009), it was possible to induce the host cells under osmotic stress and determined the effects of this stress on expression levels. From the observations, modified $a_w$ at 0.97 gave lower expression levels than those in unmodified $a_w$ (0.99 $a_w$). Increasing osmolarity can have a significant impact on some cellular processes: particularly, protein folding, ribosome biogenesis and cell wall organization (Dragosits et al., 2010).

The toxic effect of acid stress was not simply as the high concentration of $H^+$ ions at low pH. However, it was depended on the properties of the chemical used. Examining the effect of the different buffer systems on the lysozyme production, a lower enzyme production occurred in phosphate citrate buffered medium at identical pH values. This could be due to a strong chelating property of citric acid. The citric acid in a buffered medium may bind to the metal ions in the medium and sequester them in a complex

form reducing the availability of trace elements for yeast cells (Lund et al, 2000; Russell and Gould, 2003). The same effect occurred when examining the effect of pH on *P. pastoris* growth on YPD agar using this buffer system. Delayed growth and reduced populations of yeast populations were observed. This correlated with the low lysozyme production observed. This could an additional explanation for the low expression of lysozyme produced with this buffer.

# 6 Conclusions and Future Work

## 6.1 Conclusions

### (a) Taxol production by endophytes of the Yew tree

- More than 200 endophytes isolated from Yew tree samples (*Taxus baccata*) only two strains were found to be taxol producers These two strains were identified as *Parachonithyrium variabile* and *Epicoccum nigrum* according to the morphological characteristics and ITS1 and ITS2 gene sequence analysis.

- The presence of taxol by both isolated strains were found to be identical to authentic taxol which was confirmed by HPLC and LC-MS/MS analysis.

- The amount of taxol produced by P. *variabile* was found to be 0.53 to 1.75 µg/l and *E. nigrum* was 1.32 µg/l in a standard recommended M1D liquid medium.

- The ecophysiological factors including $a_w$, temperature and solute types significantly influenced the growth rate of *P. variabile* and *E. nigrum* on M1D agar media. The optimum conditions for growth were 0.99-0.98 $a_w$ in sorbitol-amended media for both strains. The optimum temperature for *E. nigrum* was at 20-25°C and was 25°C for *P. variabile*.

- The effect of pH x $a_w$ x temperature x types of solutes showed that the optimum pH for growth of both strains was 5.0 when the buffering system used was based on phosphate citrate buffer.

- The developed response surface model is the first to predict the radial growth rate of the endophytes *P. variabile* and E. *nigrum* in relation to environmental stresses ($a_w$ and temperature). These models were developed using the sorbitol-amended M1D agar. The stress factors and their interaction had significant effects on growth of both strains. From the results of validation experiment, there was good agreement between the observed and predicted values by the model ($R^2$=0.92 for *P. variabile*; $R^2$=0.86 for *E. nigrum*).

- Examination of the ecophysiological factors on fungal taxol production showed that the highest amount of taxol yield from *P. variabile* was 7.11 µg/l under mild stress conditions at 0.98 $a_w$ and 25°C in KCl-amended M1D broth medium. However, there was attenuation of fungal taxol production on this medium for *E. nigrum.*

- The use of elicitors to reverse this attenuation showed that it was possible to do this successfully for *P. variabile.* by adding salicylic acid (50 mg/l). The use of serine (85 mg/l) restored taxol productivity in *E. nigrum*.

- Combining the factors of elicitor, $a_w$ and pH were investigated. The results showed that under modified $a_w$ with KCl (0.98 $a_w$) at pH 5 and supplementation with 20 mg/l of salicylic acid resulted in the highest amount of taxol from *P. variabile*. Treatment of *E. nigrum* with serine at 30 mg/l combined with modified $a_w$ conditions with KCl (0.98 $a_w$) at pH 5 significantly stimulated taxol accumulation.

**(b) Lysozyme production by *P.pastoris***

- The optimum conditions for ecophysiological conditions for growth of *P.pastoris* was identified for the first time. Temperature and $a_w$ had a significant effect on growth of *P. pastoris*. The optimum for growth was at 15-30°C and 0.96-0.99 $a_w$. At 15°C and modified $a_w$ with glycerol (0.97 $a_w$), maximum cell numbers was obtained.

- The effect of pH x $a_w$ x types of solutes showed that the highest cell numbers were produced at 30°C, pH 7 and with $a_w$ of 0.97 modified with glycerol. Water activity had a significant effect on growth of *P. pastoris*.

- Temperature had the most significant effect on lysozyme production by *P.pastoris.* The results from this screening experiment showed that better yield could be achieved by choosing a relatively more acidic pH level, lower incubation temperature and setting the methanol concentration at a much higher level.

- MES-buffered medium resulted in the highest enzyme production at pH 6.8, 25°C, with a methanol concentration of 0.6%, and $a_w$ of 0.97.

## 6.2 Suggestions for future work

- More detailed studies are required for optimising conditions by examining interactions with C- and N- ratios and relative $O_2$ availability. This could be integrated with the identified significant factors or elicitors found in the present study. Studying the different fermentation processes, including solid state fermentation and immobilization systems should be considered.

- It would be interesting to use a synthetic biology approach to identify the key genes involved in taxol production in the present strains and perhaps introduce these into a bacteria or yeasts for the development of cell factories for further enhancing taxol production. This would also perhaps shorten the time scales of production. Scale-up strategies in fermentation processes could then be addressed.

- The factor screening experiment also performed to identify the significant factor for lysozyme production from *P. pastoris*. However, the low yields and unstable secretion of lysozyme were observed. Molecular studies need to be performed in order to have a better understanding of the gene expression under environmental stress. Conditions of fermentation such as dissolved oxygen, agitation speed and different fermentation processes need to be carried out to improve the yield.

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## Poster presentation

**Supunnika Somjaipeng**, Angel Medina and Naresh Magan. Ecophysiological approaches to enhance production of the anti-cancer drug taxol by a strain of *Parachoniothyrium variabile*. British Mycological Society, Annual British Mycological SocietyMeeting, 3-6 September 2012, Alicante, Spain.

**Supunnika Somjaipeng**, Angel Medina and Naresh Magan. Synergetic effect of elicitor, water activity and pH on the production of the anti-cancer drug taxol by a strain of *Epicoccum nigrum.* British Mycological Society Annual Scientific Meeting, September 10-13$^{th}$ 2013, Cardiff, UK . **The Best Poster Presentation Prize**

Cranfield Health

Cranfield UNIVERSITY

# Ecophysiological approaches to enhance production of the anti-cancer drug taxol by a strain of *Paraconiothyrium variabile*

**Supunnika Somjaipeng, Ángel Medina-Vayá and Naresh Magan**

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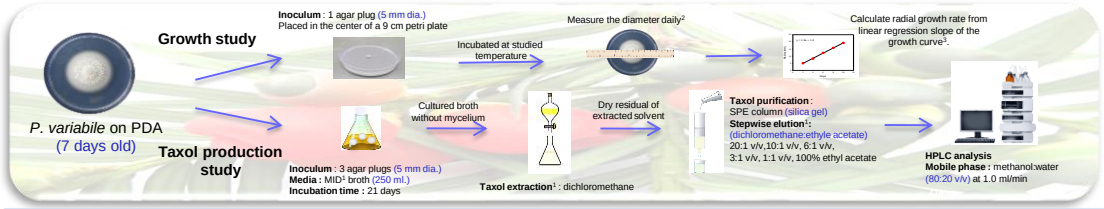
## Introduction

- One of the most interesting bioactive natural products from fungal endophytes which have anti-cancer properties is taxol.
- Taxol, the unique anticancer with high potential activity againsts advanced ovarian cancer and breast cancer.
- Endophytic fungi are known to live inside higher plant tissues, including yew trees (*Taxus baccata*) and produce taxol.
- There is interest in growing these strains and improving the titres of taxol to reduce the cost of production by fermentation strategies.

**Objectives: (1) to examine the ecophysiology factors on growth (2) to enhance taxol production under environmental stress.**

## Material and methods




## Results

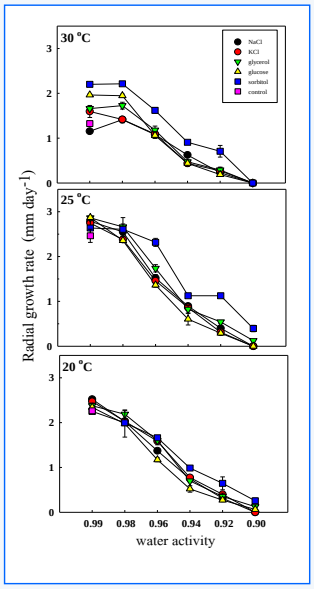



**Figure 1.** Effect of environmental stress on the growth rate of *P. variabile* .

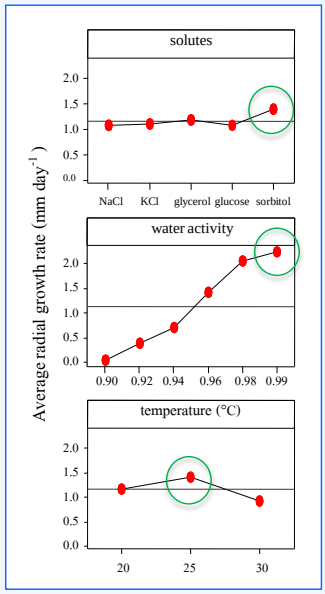



**Figure 2.** The main effect plot for the radial growth rate of *P. variabile* (mm day$^{-1}$).

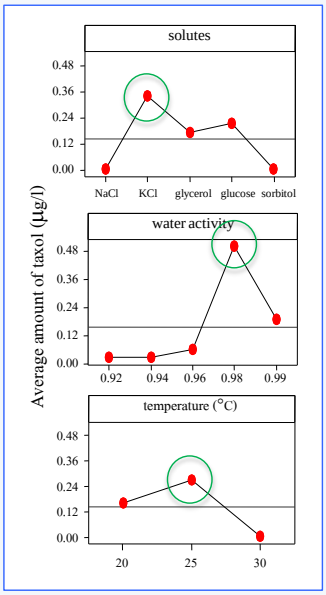



**Figure 3.** The main effect plot for the amount of taxol (µg/l ).

## Conclusions

- Under studied environmental stress, increasing titres of taxol to 7 times were observed. Thus, the factors are able to select for subsequent experiments which are in progress in order to reach the region that contains the optimal solution.
- If this can be achieved the fungal taxol could be a useful alternative to the production of this anti-cancer drug than using the yew tree.

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# Synergetic effect of elicitor, water activity and pH on the production of the anti-cancer drug taxol by a strain of *Epicoccum nigrum*

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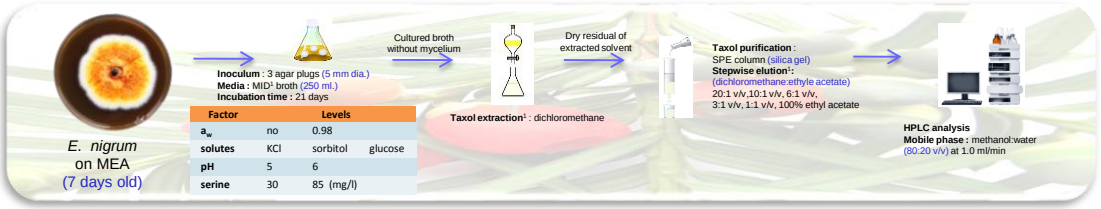
## Introduction

- One of the most interesting bioactive natural products from fungal endophytes which have anti-cancer properties is taxol.
- The low and unstable productivity of taxol from fungal endophytes are the main problems in optimized vitro conditions.
- Elicitation is one of the effective method to enhance taxol yield by using biotic and abiotic elicitors[2,3]
- There is interest in improving the titres of fungal taxol by using elicitation method combined with environmental stress conditions.

**Objectives: to examine the effect of interactions between $a_w$, pH and elicitor.**

## Material and methods




| Factor | Levels | | |
|---|---|---|---|
| $a_w$ | no | 0.98 | |
| solutes | KCl | sorbitol | glucose |
| pH | 5 | 6 | |
| serine | 30 | 85 (mg/l) | |

## Results

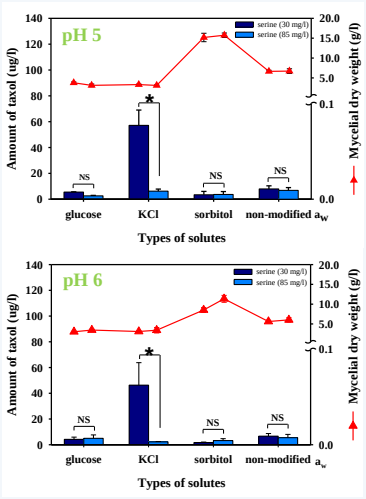



**Figure 1. Effect of serine, $a_w$, solutes and pH on taxol yield.** The highest yield was obtained after cultivated with M1D broth at pH 5, modified $a_w$ with KCl (0.98 $a_w$) and 30 mg/l of serine. (Asterisk indicates significant differences while NS represents not significant)

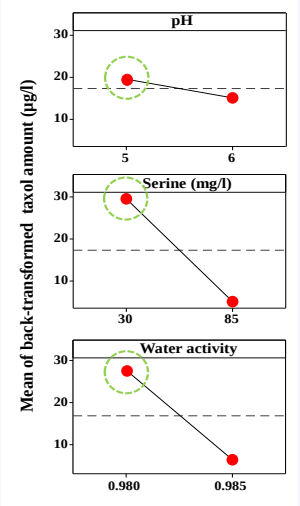
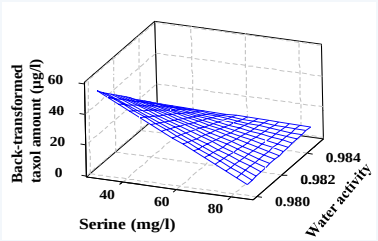



**Figure 2. Evaluation of the effect of serine, pH and $a_w$ on taxol yield (KCL was used to modified $a_w$).** The main effect plots show that all three of studied factors had a significant effect on taxol yield (P<0.05). (Green circle indicates the significant level)



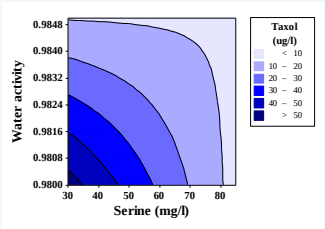




**Figure 3. The effect of the interaction between $a_w$ and serine on taxol yield (KCL was used to modified $a_w$).** An increasing trend in yield with a decrease in serine concentration was observed. The impact of water stress on the yield became more significant at lower level of serine.

## Conclusions

- The use of serine as the elicitor restored taxol productivity successfully in *E. nigrum*.
- Combining the factors of elicitors, $a_w$ and pH showed that treatment of *E. nigrum* with serine at 30 mg/l combined with modified $a_w$ conditions with KCl (0.98 $a_w$) at pH 5 significantly stimulated taxol accumulation increasing titres of taxol to 8.5 times.
- From this study, the factors are able to select for subsequent experiments which are able to reach the region that contains the optimal condition.

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