# Production of Petite Mutants of *Saccharomyces cerevisiae* by Patulin

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Patulin, an antibiotic produced by several species of *Aspergillus* and *Penicillium*, is carcinogenic in rats. The activity of this compound was investigated in an extrachromosomal mutation system of a haploid strain of *Saccharomyces cerevisiae*. The yeast cells were exposed to several concentrations of patulin for varying periods of time and the colonies produced by the survivors of such treatment were tested for their ability to reduce 2,3,5-triphenyltetrazolium chloride to its red formazan. In this manner, mutation from wild type to petite can be observed; petite colonies cannot reduce the test compound and therefore remain white. Yeast cells in exponential phase of growth were more sensitive to the toxic and mutagenic effects of patulin than were cells in stationary phase of growth. Exposure to 50 $\mu g.$ per ml. of patulin for 3 hours during exponential phase resulted in a mutation frequency of 60 to 80% with 1% survival whereas similar treatment of stationary phase cells resulted in a mutation frequency of approximately 10% with 80% survival. Toxicity, as measured by loss of viability, was apparent at the same concentration range of patulin which produced mutation.

The effects of radiation and radioactive substances on genetic material have been intensively studied; however, detection of these effects and measures for their control have been a problem of primary concern to geneticists for many years. Chemical substances which induce the same kind of genetic damage as radiation have been known for over two decades (Auerbach, 1962). If these or similar substances are released into our environment, they might possibly also represent a serious mutagenic hazard. A major problem has been the development of test systems, capable of detecting and characterizing mutagenic chemicals, which are meaningful and relevant to man. A number of useful systems are now available including cell-free studies of DNA, microbial genetic systems, cytogenetic studies in cell culture and animals, and reproductive studies in laboratory animals in which dominant lethal mutations are detected.

Patulin [4-hydroxy-4*H*-furo(3,2-*c*)pyran-2(6*H*)-one], first described in 1942 (Wiesner, 1942), has been isolated from several species of *Aspergillus* (Kent and Heatley, 1945; Waksman *et al.*, 1944; Wiesner, 1942) and *Penicillium* (Birkinshaw *et al.*, 1943; Chain *et al.*, 1942; Kent and Heatley, 1945; Wilkens and Harris, 1942, 1943) and a species of *Gymnoascus* (Karow and Foster, 1944). Its structure, an $\alpha,\beta$-unsaturated lactone, and its physical and chemical properties have been described by numerous investigators. It is stable in soil (Grossbard, 1952; Jefferys, 1952) and certain fruit juices (Scott and Somers, 1968), and is produced by fungi in apples (Brian *et al.*, 1956) and field crops (Norstadt and McCalla, 1963). Patulin may therefore represent a potential hazard from ingestion of food products contaminated by this substance. One such occurrence has been reported in Japan where mass death of cattle was traced to a patulin-producing fungus isolated from feed (Ukai *et al.*, 1954).

Dickens and Jones (1961) included patulin in their studies of a series of lactones, and found that it was carcinogenic when given to rats in repeated subcutaneous injections. Several lactones containing a 4-membered ring such as $\beta$-propiolactone, as well as some containing a 5-membered ring such as penicillic acid, 2- and 4-hexenoic lactone, and patulin, produced local tumors after prolonged periods of exposure.

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Patulin inhibits cell division, nuclear division, or both in bacteria (Babudieri, 1952), plants (Wang, 1948), and in cell culture (Keilova-Rodova, 1949). In chick embryo cell cultures, mitosis was inhibited and chromosome damage was apparent. Fragmentation of chromosomes was noted in avian eggs during mitosis (Sentein, 1955). In a more recent report, patulin among other lactones was studied in human leukocyte cell culture (Withers, 1965). Patulin caused a high percentage of polyploid cells.

Because patulin is carcinogenic, produces chromosome and mitotic abnormalities, and may be an undesirable contaminant of our environment, we investigated it for potential mutagenic activity.

Our group is currently investigating a method which utilizes the yeast *Saccharomyces cerevisiae* to test for chemical mutagens. The genetic material under investigation resides not in the nucleus of the organism but in the cytoplasm, most probably in the mitochondria. This extrachromosomal hereditary material is responsible for control of many, if not all, of the aerobic processes of mitochondria. Mutation of this genetic factor results in a cell which is unable to reduce the dye 2,3,5-triphenyltetrazolium chloride, cannot utilize carbon sources requiring oxidative degradation such as lactate, acetate, and glycerol, and has a modified cytochrome content (Nagai *et al.*, 1961).

In this investigation, the ability of patulin to cause the forward mutation to petite in *S. cerevisiae* was studied, using the tetrazolium overlay method (Ogur *et al.*, 1957) and the loss of ability to utilize glycerol as criteria in distinguishing wild type from petite mutants.

## METHODS

**Cell Cultures.** A haploid strain of *S. cerevisiae* with approximately 1% spontaneous mutation frequency was kindly donated by Fred Sherman, University of Rochester. Cultures were maintained on nutrient agar with 2% added glucose. For experiments, cells were grown in a semisynthetic liquid medium (Nagai, 1959) aerobically at 30° C. on a rotary shaker. Twenty-four hours before each experiment, the cells were diluted 1 to 100 into fresh liquid medium and the cultures were incubated as before. Preliminary studies of a growth curve under these conditions showed that stationary phase is reached in 18 to 24 hours and that early exponential phase occurs at 3 to 5 hours. Thus, for experiments utilizing stationary phase cells, a 24-hour culture was used. To obtain cells in exponential phase, a 24-hour culture was

inoculated as a concentrated suspension into fresh liquid medium and incubated 4 hours before use.

**Patulin.** Patulin as colorless crystals was kindly supplied by Odette Shotwell, Northern Regional Research Laboratory, USDA. The compound was dissolved in water and diluted so that 1 ml. of solution added to 9-ml. cell suspensions resulted in appropriate concentrations in the treatment flasks.

**Procedure.** For each experiment cells were washed twice and resuspended in 0.85% saline to give a final cell concentration of $1 \times 10^8$ cells per treatment flask. Patulin was added to the treatment flasks to final concentrations of 10, 25, 50, or 75 μg. per ml. on exponential phase cells and 25, 50, or 75 μg. per ml. on stationary phase cells. The flasks were shaken on a rotary shaker for 3 hours at 30° C.; the cells were then washed twice and resuspended in 0.85% saline. The cells were diluted in 10-fold increments; 0.1-ml. aliquots were spread on nutrient agar with 2% glucose and incubated 48 hours at 30° C. For studies of the effect of time, patulin was added to a final concentration of 25 or 50 μg. per ml. on exponential phase and 100 or 200 μg. per ml. on stationary phase cells. The flasks were shaken for 30, 60, 90, 120, or 150 minutes at 30° C., and at the end of the treatment period, the cells were washed, resuspended, diluted, and spread on nutrient agar as above.

**Detection of Survival and Mutation.** Total surviving population for each treatment was enumerated and per cent survival was calculated relative to an untreated control as 100% survival. Other plates containing 100 to 500 colonies were overlaid with tetrazolium agar as described by Ogur *et al.* (1957). After 1 to 2 hours of incubation, colonies were scored on their ability to reduce the dye to form a red color. Petite mutants are unable to do so and remain white. Number of mutants per surviving population (mutation frequency) was calculated for each treatment.

## RESULTS

Figure 1 compares survival of the cells in early exponential and stationary phases which were treated with various concentrations of patulin at 30° C. for 3 hours. Patulin, at all concentrations tested, was more toxic to cells in exponential than in stationary phase. As a specific example, 50 μg. per ml. of patulin resulted in only 1% survival during exponential phase. The same concentration of patulin on stationary phase cells resulted in approximately 80% survival.

Figure 2 compares the mutation frequencies of cells treated with patulin at early exponential and stationary phases. As in the case of toxicity, patulin is more effective at exponential phase than at stationary phase. To use the previous example, 50 μg. per ml. of patulin resulted in a mutation frequency of 65% in exponential phase cells and only 14% in stationary phase cells as compared to mutation frequencies of 2.9 and 2.6% found in the respective control cells.

Figure 3 shows survival of patulin-treated cells withdrawn at the various time intervals. As time progresses, the toxicity increases and again the exponential phase cells show a greater sensitivity to patulin than do stationary phase cells. Patulin at 200 μg. per ml. on stationary phase cells is as effective as only 25 to 50 μg. per ml. on exponential phase cells.

In Figure 4, a similar pattern is observed for mutation frequency when plotted against time. On exponential phase cells, 50 μg. of patulin per ml. produced a frequency of approximately 55% within 1 hour; on stationary phase cells, 200 μg. per ml. produced the same mutation frequency at 2 hours.

To determine whether the mutants formed had maintained their petite character after further growth, colonies arising on plates seeded with patulin-treated wild type cells were isolated and replated several times on glucose nutrient agar. At each passage, the colonies were tested for their ability to reduce triphenyl tetrazolium chloride and were restreaked on 5% glycerol nutrient agar to test for growth on a nonfermentable carbon source.

Many colonies tested were wild type, reducing tetrazolium chloride and growing on glycerol agar. This was to be expected since colonies were chosen at random. Some colonies tested, however, were mutant since they were unable to grow on glycerol agar or reduce tetrazolium chloride on the first isolation. Other colonies, after further restreaking and isolation, then failed to grow on glycerol agar or reduce tetrazolium chloride, thus demonstrating that these colonies originally contained a mixture of wild type and petite cells.

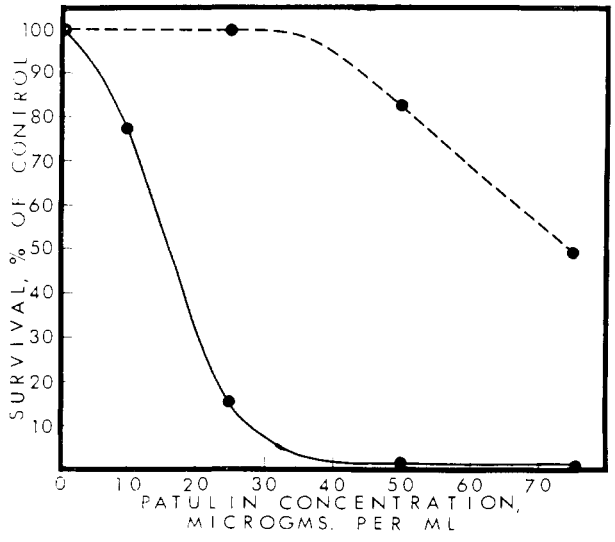


**Figure 1. Survival of *S. cerevisiae* treated 3 hours with patulin**

Stationary phase cells - - - -, exponential phase cells ——

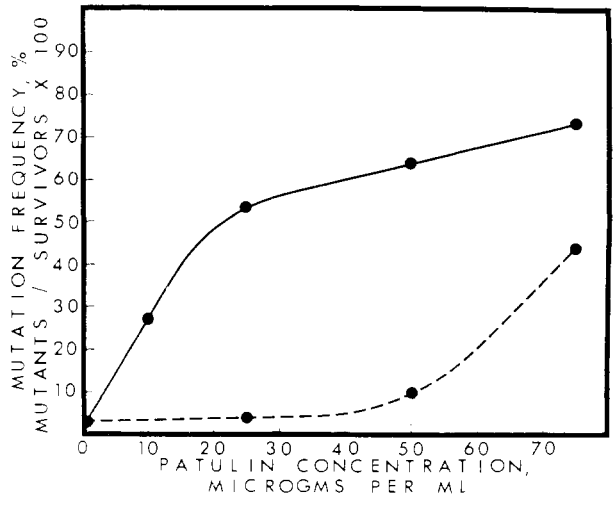


**Figure 2. Mutation of *S. cerevisiae* treated 3 hours with patulin**

Stationary phase cells - - - -, exponential phase cells ——

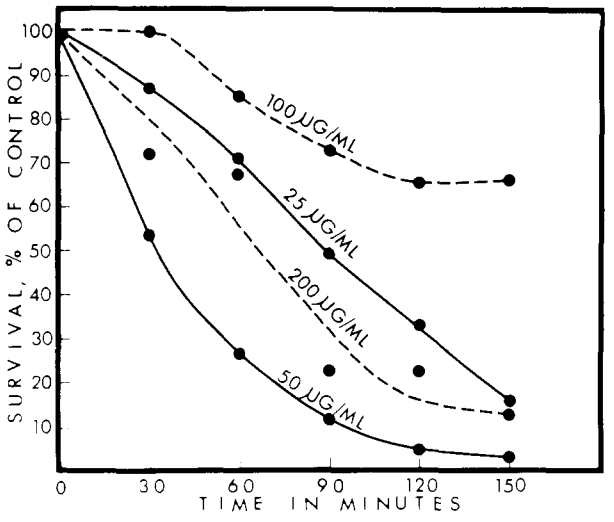


**Figure 3. Survival of *S. cerevisiae* treated with patulin**

Stationary phase cells - - - -, exponential phase cells ——

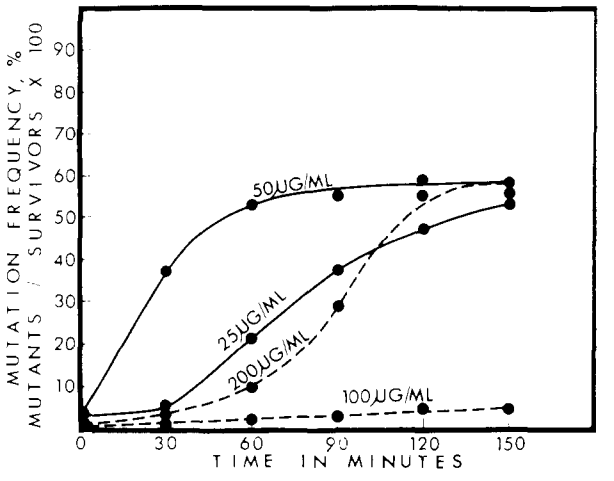


**Figure 4. Mutation of *S. cerevisiae* treated with patulin**

Stationary phase cells - - - -, exponential phase cells ——

## DISCUSSION

The toxic and mutagenic effects of patulin varied with the growth cycle phase of the treated cells. Patulin, at any concentration tested, was more effective on exponential phase cells than on stationary phase cells. The appearance of the ultrastructure of mitochondria of yeast cells during aerobic growth cycle has previously been described (Yotsuyanagi, 1962). Yeast cells in early exponential phase contain only a few mitochondria with a poorly defined inner structure. Respiration, at this time, is minimal. Mitochondrial development begins between exponential and stationary phases and cells contain many mitochondria with well-developed cristae. Respiration, at this time, reaches maximal levels. We suspect that the activity of patulin is related to these differences in mitochondrial appearance and function but the relationship is unknown.

The production of petite mutants by patulin is not due to selection of already existing mutants because the absolute number of mutant cells increased in the treated samples, as compared to control cultures which contained only mutants arising spontaneously. Furthermore, patulin and control samples were treated in 0.85% saline which imposed nongrowing conditions on the cells. Thus the increase in mutation frequency could not be due to conditions which selectively favored the growth of one or the other type. Duration of treatment was sufficiently short so that selective growth conditions for wild or mutant type, had they existed, could not have been a significant factor.

The fact that a colony arising from patulin treatment sometimes proved to be a mixture of wild and petite cells upon streaking on agar plates was at first disturbing. However, under aerobic growth conditions, many mitochondria are present per cell (Yotsuyanagi, 1962), perhaps each must be affected by patulin to produce a pure mutant cell. Further cell division after treatment would permit the segregation of mitochondria so that some cells have only nonfunctional mitochondria and are therefore mutant.

Petite mutants in yeast arise by two possible mechanisms: inactivation or mutation of the genetic material, DNA, or inactivation or elimination of one or more of the respiratory enzymes. The role of patulin in the production of petite mutants of yeast is unknown. Patulin inhibits aerobic respiration of bacteria (Chain *et al.*, 1942, 1944), fungi (Gottlieb and Singh, 1964), and mammalian cells (Andraud *et al.*, 1963; Delaunay *et al.*, 1955); however, if a genetic change is involved, this inhibition may be an effect rather than a cause. The hypothesis that an enzyme or series of enzymes is inhibited is not consistent with our finding that mutants arising from patulin treatment represent a change which is not reversed by removal of the compound.

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