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Optimization of culture conditions of *Streptomyces rochei* (MTCC 10109*)* for the production of antimicrobial metabolites

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Abstract

Fermentation and culture conditions were studied in shaken-flask culture to induce the production of greater amounts of antimicrobial metabolites by *Streptomyces rochei* (10109). Antimicrobial metabolite production started after 48 h incubation and reached its optimum level at 20% inoculum size at 120 h, at which point the metabolites showed maximum antifungal and antibacterial activity against selected human pathogenic microorganisms (*Candida albicans, Staphylococcus aureus* and *Escherichia coli*). Optimal production occurred at pH 7.5 and temperature 32°C, with 2% glycerol and 1% peptone as the carbon and nitrogen sources respectively. The effects of adding sea water (optimum 30%) and NaCl (optimum 1%) were also evaluated.

Keywords: human pathogens

Introduction

The importance of marine sources for the discovery of novel natural products with a pharmaceutical potential has been proved during the last decade, highlighted in various reviews (Faulkner 2000; Haefner 2003; Blunt *et al*. 2003). More than 70% of the naturally derived antibiotics currently in clinical use are derived from marine actinomycetes (Pimentel-Elardo *et al*. 2009), which play an important role among marine bacterial communities, because of their diversity and ability to produce novel chemical compounds of high commercial value (Hopwood 2007; Amador *et al.* 2003). They are widespread in most ecological niches (Takahashi & Omura 2003). Actinomycetes especially *Streptomyces* species are a rich source of several useful bioactive natural products with potential applications (Ubukata *et al.* 1995; Hayakawa *et al*. 1996; Xue *et al*. 1998; Jones 2000; Manteca *et al.* 2008; Atta 2009; Lakshmipathy & Kannabiran 2009) and are prolific producers of secondary metabolites, many of which have commercial importance as antibiotics, antiparasitics and antifungal agents, herbicides, pesticides, anticancer or immunosuppressive agents as well as industrially important enzymes (Curl *et al.* 1985; Choi *et al.* 1996; Osada 1998; Tskahashi & Omura 2003; Saadoun & Gharaibeh 2003; Maskey *et al*. 2003; Atta & Ahmad 2009).

The focus on the physiology and the potential of bioactive substances of non-cultivable marine microorganisms is of current problem and it becomes a great challenge to researchers to cultivate and isolate novel secondary metabolites for therapeutic applications. Improvement in the growth is carried out by manipulating the nutritional and physical parameters of the culturing conditions. Media composition plays a vital role in the efficiency and economics of the ultimate process. These not only influence the growth and metabolism of the culture, but also increase product titre and consequently process economics. The strategies that are generally adopted for selecting media composition include easy availability in the market at low cost, minimum variation from batch to batch, ability to support the growth of the organism and product formation, and limitation of production of unwanted byproducts and compounds closely related to the product of interest.

Antibiotic biosynthesis is a specific property of microorganisms and depends greatly on culture conditions. It is necessary to develop optimum conditions to make the production of an antibiotic feasible. The ability of *Streptomyces* cultures to form antibiotics is not a fixed property but can be greatly increased or completely lost under different conditions of nutrition

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and culturing (Waksman 1961), and hence the medium constitution together with the metabolic capacity of the producing organism greatly affect antibiotic biosynthesis. Changes in the nature and type of carbon, nitrogen and phosphate sources, and trace elements, have been reported to affect antibiotic biosynthesis in *Streptomyces* (Barratt & Oliver 1994; Lounes *et al.* 1996; Abbanat *et al.* 1999). Antibiotic productivity tends to decrease when metal-ion-deficient media are used and when the inocula are incubated for long periods and at high temperatures (Higashide 1984).

The present study describes the optimization of the culture conditions such as requirements of natural sea water, temperature, pH, concentrations of NaCl, and carbon and nitrogen sources for the production of antimicrobial metabolites. The study can be compared with other similar studies of the same (e.g. Chattopadhyay & Sen 1997) or different species (e.g. Saurav & Kannabiran 2010).

Materials & Methods

The producer organism *Streptomyces rochei* was isolated from seawater samples collected from the Visakhapatnam coast of the Bay of Bengal. A pure culture of this strain was maintained on starch casein agar slants kept in a refrigerator at 4ºC until used. Morphological, culture and physiological characteristics of the strain were studied using the standard methods of the International Streptomycetes Project (ISP) recommended by Shirling and Gottlieb (Mocheva *et al.* 2002). The strain was identified as *Streptomyces rochei* and deposited in the Microbial Type Culture Collection (MTCC), Chandigarh, India with accession number 10109.

For seed culture preparation, a loopful of mycelium with spores were taken from 7-day-old slant culture and used to inoculate a 250-ml flask containing 25 ml of seed medium broth of composition (g L^{-1}) as follows: soyabean meal (10), glucose (10), $CaCO_3$ (1), K_2HPO_4 (1), and a mixture (50:50) of distilled water and natural seawater aged for 30 days and adjusted to pH 7.0. The mixture was incubated at 30°C for 48 h. The production medium was prepared similarly, and 48-h-old seed culture was used as the inoculum.

Growth of *Streptomyces rochei* (10109) was measured as mycelium dry weight. The contents of the culture flask was filtered through a previously weighed dry Whatman No.1 filter paper, washed twice with distilled water and then the filter paper along with the mycelial mass was dried in a hot-air oven at 80 ºC for 18-24 h. The filter paper was then cooled in a desiccator and weighed. At 24-h intervals the flasks were harvested and the biomass determined in terms of total dry cell weight.

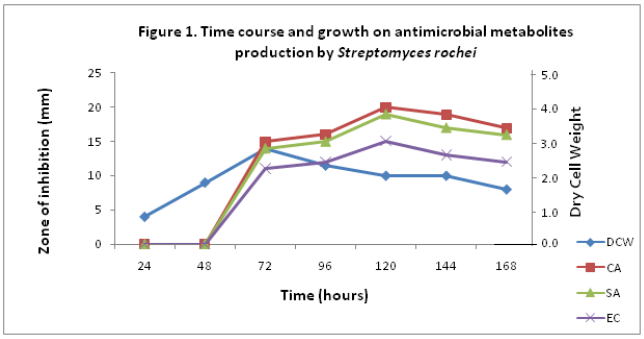
Antimicrobial metabolite production was carried out in production medium. Five ml of seed culture were used to inoculate a 250-ml flask containing 50 ml of production medium broth, which was then cultured in submerged shaken-flask fermentation and adjusted to pH 7.0. It was then incubated for an additional 120 h at 30 °C on the rotary shaker at 110 rpm. The culture broth was then centrifuged at 10,000 rpm for 15 min to separate the cells from the fermented broth, and the clear supernatant broth used as the source of crude antimicrobial metabolites.

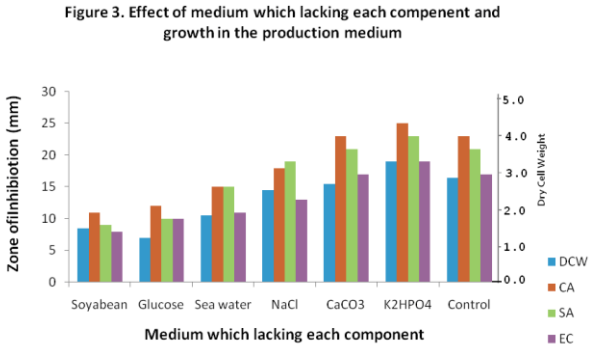
The antimicrobial activity of the fermented broth was examined against selected human pathogenic microorganisms: the fungus *Candida albicans* (MTCC 183) (‘CA’ on the figures), the gram-positive bacterium *Staphylococcus aureus* (MTCC 1771) (‘SA’ on the figures) and the gram-negative bacterium *Escherichia coli* (MTCC 443) (‘EC’ on the figures). Pathogenic microorganisms were obtained from IMTECH, Chandigarh, India. Potato dextrose broth and nutrient broth media were used for the cultivation of test fungi and bacterial species respectively, and all the cultures were kept at 4 ºC. Antimicrobial activity was tested by the agar-well diffusion method (Saadoun & Muhana 2008). Wells of 6 mm diameter were prepared in nutrient-agar plates and the test pathogenic bacterial and fungal cultures swabbed onto the surface (Mitra *et al.* 2008). The wells were filled with 50 µl of crude culture

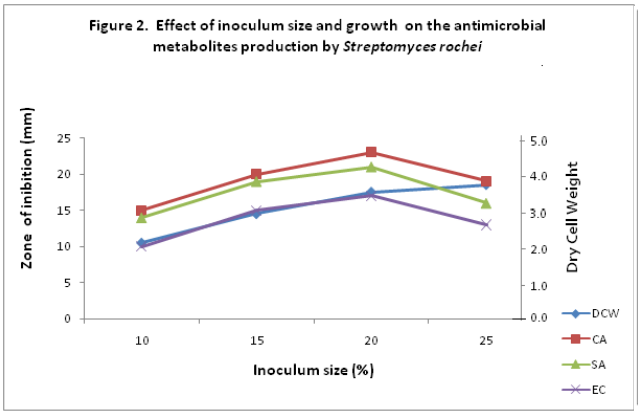
supernatant, and the diameter of inhibition zones measured after incubation for 24 h at 37 ºC (bacterial species) and 48 h at 28 ºC (fungal species).

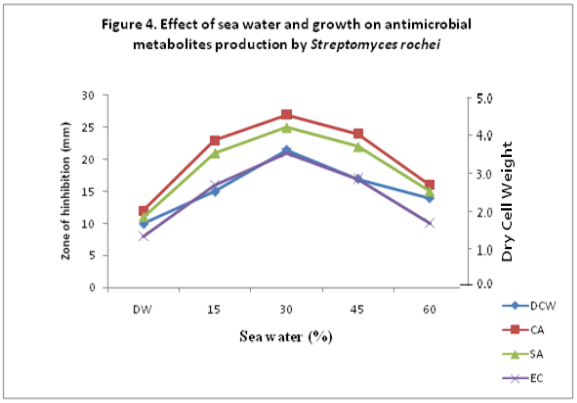
In order to enhance the antimicrobial metabolites production, optimization of the production medium was essential. The classical method of experimentation with one factor varying at a time, with all other factors held constant at a specific level, was carried out as described by Strobel & Sullivan (1999). The effect of the medium components on antimicrobial metabolite production in submerged flask culture was studied by removing medium components one by one and observing their effect on the production of metabolites.

In order to study the enhancement or inhibitory effect of nutrients on antimicrobial metabolite production, supplementation experiments were performed. Several sources of carbon and nitrogen were supplemented in the production medium, and the best sources were also tested for optimal antimicrobial metabolite production at different concentrations. In addition, the effects of supplements such as inoculum size, seawater and NaCl were studied at the optimal incubation time of 120 h. Various inoculum sizes (10%, 15%, 20% and 25% v/v) were tested, as were different dilutions of seawater (15%, 30%, 45% and 60%) with distilled water as a control. Different temperatures (28, 32, 37, 42 and 50 °C) and pHs (6.0, 7.0, 7.5, 8.0, 9.0 and 10.0) were tested, as were concentrations of NaCl (1, 1.5, 2, 3 and 4.5%). Various carbon sources (starch, glucose, glycerol, xylose, arabinose, sucrose, lactose, galactose, ribose, meso-inositol and citric acid) were tested to determine the best one, which was further studied at different concentrations (1, 2 and 3%) to determine the optimum. Various nitrogen sources (peptone, potassium chloride, casein, yeast extract, meat extract, ammonium chloride and sodium nitrite) were also tested, and again the best one studied at different concentrations (1, 2 and 3%).





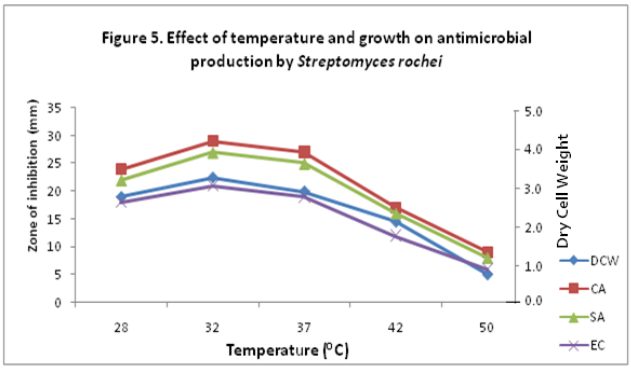


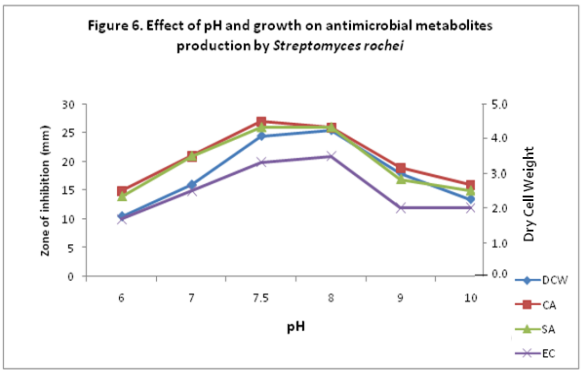


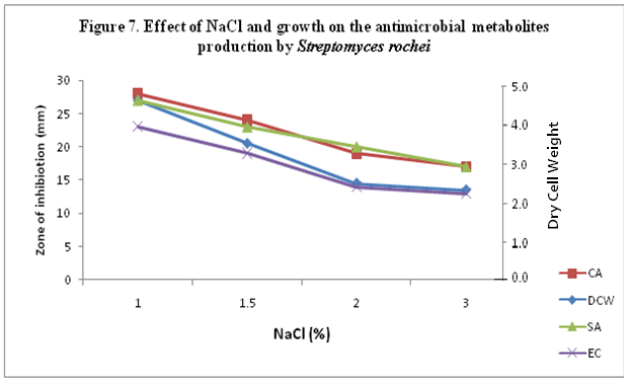
Results

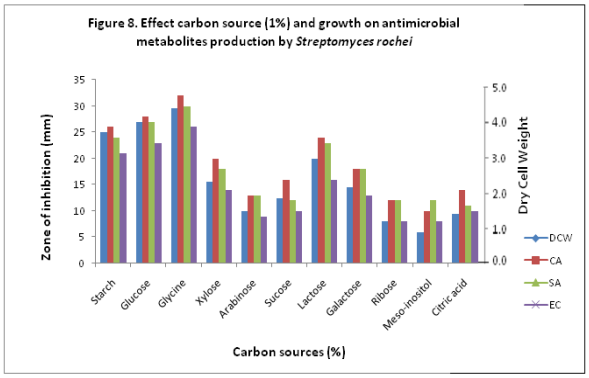
On the basis of morphological, physiological and culture characteristics, and chemotaxonomic studies, the strain investigated was identified as *Streptomyces* (Reddy *et al.* 2010). It was

further identified on the basis of 16S rRNA homology studies: the gene sequence was submitted to NCBI GenBank (accession number HQ 400987) and identified as *Streptomyces rochei*. It has been deposited at the Microbial Type Culture Collection (MTCC), Chandigarh, India with accession number 10109.









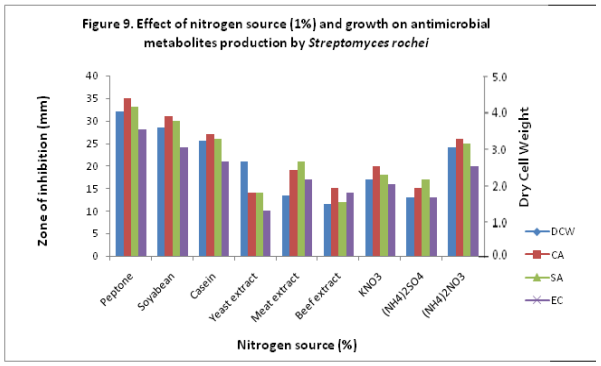
A typical time course of antimicrobial metabolite production (Fig 1) showed mycelium growth gradually increasing up to 72 h, and then entering a stationary phase. Antimicrobial metabolite production was detected in the culture broth after 48 h of incubation, and reached a maximum in the late stationary phase (120 h incubation). Optimum inoculum size was observed at 20% (*v/v*) (Fig 2), and further increases in the inocululm size resulted in decreased production of antimicrobial metabolites.

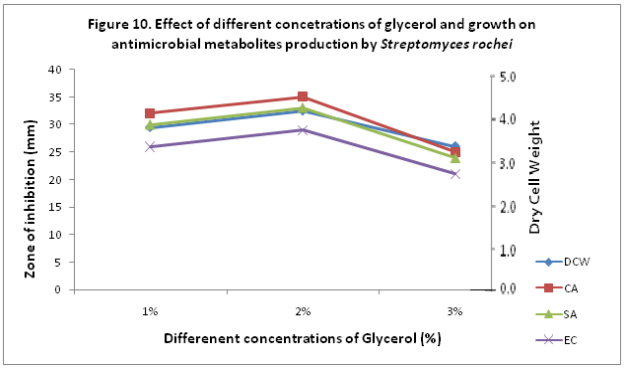
For optimizing the antimicrobial metabolite production, experiments with the removal and supplementation of nutrients based on single–dimension optimization were carried out. As shown in Fig 3, glucose and soybean were very important carbon and nitrogen sources, whereas natural seawater and NaCl were less essential. K_2HPO_4 showed a slightly inhibitory effect, and $CaCO_3$ only helped increase growth, but did not influence the production of antimicrobial metabolites.

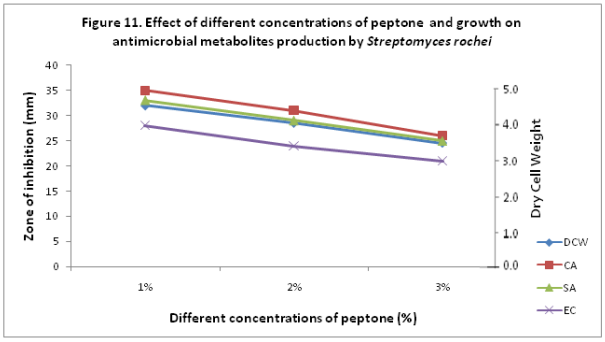
Tests of seawater dilutions showed that 30% was optimal for maximal production of antimicrobial metabolites (Fig 4); further dilution gradually decreased the metabolite production. The absence of sea water greatly inhibited production, clearly indicating that seawater is essential in *Streptomyces rochei*. 32°C proved to be the optimal temperature for production of metabolites (Fig 5); higher temperatures had an adverse effect on both growth and metabolite production. The pH of the culture broth was a significant influence on both metabolite production and mycelium growth. There was a broad range of optimum pH levels ranging from 7.5 to 8.0 (Fig 6), while acidic conditions supported neither growth nor metabolite production. 1% NaCl was the optimum concentration production as well as growth (Fig 7); further increase in the concentration gradually decreased growth and productivity.

Antimicrobial metabolite production was greatly influenced by the nature, type and concentration of the carbon source. This strain of *Streptomyces rochei* was able to grow in all

carbon sources tested (Fig 8), but maximum production was obtained in cultures supplemented with glycerol, followed by glucose and starch. 2% glycerol was the optimum concentration for metabolite production (Fig 10).







Metabolite production was also greatly influenced by the nature, type and concentration of the nitrogen source. The strain was able to grow in all the nitrogen sources tested (Fig 9), but the maximal productivity was obtained with peptone as the nitrogen source, followed by casein and $(NH_4)_2NO_3$. 1% peptone was the optimum concentration for productivity (Fig 11).

Discussion

Optimization of media is generally done by studying the effects of the ingredients/nutrients on growth using fermentation studies, selecting and optimizing a few parameters (Gresham & Inamine 1986). Actinomycete cell metabolism under conditions of nutritional excess is directed towards the generation of cell mass rather than the production of secondary metabolites, and when depletion of key nutrients occurs, it shifts the cell cycle to the stationary phase and signals the transition from primary to secondary metabolism in which these bioactive metabolites are produced (Abanat *et al*. 1999l; Abd-Allah & El-Mehalawy 2002). In the present investigation, the time course of fermentation of *Streptomyces rochei* (MTTC 10109) showed mycelial growth up to 72 h followed by a stationary phase; antimicrobial metabolite production was detected after 48 h incubation, and reached optimum yields in the late exponential and stationary phase after 120 h incubation, indicating mainly secondary metabolism (Demain & Fang 1995; Bibb 1996; Vineeta *et al*. 2008).

The inoculum size was important for metabolite production (Grag & Neelakantan 1981; Maha *et al*. 2001) with an optimum at 20% (*v/v*). Marine *Streptomyces* are particularly attractive because they have not been extensively exploited compared to their terrestrial counterparts, and show high titers of antimicrobial metabolite production due to the diluting effect of seawater in the marine environment (Krish *et al*. 2010). In the present study seawater greatly enhanced growth as well as metabolite production in *S. rochei*. Environmental factors

such as temperature, pH and incubation period are known to have profound influences on growth and antibiotic production in *Streptomyces* species (Srinivasan *et al.* 1991). Changes in external pH affect many cellular processes such as regulation and biosynthesis of secondary metabolites (Chang *et al.* 1991; Datta & Kothary 1993; Sole *et al.* 1994; Sole *et al.* 1997). In the present study, maximal antimicrobial metabolite production resulted at a pH of 7.5, a temperature of 32°C and 1% NaCl concentration.

In the production of secondary metabolites, the interaction between growth metabolism and product secretion is critically influenced by growth-limiting nutrient concentrations (Kumar & Kannabiran 2010). Optimal production has generally been achieved by cultivating organisms in media containing slowly utilized nutrient sources, or under conditions which allow a slow supply of these nutrients (Drew & Demain 1977; Jonsbu *et al.* 2002). For *S. rochei* (10109), 2% glycerol was the best carbon source for optimal metabolite production. Glycerol is known to be an important medium component for the production of antimicrobial metabolites from microorganisms (Minambres *et al.* 1992; Sengupta & Paul 1992; Qureshi *et al.* 2001; Fukuda *et al.* 2005; Fguira et al. 2005; Mehdi *et al.* 2006). Secondary metabolite production in actinomycetes is often stimulated by slowly assimilated complex carbohydrates in the production media, and decreased when more rapidly utilized monosaccharides such as glucose are present (Bertasso *et al.* 2001). A possible explanation of this phenomenon is that glucose causes catabolite repression, in which the production of enzymes of secondary metabolite biosynthesis is inhibited (Drew & Demain 1977; Iwai & Omura 1982).

Lower concentrations of peptone (1%) greatly favoured higher antimicrobial metabolite yield in the present investigation. Similar observations have been reported by many investigators (Khaoua *et al.* 1991; Mansour *et al.* 1996; Chattopadhyay & Sen 1997; Vandana *et al.* 2008). In the present study, phosphate (KH_2PO_4) was not favorable for metabolite production; an excessive amount of inorganic phosphate suppresses the production of antibiotics (Zinzina & Efimova 1979; Kishimoto *et al.* 1996).

We conclude that the ability to produce antimicrobial metabolites changes greatly under different culture and nutritional conditions. Optimal levels of culture conditions were determined for *Streptomyces rochei.*

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الملخص العربي

تهيئة الظروف المثلي لاستنبات بكتريا ستربتوميسيس روشي [*Streptomyces rochei* (MTCC 10109)] لإنتاج نواتج أيض مضادة للميكروبات.

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درست عمليات التخمر والاستنبات في قارورة هزازة للحث على إنتاج كميات أكبر من نواتج أيض مضادة للميكروبات بواسطة بكتيريا ستربتوميسيس روشي [*Streptomyces rochei* (10109)]. بدأت عمليه انتاج نواتج أيض مضادة للميكروبات بعد إنقضاء 48 ساعة من فترة الحضانة ووصلت إلى أعلى مستوى لها وهو 20 ٪ في 120 ساعة ، وعند هذا الحد أظهرت نواتج الأيض أقصى نشاط لها كمضادات للفطريات والبكتيريا ضد بعض الميكروبات المختارة والمسببة للأمراض بالنسبة للإنسان [فطر الكانديدا (*Candida albicans*)، بكتيريا ستافيكوكس أوريس (*Staphylococcus aureus*)، بكتيريا ايشريشيا كولاي (*Escherichia coli*)]. ظهر الإنتاجية المثلي لتلك المواد عند درجة الحموضة البالغة 7.5 ودرجة حرارة 32 درجة مئوية ، وعند إضافة 2 ٪ من الجلسرين و 1 ٪ من الببتون كمصادر للكربون والنيتروجين على التوالي. كما تم تقييم آثار إضافة مياه البحر (النسبة الأمثل هى 30 ٪) وكلوريد الصوديوم (النسبة الأمثل هى 1 ٪).