The Journal of Natural Science,
Pai Chai University, Korea
Volume 8: 33~39, 1995

Production of High Viscous Hyaluronic Acid Complex from *Klebsiella* sp. L-10 NTG 50

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Klebsiella sp. L-10의 NTG 50 변이주에 의한 고점성 히아루론산 복합체의 생산

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Klebsiella sp. L-10 was treated with physical and chemical mutagens, and one of the NTG mutant which increased hyaluronic acid complex yield 2.5 folds was selected. The yield of hyaluronic acid complex from *Klebsiella* sp. L-10 NTG 50 mutant reached maximum level in the YTD medium containing 0.1% yeast extract, 3% Bacto-tryptone, 3% dextrose, each 30mM of K_2HPO_4 and KH_2PO_4 (pH 6.0~6.5) with shaking culture at 37℃ for 24 hrs, and 2900㎎ of hyaluronic acid complex per litre of culture was produced under the above condition.

Klebsiella sp. L-10을 N-methyl-N′-nitro-N″-nitrosoguanidine 과 ethylmethane sulfonate 및 자외선 등으로 돌연변이시켜 히아루론산 복합체의 수율이 친주보다 약 2.5배 증대된 NTG 50 변이주를 얻었다. NTG 50변이주를 이용한 히아루론산 복합체의 최적 생산배지 조성은 효모 추출물 0.1%, 트립톤 3%, 포도당 3%, K_2HPO_4와 KH_2PO_4 각각 30mM 이었고 배양조건은 배지의 초기 pH 6.0~6.5, 배양온도 37℃, 24시간의 진탕배양이었으며 이때 배양액 리터당 2900㎎의 히아루론산 복합체가 생산되었다.

Key words : High Viscous, Hyaluronic Acid Complex, *Klebsiella* sp. L-10 NTG 50 Mutant.

I. Introduction

Hyaluronic acid is composed of repeating subunits of glucuronic acid β-1,4 bonds and it is a naturally distributed in connective tissue and skin of mammalians, eye, articular sac, cockscomb, placenta wall of main antesy, and capsule of *Streptococcus* Hemolytics A and C group.[1)]

Beacause it lubricates joints and absorbs the external shock and also have high viscosity and water retention capacity in skin, hyaluronic acid is widely used in medical industry (such as drug delivery, orthopedics, ophthalmic surgery, cardiovascular aids, wound healing etc.) and cosmetic industry.[1,2)]

Extraction, purification and utilization of hyaluronic acid from connective tissue or cockcomb and biosynthesis, seqencing of hyaluronic acid synthase, its structure and function by using *Streptococcus* sp. A and C group have been

extensively studied.[3-19] However, there have been inverstigated few on production of hyaluronic acid or strain improvement for increasing of productivity from other microbes except *Streptococcus zooepidemicus*,[5,7,9] *Streptococcus equi*[5] and its chemical mutants,[6] *Streptococcus pyogenes*.[4]

In previous paper,[20] we reported on isolation and identification of high viscous hyaluronic acid complex-producing *Klebsiella* sp. L-10 and optimization of production conditions, and this paper discribes on chemical mutagenesis of *Klebsiella* sp. L-10 and optimization of production condition in order to increase the yield of hyaluronic acid complex using mutant of *Klebsiella* sp. L-10.

II. Materials and Methods

1. Strain and cultivation

The strain used in this work was *Klebsiella* sp. L-10 screened from soil previously in our laboratory.[20] The cultivation was carried out in YTD medium containing 0.1% yeast ext., 3.0% Bacto-tryptone, 5.0% dextrose, each 10mM K_2HPO_4 and KH_2PO_4 (pH 6.5) at 37℃ with 150 rpm in 250㎖ shake flask.

2. Mutagenesis and selection of mutant.

Klebsiella sp. L-10 was cultured in YPD midium at 37℃ for 1 hour with shaking, and 10㎎/㎖ of NTG and 5ng/㎖~5㎎/㎖ of EMS dissolved in 10mM phosphate buffer (pH 8.0) was added, respectively and then further incubated for 30 min. The treated cells were harvested, and plated onto YPD, and viscous colonies were selected first and the other colonies screened randomly. The selected colonies were recultivated in YPD media for 72 hrs. The cell that can produce hyaluronic acid complex potently were selected as mutants. Meanwhile, the cell grown to exponential phase in YPD media were harvested, and suspended in 1㎖ of 10mM phosphate buffer (pH 8.0), and then irradiated UV at 10cm distance for 30 sec or 60 sec. The treated cells were plated onto YPD and mutants were selected as decribed in chemical mutagenesis.

3. Measurement of cell growth and hyaluronic acid complex

Cell concentration and hyaluronic acid complex were determined as the method described in previous paper.[20]

III. Results and Discussion

1. Selection of mutant

Klebsiella sp. L-10 was treated with chemical and physical mutagens and compared with their yield of hyaluronic acid complex. As shown in Table 1, one of NTG mutant produced 660㎎ of hyaluronic acid complex per litre of cultures, 2.5 fold higher than that of the parent strain(285㎎/litre of cultures), however the amounts of hyaluronic acid complex produced from the other mutants were not increased compared to the parent strain. Therefore, the NTG mutant was selected finally as mutant used for the follow-up work and named as NTG 50.

2. Production condition of hyaluronic acid complex

1)Effect of carbon source

To investigate the effect of carbon sources on production of hyaluronic acid complex from *Klebsiella* sp. L-10 NTG 50 mutant, 3% or 5% of carbon sources were added in YPD medium containig yeast extract 0.1%, peptone 2.0%, K_2HPO_4 0.1% and KH_2PO_4 0.3% and incubated at 37℃ for 48 hrs.

As shown in Table 2, glucose served as the most effective carbon source, similar to that of parent strain, *Klebsiella* sp. L-10 and the mutant was not also utilized organic salts such as sodium citrate, oxalate and lactic acid.

Table 1. Production of hyaluronic acid complex from *Klebsiella* sp. L-10 and mutants.

Strain	Growth (Dry cell weight, g/ℓ)	HA complex (mg/ℓ)	Yield coefficent (mg HA complex/g cell)
Klebsiella sp. L-10	0.84	285	339
NTG-50M	0.86	660	773
EMS-M	0.79	55	69
UV-M	0.81	78	96
Klebsiella pneumonia	0.62	48	77
Klebsiella aerogens	0.71	31	43

Table 2. Effect of carbon source on the production of hyaluronic acid complex from *Klebsiella* sp. L-10 NTG 50 mutant

Carbon source	Growth (g/ℓ)	HA complex (mg/ℓ)	Yield coefficent (mg HA complex/g cell)
Glucose	0.95	940	995
Galactose	0.07	-	-
Sucrose	1.19	546	459
Maltose	0.83	426	513
Soluble starch	1.10	-	-
Ribose	0.41	364	885
Glycerol	0.71	98	138
Ethanol	0.71	91	128
Sodium acetate	0.42	320	762
Sodium citrate	-	-	-
Sodium oxalate	-	-	-
Lactic acid	-	-	-
Control	0.65	-	-

The effect of glucose concentration on the production of hyaluronic acid complex from the NTG 50 mutant is shown in Fig. 1.

When glucose was added to 3.0%, the hyaluronic acid complex was produced 1,100㎎ per litre of cultures, wherease it was decreased in high concentration than 3.0% of glucose. The glucose effect was quite different that of parent strain and we guess it is because synthesis of hyaluronic acid complex was repressed by enlargement of cellular diffusion caused by addition of high concentration of glucose.

Chang reported hyaluronic acid was produced 0.105㎎ per ㎖ cultures in *Streptococcus zooepidemicus* by addition of 3% glucose in basal medium and 5g per litre of cultures in *Streptococcus equi* PCI 1988 mutant by addition of 8% glucose.[5,21)]

2) Effect of nitrogen source

Effect of nitrogen source on the production of hyaluronic acid complex from NTG 50 mutant was investigated by adding single or mixture in YPD medium containing 3% glucose, 0.1% K_2HPO_4 and 0.3% KH_2PO_4 and culturing at 37℃ for 48 hrs(Table 3).

Table 3. Effect of nitrogen sources on the production of hyaluronic acid complex from *Klebsiella* sp. L-10 NTG 50 mutant.

Nitrogen source (%)		Growth (g/ℓ)	HA complex (mg/ℓ)	Yield coefficent (mg HA complex/g cell)
Tryptone	1.0	1.26	135	107
Peptone	1.0	0.51	210	412
Casamino acid	0.5	0.17	63	370
Urea	0.5	0.05	-	-
Yeast ext.	0.2	0.77	408	538
Casein	0.2	0.31	106	342
Beef ext.	0.2	1.10	2	20
NH_4Cl	0.1	-	-	-
$(NH_4)_2SO_4$	0.1	-	-	-
Ammonium acetate	0.1	-	-	-
Ammonium citrate	0.1	-	-	-
NH_4NO_3	0.1	-	-	-
$(NH_4)_2\ H_3PO_4$	0.1	-	-	-
Ammonium oxalate	0.1	-	-	-
$NaNO_3$	0.2	-	-	-
KNO_3	0.2	-	-	-
Yeast ext. + Tryptone				
0.05%	1.0	0.63	75	119
	2.0	0.94	106	113
	3.0	0.98	61	62
0.10% + Tryptone				
	1.0	1.30	1,602	817
	2.0	1.26	1,230	976
	3.0	1.36	1,881	1,368
0.10% + Peptone				
	1.0	1.10	720	638
	2.0	1.16	336	290
	3.0	1.18	795	674
0.30% + Tryptone				
	1.0	1.30	1,740	638
	2.0	1.30	1,313	1,010
	3.0	1.38	1,881	1,363
0.30% + Peptone				
	1.0	1.22	475	389
	2.0	1.23	1,590	1,293
	3.0	1.20	637	530
Yeast ext. 0.1% + Tryptone 3.0% + Peptone 1.0%		1.20	1,638	1,353
Control		0.03	-	-

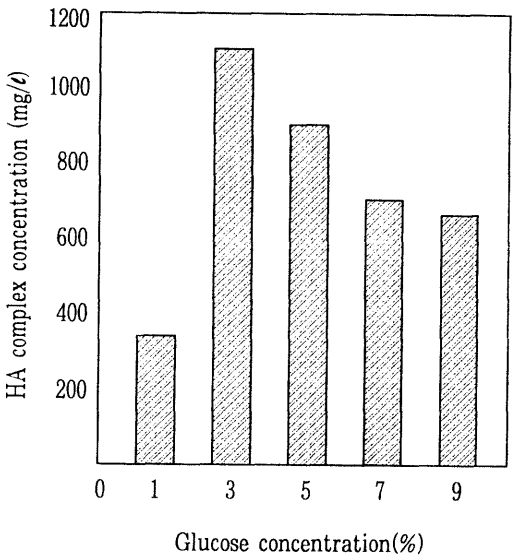

Fig. 1 Effect of concentration of glucose on the production of hyaluronic acid complex from *Klebsiella* sp. L-10 NTG 50 mutant.

The mutant were not utilized almost inorganic nitrogen sources and urea, while organic nitrogen sources such as yeast ext, tryptone, peptone and beef extract were very effective in the production of hyaluronic acid complex. Furthermore, when both mixture of 0.1% yeast ext. and 3% tryptone was added in YTD medium containing 3% glucose, 0.1% K_2HPO_4 and 0.3% KH_2PO_4, 1881mg of hyaluronic acid complex per litre of cultures was produced.

3) Effect of buffer solution, inorganic salts and minerals.

Hyaluronic acid complex was produced about 2300mg per litre of cultures from the NTG 50 mutant when each 30mM of K_2HPO_4 and KH_2PO_4 were added in YTD medium containing 3% glucose, 0.1% yeast ext. and 3% tryptone, but inorganic salts and minerals was not affect in the production of hyaluronic acid complex(data not shown).

4) Effect of pH and temperature

The effect of initial pH of medium on production of the hyaluronic acid complex was examined in YTD medium containing 3% glucose, 0.1% yeast ext, 3% tryptone, each 30mM K_2HPO_4 and KH_2PO_4 of various pH.

As shown in Fig 2, high yield (2700~2800mg /litre of cultures) was observed in cultures from medium of initial pH 6.0~6.5 and about 2100mg of hyaluronic acid complex per litre of culture was produced in pH 7.0.

Generally, it is known that if the cultivation is performed without pH control, pH of the cultures is decreased to 5.0, following growth and production of hyaluronic acid is ceased.

Optimal temperature for production of hyaluronic acid complex from NTG 50 mutant was 37℃ (Fig.3) and about 2300mg per litre of cultures was produced at 33℃.

From all data presented above, optimum production medium of hyaluronic acid complex form *Klebsiella* sp. L-10 NTG 50 mutant was composed of 3.0% glucose, 0.1% yeast extract, 3.0% Bacto-tryptone, each 30mM of K_2HPO_4 and KH_2PO_4, which was adjusted to pH 6.0~6.5 and the culture temperature was 37℃.

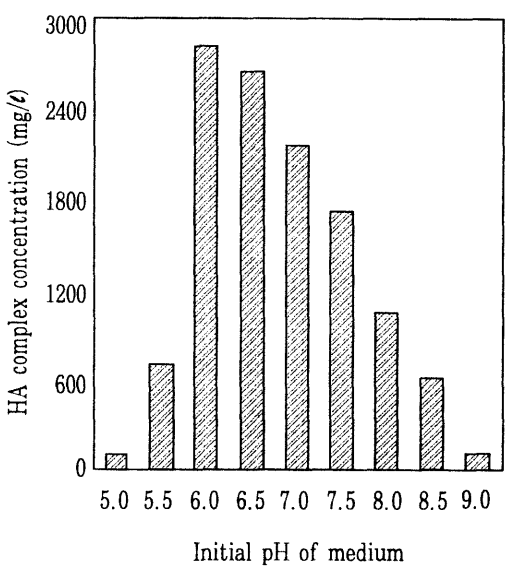

Fig. 2 Effect of initial pH of medium on the production of hyaluronic acid complex from *Klebsiella* sp. L-10 NTG 50 mutant.

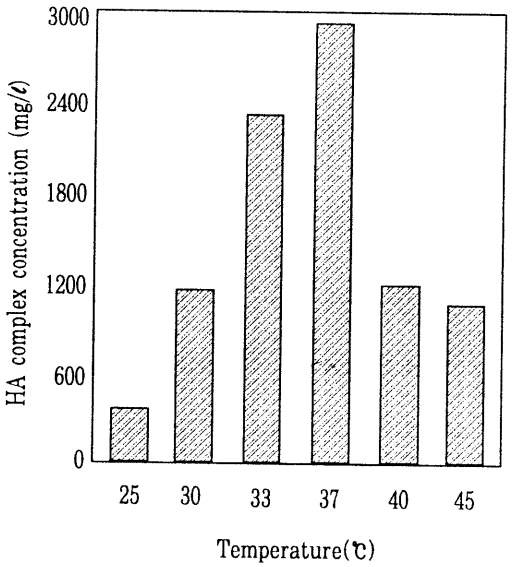

Fig. 3 Effect of temperature on the production hyaluronic acid complex from *Klebsiella* sp. L-10 NTG 50 mutant.

Aknowledgement

This study was supported by a research grant from Paichai University (93-11-07).

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