Biotechnological Production of High Specific Activity L-^{35}S-Cysteine and L-^{35}S-Methionine by Using a Diploid Yeast *Saccharomyces cerevisiae*

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SUMMARY

High specific activity L-^{35}S-cysteine and L-^{35}S-methionine were synthesised by using a wild type diploid strain of baker's yeast-*Saccharomyces cerevisiae*. Yeast cells were grown in a sulphur depleted synthetic medium in which $Na_2{}^{35}SO_4$ (50 mCi/ml) was supplemented as the sole sulphur source. The level of incorporation was 60% on an average. The protein hydrolysate of the cultured cells was subjected to paper and column chromatographic separations to get the individual L-^{35}S-aminoacids. The radiochemical yields of cysteine and methionine were 6-7% and 18-20% respectively. The radiochemical purity of the products was >95%. The highest specific activity for the products obtained by employing this method was 1100 Ci/mmole from the starting material, $Na_2{}^{35}SO_4$, with a specific activity of 1350 Ci/mmole.

Key words: ^{35}S, L-^{35}S-cysteine biosynthesis, L-^{35}S-methionine biosynthesis, *Saccharomyces cerevisiae*

INTRODUCTION

^{35}S is a pure soft β emitting radioisotope ($t_{1/2}$,87.4 d; E_{max}, 167 keV) which is ideal for tracer studies. Its increasing application in the current molecular biology research (particularly in the form of L-^{35}S-methionine and ^{35}S-nucleotides)[4,7] necessitates its uninterrupted production and

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supply. Several attempts were made earlier to obtain ^{35}S-labelled aminoacids through biosynthesis[3,5,6,10,11]. In order to obtain the highest specific activity in ^{35}S-labelled biomolecules, using microorganisms, it is necessary to limit the sulphur contribution from naturally existing ^{32}S components of the inoculum and the nutrient medium to a minimum proportion so as to avoid the dilution of specific activity during the microbial growth process. The addition of ^{35}S-sulphate in growth medium as the sole sulphur source often leads to inhibition of its incorporation due to inability of the system to proliferate in the radiation field as a result of the radiation dose received. Thus, the radioresistance of a given organism is considered to be the major determining factor in achieving the end product with high specific activity. Generally, *Saccharomyces cerevisiae* (baker's yeast) is known to be far more radioresistant as compared to the commonly used *Escherichia coli* strains. By using yeast, Albahari and Skakun-Todorovic[1], obtained L-^{35}S-aminoacids with a specific activity of 1-10 Ci/mmole only, whereas this paper presents a modified method to get L-^{35}S-cysteine and L-^{35}S-methionine of specific activity as high as 1100 Ci/mmole.

MATERIALS AND METHODS

For this study, a diploid strain of yeast, *Saccharomyces cerevisiae*, obtained from Yeast Genetic Stock Centre, Berkeley, USA was used[8,9]. The ^{35}S source of specific activity 1350 Ci/mmole was obtained from M/s. ICN Radiochemicals,USA and that of specific activity 525 Ci/mmole, from RC & RSO/BRIT, Bombay, India. The specific activity of ^{35}S sulphate was found out by a modified procedure employing the chloranilate method[2]. In order to conserve the radioactive source for the main production, only 10-20 mCi of ^{35}S-sulphate was used for the specific activity

assay. The radioactivity was assayed by liquid scintillation counting.

The composition of the sulphur omission medium per litre was as follows: glucose 20g, $(NH_4)_2HPO_4$ 2g, citric acid (trisodium) 1g, asparagine monohydrate 2.5g, KH_2PO_4 875mg, K_2HPO_4 125mg, NaCl 100mg, $MgCl_2.6H_2O$ 100mg, zinc acetate 0.4mg, $FeCl_3$ 0.15mg, $CuCl_2$ 0.025mg, biotin 0.01mg, pantothenic acid (calcium salt) 0.5mg, thiamine.HCl 0.6mg, pyridoxine 1mg and inositol 10mg. The pH of the medium was 7.0. Sterilization was done by Millipore filtration. The medium was subjected to sulphur depletion prior to the addition of radioactive sulphate. Yeast cells were added to the medium at a final titre of 10^6/ml and incubated aerobically at 30 °C. The sulphur depletion was revealed by the cessation of growth after 24 hr. The microbial population during this process increased from 10^6 to about $5X10^6$ cells/ml. A portion of this culture was filtered through a Millipore filter of pore size 0.45 μm and the filtrate/cell pellet was used to adjust the cell titre of the culture to the desired level (2.5-$5x10^6$ cells/ml). The culture was further incubated for 8 hr after the addition of ^{35}S-sodium sulphate at radioactive concentrations ranging from 20-100 mCi/ml. The protein cells were hydrolysed by heating *in vacuo* with 6N HCl at 110 °C for 18 hr. The per cent incorporation was determined by finding out the radioactivity in the protein hydrolysate. L-^{35}S-aminoacids were identified by paper chromatography (n-butanol: acetic acid: water, 4:1:5) coupled with autoradiography. Aqueous β-mercaptoethanol (0.1%) was used as an antioxidant and preservative for both the aminoacids. Methionine sulphoxide and cystine formed were reduced to methionine and cysteine respectively, by refluxing separately with 20% aqueous β-mercaptoethanol solution at 60 °C under nitrogen atmosphere. Purification of the L-^{35}S-aminoacids was carried out by using ion

exchange resin columns taking utmost care to minimise oxidation. The specific activity of the L-^{35}S-aminoacids was determined by MP 3000 (LDC, Milton Roy) aminoacid analyzer, using post-column *o*-phthalaldehyde derivatization method.

RESULTS AND DISCUSSIONS

Biological depletion of residual non-radioactive sulphur from synthetic medium offers an attractive and effective method to achieve high specific activity end products. Since radiation damage is considered to be the limiting factor in achieving high incorporation, it is necessary to keep the concentration of ^{35}S to the lowest possible limit. At the same time, enough ^{35}S-sulphate has to be provided for the cells to multiply by a few generations in order to achieve high specific activity end product. Experiments performed with non-radioactive sodium sulphate revealed that the concentration of 2 mg/l of medium supported 5x10^{6} cells/ml to undergo divisions by two generations. When $Na_2{}^{35}SO_4$ was supplied at equivalent concentration i.e., 20 mCi/ml, the incorporation was >90% in 8 hr. But the specific activity of the resultant L-^{35}S-aminoacids was found to be reduced. However, high specific activity end products were obtained when the radioactive concentration was increased to 50 mCi/ml (Table 1). The kinetics of ^{35}S incorporation into protein was influenced by the amount of inoculum and ^{35}S activity. The radiochemical yields of cysteine and methionine were 5-7% and 18-20% respectively. The radiochemical purity of the end products obtained was >95%.

By reducing the starting cell concentrations in the cultures, it is possible to obtain L-^{35}S-cysteine and L-^{35}S-methionine of very high specific activity, but with reduced radiochemical yield. This results when the cells cease to multiply because of radiation damage prior to incorporation of all ^{35}S atoms into the protein chain. On the other hand, a large inoculum will

incorporate all ^{35}S, yielding cysteine and methionine of low specific activity by introducing large amounts of unlabelled aminoacids from the inoculum itself. Hence, maintaining optimum inoculum size and ^{35}S concentration is important for achieving moderate yield with acceptable specific activity.

Radionuclides can bring about biological damage by irradiation, transmutation and recoil processes. Our recent studies[1,2] (proceedings of 10th NSRP,1993, Madras, India. pp:257-258) have demonstrated the dose dependent DNA damage and cell inactivation in yeast when exposed to ^{35}S-β particles. The radiotoxicity of ^{35}S has importance in the incorporation of ^{35}S into proteins. If a system is radiosensitive, high levels of incorporation may be impeded by the radiation induced damage to the cellular DNA. On the other hand, a very radioresistant organism with a short generation time should prove useful for such a purpose. We have shown earlier that the diploid yeast *Saccharomyces cerevisiae* requires 140 Gy of ^{35}S- β radiation as the mean lethal dose. With the generation time of about 2.5 hr in synthetic medium, the organism obviously can undergo few divisions in ^{35}S milieu at the concentration of 50 mCi/ml, before being inactivated fully. When considering 1100 Ci/mmole of L-^{35}S-cysteine and L-^{35}S-methionine obtained from the starting specific activity of 1350 Ci/mmole of $Na_2{}^{35}SO_4$, the net dilution in specific activity was only 18.5%. It is our observation that diploid yeast, *Saccharomyces cerevisae*, can be exploited for the production of high specific activity ^{35}S- labelled biomolecules.

Table 1. Biosynthesis of ^{35}S-labelled L-cysteine and L-methionine by using diploid yeast, *Saccharomyces cerevisiae*

Expt. No.	Specific activity of $Na_2{}^{35}SO_4$ (Ci/mmole)	Radioactive concentration (mCi/ml)	% incorporation	Cells per ml (inoculum)	Specific activity of end products (Ci/mmole)
1.	525	20	>90	10^7	150
2.	480	40	63	$5x10^6$	350
3.	440	50	60	$5x10^6$	400
4.	420	50	60	$5x10^6$	ND
5.	420	100	30	$5x10^6$	ND
6.	400	50	32	$2.5x10^6$	ND
7.	400	50	65	$5x10^6$	360
8.	1350	50	60	$5x10^6$	1100
9*.	1350	20	90	$5x10^6$	1000
10.	1350	50	40	$4x10^6$	1100
11*.	1350	30	67	$5x10^6$	1050

ND = Not Determined

*Unincorporated ^{35}S from experiments 8 and 10 was reused in experiments 9 and 11 respectively by adding fresh inoculum.

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