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Production of 1-Deoxynojirimycin by *Streptomyces* sp. SID9135

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To increase the high production of 1-deoxynojirimycin (DNJ) from *Streptomyces* sp. SID9135, the effect of various carbon sources, nitrogen sources, cationic metal ions, the initial pH of the medium, and agitation speed were investigated. The most effective carbon and nitrogen sources were found to be lactose 2.5% (w/v) and soybean meal 2.0% (w/v), respectively. None of the cationic metal ions examined had any detectable stimulating effect on DNJ production except Fe^{+2} ion. The initial optimum pH for DNJ production ranged from 6~8 and agitation speed was most effective at 400 rpm. In the jar fermentor experiments under optimal culture conditions, the accumulation of DNJ reached about 640 μg/ml after 5 days of cultivation and the level remained the same for a further two days.

DNJ has been shown to inhibit intestinal α-glucosidases both *in vitro* and *in vivo*. It was first obtained from nojirimycin by catalytic hydrogenation at the platinum contact or by reduction with $NaBH_4$ (7). In the course of screening for inhibitors of the mammalian intestinal α-glucosidase, DNJ was isolated from the root bark of a *Morus* species (20) and was also obtained from the culture filtrates of a *Bacillus* species and *a Streptomyces* species (14). DNJ and its derivatives have great therapeutic potential due to their low cytotoxicity, at relatively high concentrations. There have been many reports on their therapeutic uses for diabetes (8, 21), viral infections (17, 18), tumor metastasis (19) and glycogenolytic disorders (1, 2). In addition, DNJ was used in the enzymatic synthesis of high purity maltooligosaccharides (11, 12), in the synthesis of 4-O-α-D-glucopyranosyl moranoline (4, 6), as an enzyme stabilizer (13), as an oligosaccaride processing modifier (10), and as an inhibitor of glucotransferase in oral *Streptococci* (15). In expectation of the various uses of DNJ and its derivatives, we investigated the optimal cultural conditions for the production of DNJ by *Streptomyces* sp. SID9135.

MATERIALS AND METHODS

Microorganism and Culture Conditions

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Streptomyces sp. SID9135 isolated in our laboratory from soil was used for this work (16). The medium used for maintenance of the microorganism consisted of 0.4% glucose, 0.4% yeast extract, 1% malt extract and 2% agar (pH 7.3). The strain SID9135 was cultured in a 250-ml baffled flask containing 50 ml of the basal medium (0.4% yeast extract, 0.2% $CaCO_3$ pH 7.0) supplemented with carbon and nitrogen sources at 29°C for 5 days on a rotary shaker at 200 rpm.

Fermentation in a Jar Fermentor

Tryptic Soy broth (Difco) was used as the seed medium which had been inoculated with the cells of SID9135 grown on yeast-malt agar slants and cultured on a rotary shaker at 29°C for 2 days. This seed culture (150 ml) was transferred to a 6.6-liter jar fermentor (New Brunswick Scientific Co. Ltd., BIOFLO IIc) containing 3.0 liters of the culture medium. The composition of the culture medium consisted of 2.5% lactose, 2.0% soybean meal, 0.4% yeast extract, 0.2% $CaCO_3$ and 0.005% $FeSO_4 \cdot 7H_2O$ (pH 7.0). Fermentation was carried out at 29°C with an aeration rate of 1 vvm and different agitation speeds. The DO and pH of the culture broth were monitored with a DO electrode (Ingold Electrodes, Inc) and a pH electrode (Ingold Electrodes, Inc), respectively.

Enzymatic Determination of DNJ

The inhibitory effect of DNJ on porcine intestinal maltase was assayed according to the method described by Dahlqvist (3). DNJ in the culture broth of the flask was quantified by comparing the IC_{50} of the diluted broth against porcine intestinal maltase with that of the stan-

dard solution of DNJ (Sigma). The IC_{50} value of DNJ against the maltase was usally 0.05 μg/ml.

Determination of DNJ by High Performance Liquid Chromatography (HPLC)

DNJ produced in the jar fermentor was measured by HPLC. Culture broth (20 ml) was centrifuged at 10,000 rpm for 10 min and the supernatant obtained was treated with the Diaion HP-20 resin, and then applied on a Dowex 1×2-100 (OH^-) column and eluted with water. The eluate was adsorbed on Dowex 50W×4-100 (H^+) which had been washed with water, and then was eluted with 0.5 N NH_4OH. The active fractions containing DNJ were collected and evaporated under reduced pressure and the powder obtained by evaporation was dissolved in water and assayed by HPLC (column: NH2, ϕ: 4.6× 250 mm, YMC pack; mobile phase: 85% acetonitrile; flow rate: 1 ml/min; detection: UV 195 nm). The content of DNJ was calculated on the basis of the peak area of the standard solution (Fig. 1).

Determination of Viable Count

Fermentation broth (1 ml) was successively diluted with sterile saline. Viable colonies were counted after incubation for 6 days at 29°C on Tryptic Soy agar (Difco).

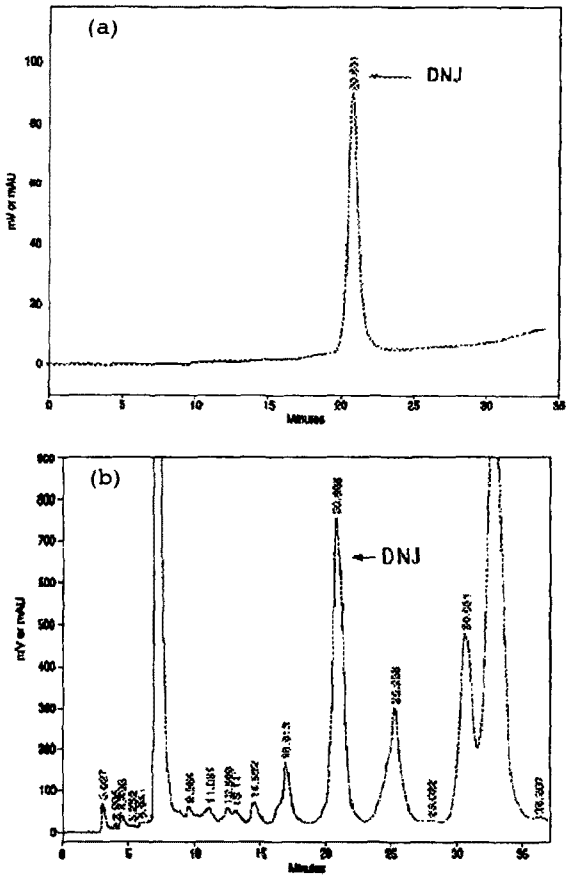

Fig. 1. HPLC analysis of DNJ.
panel a: standard DNJ, panel b: DNJ of partial purified culture broth.

RESULTS AND DISCUSSION

Effects of Carbon Sources

Production of DNJ by the strain SID9135 was examined in the presence of various carbon sources. Each of the carbon sources tested was added to the basal medium at the concentration of 10 g/l. The amount of DNJ produced was determined by using the enzymatic assay method. As shown in Table 1, the highest DNJ production was observed with lactose, whereas fairly low levels of DNJ production were detected in the culture media supplemented with other carbon sources such as cellobiose, melibiose, melizitose, rhamnose, raffinose, xylose, mannitol and sorbitol. Next, to investigate the effect of lactose concentrations on DNJ production, lactose was added to the basal medium at various concentrations as indicated in Fig. 2. DNJ production was found to be the highest at a lactose concentration of 25 g/l and the amount of DNJ produced was 275 μg/ml.

Effects of Nitrogen Sources

Production of DNJ was measured in the presence of various nitrogen sources at the concentration of 10 g/l in the basal medium supplemented with 2% lactose as a sole carbon source. As shown in Table 2, organic nitrogen sources were generally more effective than inorganic nitrogen sources for the production of DNJ. Futhermore, $NH_4H_2PO_4$ appeared to greatly inhibit DNJ production. The highest DNJ production levels were

Table 1. Effects of various carbon sources on DNJ production.

Carbon sources	Final pH	DNJ (μg/ml)
Arabinose	8.1	45
Cellobiose	8.0	27
Fructose	8.0	60
Galactose	8.2	53
Glucose	8.1	158
Lactose	8.1	164
Maltose	8.1	93
Mannose	8.1	43
Melezitose	8.4	20
Raffinose	8.4	24
Rhamnose	8.4	25
Sucross	8.4	58
Xylose	8.1	10
Mannitol	8.4	23
Sorbitol	8.4	27
Corn starch	8.4	156
Potato starch	8.3	150
Soluble starch	8.1	144

Streptomyces sp. SID9135 was cultured for 5 days in the basal medium supplemented with 1% carbon sources.

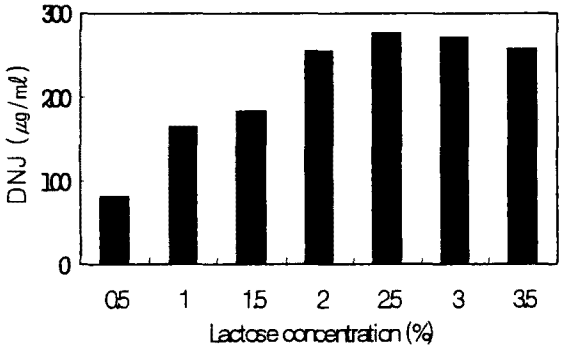

Fig. 2. Effects of lactose concentration on DNJ production.
Streptomyces sp. SID9135 was cultured for 5 days in the basal medium supplemented with several lactose concentration.

observed with soybean meal. To examine the effect of soybean meal concentrations on DNJ production, soybean meal was added to the culture medium (the basal medium supplemented with 2.5% lactose) at various concentrations as shown in Fig. 3. At the concentration of 20 g/l, the amount of DNJ produced was 784 μg/ml. However, at concentrations higher than 20 g/l, DNJ production was markedly reduced (Fig. 3). The optimum concentration of soybean meal for DNJ production was thus determined to be 20 g/l.

Effects of Metal Ions

The effects of metal ions on the production of DNJ were also investigated. The metal ions listed in Table 3 were added at a concentration of 0.05 g/l to the medium consisting of 2.5% lactose, 2% soybean meal, 0.4% yeast extract and 0.2% $CaCO_3$. DNJ production was slightly increased to 814 μg/ml only by the presence of Fe^{+2} ion.

Table 2. Effects of various nitrogen sources on the DNJ production.

Nitrogen sources	Final pH	DNJ (μg/ml)
None	8.1	275
Beef extract	8.4	402
Malt extract	8.2	543
Oat meal	7.4	536
Peptone	8.1	517
Soybean meal	8.2	631
Soytone	8.1	529
Tryptone	8.1	501
Tryptose	8.0	517
NH_4Cl	7.9	354
$(NH_4)_2SO_4$	8.1	305
NH_4NO_3	7.8	374
$NH_4H_2PO_3$	6.8	63

Streptomyces sp. SID9135 was cultured for 5 days in the optimal lactose medium (2.5% lactose, 0.4% yeast extract, 0.2% $CaCO_3$) supplemented with 1% of nitrogen sources.

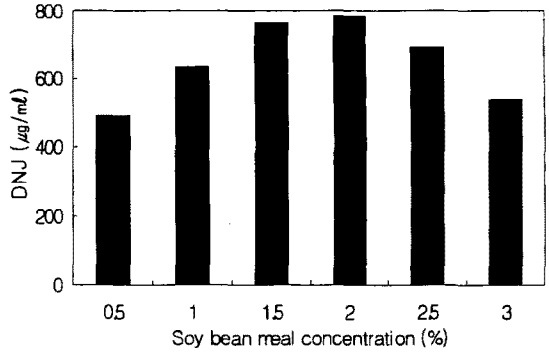

Fig. 3. Effect of soybean meal concentration on DNJ production.
Streptomyces sp. SID9135 was cultured for 5 days in the optimal lactose medium (5% lactose, 0.4% yeast extract, 0.2% $CaCO_3$) supplemented with several soybean meal concentrations.

Table 3. Effects of various metal ions on the DNJ production.

Metal ion sources	Final pH	DNJ (μg/ml)
None	8.1	784
$MnSO_4 \cdot 5H_2O$	8.8	791
$FeSO_4 \cdot 7H_2O$	8.2	814
$CoSO_4 \cdot 7H_2O$	8.7	777
$CuSO_4 \cdot 5H_2O$	8.4	785
$ZnSO_4 \cdot 7H_2O$	8.4	780
$LiSO_4 \cdot H_2O$	8.4	780
$MgSO_4 \cdot 7H_2O$	8.7	792

Streptomyces sp. SID9135 was cultured for 5 days in the optimal soybean meal medium (2.5% lactose, 2% soybean meal, 0.4% yeast extract, 0.2% $CaCO_3$) supplemented with 0.005% of metal ion sources.

Effects of Culture pH and Temperature

As shown in Table 4, DNJ production was not changed when the initial pH of the culture medium (2.5% lactose, 2% soybean meal, 0.4% yeast extract, 0.2% $CaCO_3$ and 0.005% $FeSO_4 \cdot 7H_2O$) was adjusted to be between pH 6 and 8. The cells grew poorly at the initial pHs of 5 and 9 (data not shown) and also produced significantly low levels of DNJ. The optimum culture temperature was determined to be 29°C. At temperatures higher than 35°C, DNJ production as well as cell growth was markedly inhibited (data not shown). On the basis of the

Table 4. Effects of initial pH on the DNJ production.

Initial pH	Final pH	DNJ (μg/ml)
5	8.0	544
6	8.2	802
7	8.2	814
8	8.3	814
9	8.5	507

Streptomyces sp. SID9135 was cultured for 5 days in the optimal medium (2.5% lactose, 2% soybean meal, 0.4% yeast extract, 0.2% $CaCO_3$, 0.005% $FeSO_4 \cdot 7H_2O$) with various initial pH.

Table 5. Effects of agitation on DNJ production.

Culture time (days)	DNJ (μg/ml)			
	Agitation (rpm)			
	200	300	400	500
1	–	–	–	–
2	–	–	80	80
3	–	–	213	213
4	–	96	360	360
5	85	128	640	640
6	160	160	640	640
7	160	160	640	584

Fermentation was carried out on 6.6-liter jar fermentor containing 3.0-liter optimal medium (pH 7.0) under aeration (1 vvm) with various agitation. –, not detected.

results described above, the optimum medium for the production of DNJ was established to be 2.5% lactose, 2% soybean meal, 0.4% yeast extract, 0.2% $CaCO_3$ and 0.005% $FeSO_4 \cdot 7H_2O$. This medium was employed in the following jar fermentor experiments.

Effects of Agitation Speed

The strain SID9135 was cultured in a 6.6-liter jar fermentor as described in Materials and Methods. As shown in Table 5, high DNJ production was achived when the agitation speed was increased to 500 rpm. At 400 rpm, the accumulation of DNJ reached about 640 μg/ml after 5 days of cultivation. The DNJ yeild at 500 rpm was similar to that at 400 rpm, but higher agitation speeds were found to injure mycellium.

Time Course of DNJ Production

During jar fermentation at an agitation speed of 400 rpm, DNJ production was monitored every 24 h (Fig. 4).

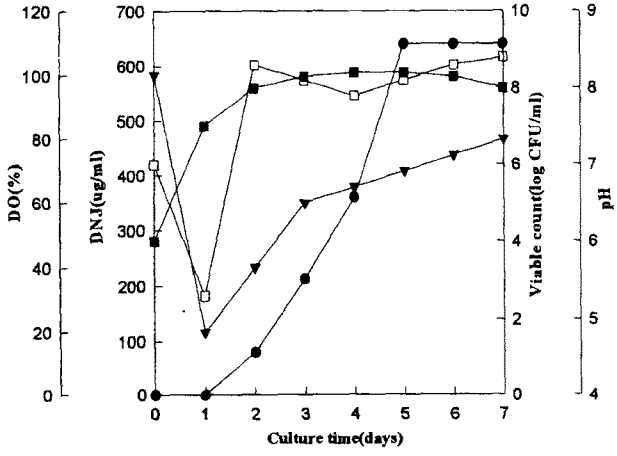

Fig. 4. Time course of DNJ production by *Streptomyces* sp. SID9135.

Cultivation was carried out as described in materials and methods at 29°C for 7 days in optimal medium of a 6.6-liter jar fermentor. ●, DNJ; □, pH; ▼, DO; ■, Viable count.

With the optimum medium, accumulation of DNJ in the culture fluid reached about 640 μg/ml after 5 days of cultivation and remained at the same level for a further 2 days of fermentation. This indicates that DNJ is a stable substance under these culture conditions. The culture time for the maximum DNJ production was estimated to be about 1.8 times faster than that reported by Kojima *et al.* (9). The pH of the culture medium showed a sharp drop and subsequently a rapid rise during the first two days of fermentation but thereafter it stayed at a nearly constant level of pH 8. This pattern of pH change was quite different from that of the fermentation for *Streptomyces lavendulae* (5). The DO change during fermentation showed a rapid decrease at the initial culture time and thereafter a gradual increase to 80%. Interestingly, the initial rapid decrease of pH and DO was observed to occur in the same time course. The strain SID9135 appeared to be viable until the end of cultivation without any decomposition of DNJ already produced. From these results, we could establish relatively simple medium and culture conditions for the efficient production of DNJ by the strain SID9135 in a short culture period.

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