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Fermentation Studies for the Production of Dibutyl Phthalate, an Ester Bioactive Compound from *Streptomyces albidoflavus* MTCC 3662 Using Low-Priced Substrates

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Abstract

Attempts were made to evaluate the limiting nutritional parameters for production of dibutyl phthalate from *Streptomyces albidoflavus* MTCC 3662. The compound was found as a broad spectrum bioactive compound that acts as an antimetabolite of proline. Among the carbon and nitrogen sources tested in batch fermentation, glycerol (1.75%) and arginine (0.075%) supported best production. Arginine was replaced with sodium nitrate (0.025%) in the later studied due to its low cost, easy availability and as well as for good production capability. Other limiting nutrients, KH_2PO_4 0.05%, NaCl 0.15% and $MgSO_4$ 0.03% were found best for production. Effect of trace salts in the basal medium was also tested. Medium supplementation with casein hydrolysate, jaggery and yeast extract (0.001%, each) enhanced the production. Yield enhancement was also observed with Tween-80 (0.2µg/ml). Optimization of nutritional parameters imparts enhancement of production by 2.4 fold, using low cost substrates.

Keywords: Fermentation, Optimization, Dibutyl Phthalate, Bioactive Compound, Antimetabolite, Proline, *Streptomyces albidoflavus* MTCC 3662

1. Introduction

Dibutyl phthalate (DBP) is bioactive ester produced by bacteria (Morsi *et al*., 2010), actinomycetes (Roy *et al*., 2006), fungi (Mabrouk *et al*., 2008), algae (Namikoshi *et al.*, 2006; Babu and Wu, 2010) and also by higher plants (Ruikar *et al.*, 2011). DBP varies qualitatively in terms of ^{14}C content of various sources between petrochemicals and biologicals. In case of biological source, ^{14}C level is more than 50% whereas of petrochemical source, it is beyond the detectable level (Namikoshi *et al*., 2006). The *de novo* synthesis of DBP by plant cell culture using medium containing $NaH^{13}CO_3$ has been reported (Babu and Wu, 2010). The bioactive compound produced by *Streptomyces albidoflavus* MTCC 3662 was structurally similar to DBP having antimicrobial activity against Gram positive and Gram negative bacteria, and also against unicellular and filamentous fungi (Roy *et al.*, 2006). It is also a pH and thermo tolerant antimetabolite of proline (Roy *et al*., 2006, Roy and Sen, 2007). Antimetabolites, the potential antimicrobial, are organic compounds having deleterious effect on growth and viability of an organism while the effect can be reverted by concurrent administration of one or more common biochemicals (Pruess and Scannell, 1974). They act as anticancer and antitumour (Ogawa *et al*., 1998) and herbicidal (Lee *et al*., 2003) compounds and also in studies of various physiological processes. Cytotoxic activity of DBP against tumor cell lines has been worked out (Mabrouk *et al*., 2008). Thus, this necessitates further studies on DBP production.

There is no common phenomenon or regulation connected with the growth and production ability of microorganisms. Under varying conditions of cultivation, the production may be stimulated. Suitable alterations of carbon, nitrogen or phosphate sources and supplements have been reported to affect metabolic processes in streptomycetes (Barratt and Oliver, 1994; Loun'es *et al*., 1996; Abbanat *et al*., 1999). Therefore, it is essential to pay special attention for the optimization of production parameters for cost effective production of DBP. Optimization of physical conditions of the selected medium for the production of DBP by *S. albidoflavus* MTCC 3662 has been reported (Roy and Sen, 2011). This communication evaluates the effect of nutritional factors for production of DBP by *S. albidoflavus* MTCC 3662.

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2. Materials and Methods

2.1. Microorganisms Used

The *Streptomyces albidoflavus* 321.2 (MTCC 3662), a soil isolates (Roy and Sen, 2002), was maintained in glucose-asparagine agar containing (g/l): glucose, 10; asparagine 0.5; K_2HPO_4, 0.5; pH 6.8; agar, 20. Test organism *Escherichia coli* ATCC 25922 was maintained in nutrient agar, containing (g/l) peptone, 5; NaCl, 5; beef extract, 1.5; yeast extract 1.5 pH 7.2; agar, 20. Organisms were stored at 4°C.

2.2. Inoculum

Spore suspension ($1.2x10^7$spores/ml) of 6 day old culture was used as inoculum**.**

2.3. Basal Production Medium

Arginine glycerol salt (AGS) medium, containing (g/l) arginine, 1; glycerol, 12.5; K_2HPO_4, 1; NaCl, 1; $MgSO_4$, $7H_2O$ 0.5; $Fe_2(SO_4)_3$, $6H_2O$ 0.01; $CuSO_4$, $5H_2O$ 0.001; $ZnSO_4$, H_2O 0.001; $MnSO_4$, H_2O 0.001; agar 20; pH 6.75. All chemicals used were of analytical grade.

2.4. Fermentation Condition

Batch experiments were carried out in basal medium (25 ml in 100 ml Erlenmeyer flask) at 32°C temperature for 6 days at stationary condition using 6% inoculum. Other test chemicals for individual experiments were sterilized separately and added to the fermentation medium in required quantity aseptically. The optimized parameter of an experiment was considered for the designing of subsequent experiments keeping all other conditions constant. All the experiments were made in five set in all treatments and each test case.

2.5. Assay of Antimetabolite

Yield of antimetabolite was determined by agar cup method (Higashida *et al*., 1971), using glucose-asparagine agar and 1ml suspension ($1.2X10^6$ CFU/ml) of the test organism. Each cup was filled with cell-free fermented broth (crude sample, 0.1 ml) and incubated at 30°C for 24 hs. The yield was determined by measuring the zone of inhibition against a standard curve of authentic sample.

2.6. Statistical Analysis

Standard error (±) of mean was calculated from five determinations using Microsoft Office Excel 2007.

3. Results and Discussion

3.1. Effect of Carbon Source

In batch experiments production kinetics were observed under growth-limiting conditions. Carbon being the prime factor for growth, several carbon sources was tried, taking 1% of each along with 0.32 g/l of nitrogen, as recommended in AGS medium. The isolate was able to grow in all the tested carbon sources, however; maximum production was obtained in medium containing glycerol as a sole source of carbon followed by Ca-gluconate and lactose (Table 1). In medium, containing maltose, meso-inositol, Na-acetate and xylose, the production was not detectable. It was further observed that increase of glycerol amount above 1.75% influenced the yield, causing inhibition (Figure 1). Authors worked for secondary metabolite production by *Streptomyces* spp. found glycerol limitation at 1% (Kojima *et al*., 1995), 1.15% (Bhattacharyya *et al*., 1998) and 2% (Poetsch *et al*., 1985).

Table 1. Suitability of carbon source for the production of DBP by *Streptomyces albidoflavus* MTCC 3662

Carbon sources (1%)	Yield (µg/ml)
Na-acetate	ND
D-Ribose	75 ±1.58
Xylose	ND
Raffinose	140±2.74
Ca-gluconate	175±3.54
Dextrose	75±1.58
Meso-inositol	ND
Mannitol	95±1.58
Glucose	110±2.74
Fructose	70±1.58
Mannose	90±2.24
Galactose	125±3.54
Glycerol	250±3.87
Sucrose	110±2.24
Lactose	145±3.16
Maltose	ND
Starch	070±1.58
Control (without carbon source)	ND

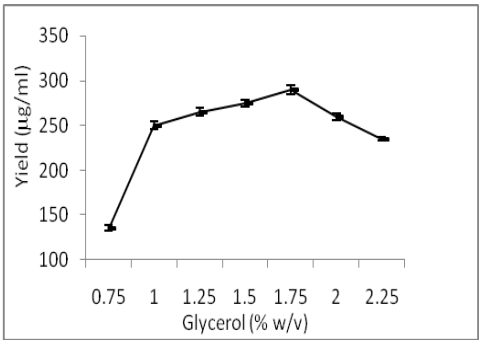

Figure 1. Effect of glycerol concentration on the production of DBP by *Streptomyces albidoflavus* MTCC 3662.

3.2. Effect of Nitrogen Source

Preferential utilization of nitrogen is a distinguishing phenomenon of *Streptomyces.* Nitrogen sources (0.32N g/l) were tested with optimum level of glycerol. It was revealed that the yield was greatly influenced by the nature of the nitrogen source (Table 2). Arginine was the best source for yield enhancement followed by asparagine and sodium nitrate. Subsequent repeat experiment was performed with the best two nitrogen sources. It was found that 0.075% arginine and 0.025% sodium nitrate were the most favored sources for yield (Figure 2). Of the two nitrogen sources, sodium nitrate was selected as a cheap and easy availability. Several researchers (Furumai *et al*., 1993; Haque *et al*., 1995, Bhattacharyya *et al*., 1998) studied the nitrogen limitation and secondary metabolite production by *Streptomyces* spp.

Table 2. Suitability of nitrogen source for the production of DBP by *Streptomyces albidoflavus* MTCC 3662

Nitrogen sources (~ 0.32 N g/l)	Yield (µg/ml)
Ammonium nitrate	125±2.24
Ammonium chloride	140±1.58
Ammonium dihydrogen phosphate	110±2.24
Ammonium sulphate	145±1.58
Arginine	290±2.24
Asparagine	190±2.74
Di-ammonium hydrogen orthophosphate	145±2.24
Potassium nitrate	145±1.58
Sodium nitrate	175±3.16
Tyrosine	175±3.16
Urea	75±2.24
Control	110±2.74-

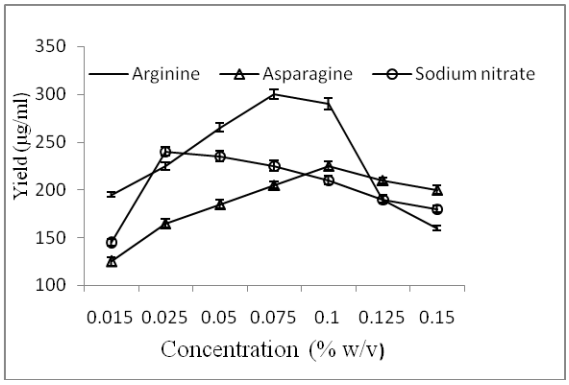

Figure 2. Effect of nitrogen on the production of DBP by *Streptomyces albidoflavus* MTCC 3662

3.3. Effect of Phosphate

The limitation of phosphate can regulate the metabolic rate; hence, the source and amount were varied. Thus, the production was maximum at 0.05% KH_2PO_4 (Figure 3). In transcriptional regulation the involvement of phosphate is well established for biosynthesis of secondary metabolites (Reeve and Baumberg, 1998). Phosphate limitations were also observed (Kishimoto *et al*., 1996). Data of this experiment could well corroborate with the observation of Ochi *et al*. (1988) and Ogawa *et al*. (1998).

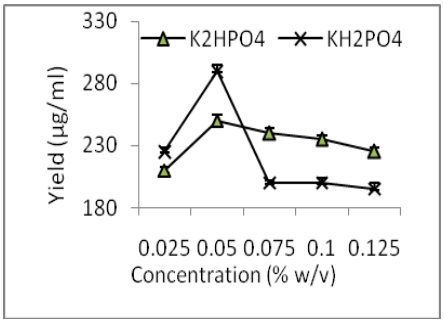

Figure 3. Effect of phosphate on the production of DBP by *Streptomyces albidoflavus* MTCC 3662.

3.4. Effect of Chloride

Apparently, chlorine may not play an important nutritional role, but its limitation concerns. Sometimes, range of osmotic role for growth is quite narrow and varies with the habitational limitations of the species; hence, the adjustment of NaCl concentration was recommended (Stanbury *et al*., 1997). Commonly used chlorine salts, like NaCl and KCl, were used to determine their optimum concentration and was found 0.15% NaCl (Figure 4). This is closely related to the reported range of 0.1% (Kojiri *et al*., 1992) to 0.25% (Harindran *et al*., 1999). Even though KCl was also used by several workers (Tunac *et al*., 1985; Nakamura *et al*., 1986) the present experiment did not support.

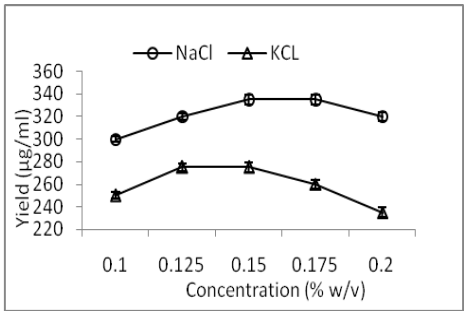

Figure 4. Effect of chloride on the production of DBP by *Streptomyces albidoflavus* MTCC 3662

3.5. Effect of Sulphate

Sulphur is a component part of protein, enzyme and coenzyme A. Quite often, adequate synthesis of enzyme depends on the availability of sulphur. Therefore, experiment was conducted with different concentrations of $MgSO_4$ as the source of sulphur and 0.03% was found best for the yield of antimetabolite (Figure 5). Role of $MgSO_4$ was reported for production of antimicrobials by *Streptomyces* spp. and optimized at 0.03% (Ochi *et al*., 1988) and 0.05% (Ogawa *et al*., 1998).

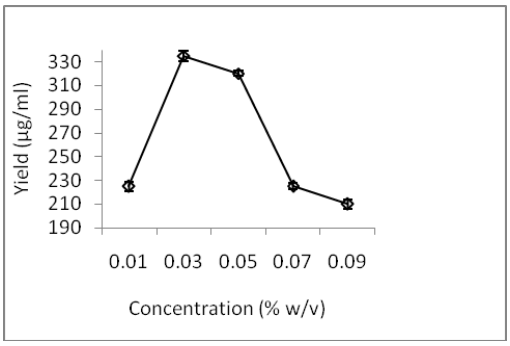

Figure 5. Effect of $MgSO_4$ on the production of DBP by *Streptomyces albidoflavus* MTCC 3662

3.6. Effect of Trace Element

Solubility and ionization often regarded as important factors for influencing the yield of secondary metabolites. Martin and McDaniel (1977) suggested that the metal ions probably activate the enzymes involved in synthetic steps of secondary metabolism. The salts of manganese, iron and zinc were found to play critical role in secondary metabolism (Weinberg, 1970). While studying the effect

of trace salts with *Streptomyces albidoflavus* 321.2 medium was prepared out using triple distilled water and tap water. The results showed that Mn^{++} (as $MnSO_4$, 0.001g/l) alone could enhance yield. But the yield was further enhanced when all the trace salts were used together (Table 3). Haque *et al*. (1995) observed the importance of manganese and zinc for the production of antimicrobial compound from *Streptomyces.*

Table 3. Effect of trace elements for the production of DBP by *Streptomyces albidoflavus* MTCC 3662

Trace salts	Concentration (%)	Yield (µg/ml)
Fe ($FeSO_4$)	0.01	265±3.54
	0.001[a]	290±2.74
	0.0001	250±2.24
Cu ($CuSO_4$)	0.001	135±4.48
	0.0001	175±4.48
	0.00001[b]	225±3.16
Mn ($MnSO_4$)	0.01	240±5.24
	0.001[c]	400±5.70
	0.0001	265±3.87
Zn ($ZnSO_4$)	0.001	290±4.48
	0.0001[d]	320±4.18
	0.00001	290±3.16
Tap water	--	265±4.18
a+b+c+d	--	450±6.12
Control (as recommended in basal medium)	--	335±5.24

3.7. Effect of Supplement

Supplementation of synthetic medium with natural compounds increases nutrient support viz; vitamins that commonly play notable role in cell metabolism. While checking the effect of supplementation, the yield was enhanced only with yeast extract (0.001%) and in combinations (Table 4). The role of yeast extract for the production of antimicrobial compound from *Streptomyces* was found both stimulatory (Haque *et al*., 1995) and inhibitory (Raytapadar and Paul, 2001).

Table 4. Effect of supplement for the production of DBP by *Streptomyces albidoflavus* MTCC 3662

Supplement	Concentration (%)	Yield (µg/ml)
Casein hydrolysate	0.1	240± 2.74
	0.01	290± 2.24
	0.001[a]	450± 4.18
	0.0001	450±5.24
Jaggary	0.1	265± 3.87
	0.01	335±4.18
	0.001[b]	450±4.48
	0.0001	450±5.24
Yeast extract	0.1	225±3.16
	0.01	240±4.18
	0 .001[c]	500±6.32
	0.0001	500±6.12
Control (without supplement)	_	450±5.70
a+b+c+d	-	550±6.32

3.8. Effect of Surfactant

With the use of surfactants, yield enhancement was observed only at 0.2 µg/ml of Tween-80 (Table 5). Surface acting agents are known to increase the membrane permeability and to influence in flow and out flow for biosynthesis (Abbott and Gledhill, 1971). Medium supplemented with fatty acids and detergents were found to increase yield of antimicrobial compound by *Streptomyces* (Mouslim *et al*., 1997).

Table 5. Effect of surfactants for the production of DBP by *Streptomyces albidoflavus* MTCC 3662

Surfectants	Concentration (µg/ml)	Yield (µg/ml)
Teepol	0.02	550±6.32
	0.2	500±4.48
	2	90±2.24
SDS	0.02	350 ±3.87
	0.2	225±4.18
	2	100±3.16
EDTA	0.02	500 ±6.12
	0.2	350±3.16
	2	135±2.74
Tween-80	0.02	550±5.24
	0.2	600±6.32
	2	225±3.16
Control (without surfectant)	-	550±5.7

4. Conclusions

The synthesis of secondary metabolite in excess is controlled by the genetic makeup and its fullest expression is possible only by the identification of limiting conditions. However, at least part of what can be substantiated by the above experimental results from using *Streptomyces albidoflavus* MTCC 3662 as producer, and the yield is increased to 2.4 fold, at its wild state of the producer.

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