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# SCREENING OF KILLER-SENSITIVE-PATTERN (KSP) FOR BIOTYPING YEAST STRAINS ISOLATED FROM SLIME FLUXES OF TREES AND FLOWERS' NECTAR

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**Abstract**

Killer-Sensitive Pattern (KSP) was screened by cross reactions in 23 yeast species belonging to 13 genera which were previously isolated from slime fluxes of trees and 56 yeast species belonging to 23 genera from nectar of different flowers. Among the yeasts species from slime fluxes, *Bullera pseudoalba*, *Pichia anomala* and *Sporidiobolus ruineniae* appeared to be the most killer and *Pichia strasburgensis* as the most sensitive strains. Similarly among the yeasts species from flowers' nectar *Bensingtonia miscanthi* and *Bullera megalospora* were found to be as the most killer strains, whereas *Candida valdiviana*, *Cryptococcus laurentii*, *Mrakia frigida*, *Pichia fabianii*, *Pichia jadinii*, *Saccharomyces kluyveri* and *Williopsis californica* were found as the most sensitive strains. The Killer-Sensitive Pattern (KSP) appeared as strain level character rather than species.

## Introduction

Certain yeasts produce killer toxins (mycocins), which are lethal to closely related strains but the killer yeast itself has a killer resistant phenotype (Bevan & Makower, 1963; Woods & Bevan, 1968; Bussey, 1972; Pfeiffer & Radler, 1982; Spencer & Spencer, 1997). The killer phenomenon provides an excellent model system to study host-virus interactions in eukaryotic cells (Wickner, 1979) and to investigate the mechanisms of protein processing and secretion (Douglas *et al*., 1988). Possible uses of killer phenomenon, which aroused great interest, include the differentiation of pathogenic strains (Morace *et al*., 1984) and their possible role in ecosystems mainly in natural fermentation processes (Starmer *et al*., 1987; Vagnoli *et al*., 1993; Hidalgo & Flores, 1994). Killer activity is one of the mechanisms of antagonism among yeasts during spontaneous fermentations and because of this mechanism killer strains could dominate at the end of the wine fermentation (Bussey *et al*., 1988; Jacobs et *al*., 1988; Longo *et al*., 1990).

Killer toxins are protein in nature and active at low pH (Young & Yagiu, 1978; Pfeiffer & Radler, 1982; Radler *et al*., 1985). They are secreted in an inactive glycosylated form that once secreted to the cell plasma membrane, becomes cleaved (Zhu *et al*., 1993). A portion of the toxin with the glycosylated site remains associated with the membrane and conveys immunity to the cell. The cleaved mature toxin is available to bind at the sites located on the cell wall and the plasma membrane of sensitive yeasts. However the phenomenon of insensitivity towards killer toxins generally occurs at the cell wall level. Resistant yeasts lack receptors necessary for the formation of the link and thus for the action of the killer toxin (Marquina *et al*., 2002; Golubev, 2006). As a result, if different cell wall chemical compositions are taxon-associated; resistance, causing insensitivity could be a taxon-related property as well (Golubev, 1998, 2006). Based on

evidence that the chemical composition of yeast cell walls is a taxon related characteristic, Golubev (2006) hypothesized that KSP profiles may have taxonomic relevance. The theoretical rationale supporting this conclusion is related to the resistance mechanism. In the present study Killer-Sensitive-Pattern (KSP) has been screened by cross reactions in yeast species previously isolated from slime fluxes of different trees (Mushtaq *et al*., 2005; Mushtaq *et al*., 2008) and flower's nectar (Mushtaq *et al*., 2007; Mushtaq *et al*., 2008).

## Materials and Methods

A modified method of Abranches *et al*., (1997) was used to screen Killler-Sensitive-pattern (killer, sensitive and neutral phenotypes) in 23 yeasts species belonging to 13 genera previously isolated from slime fluxes of trees and 56 yeast species belonging to 23 genera from flowers' nectar, on yeast extract-malt extract agar supplemented with 0.003% methylene blue (YM-MB Agar). Twenty-four h old yeast culture grown on YM agar (Kreger-van Rij, 1984) was diluted in double distilled sterile water to obtain a suspension of $4x10^5$ cells/ml and spread with a sterile cotton swab as seeded (lawn) cultures on the surface of YM-MB agar in Petri plates and dried. Fresh cultures of the yeasts to be tested were grown on YM agar for 24 h and each inoculated in a single streak on plates seeded with the yeast culture and incubated at 25±1°C for 10 days and observed daily. The seeded yeast was considered as killer if a blue colored killing zone appeared on streak and sensitive if killing zone appeared around the streak on lawn. Intensity of the killer activity was recorded as $K^{+1}$ (very light blue killing zone), $K^{+2}$ (blue killing zone), $K^{+3}$ (dark blue killing zone) and $K^{+4}$ (intense dark blue killing zone) reaction. The sensitivity of the yeast was also recorded in the same manner as $S^{+1}$, $S^{+2}$, $S^{+3}$ and $S^{+4}$. A negative reaction indicated by (-) when yeasts did not show any reaction as designated as neutral strains. Percentages of killing activity and sensitivity of yeast species were calculated. Strains that showed >50% killing activity or sensitivity were considered as super killers and super sensitive.

## Results

Killer-Sensitive-Pattern (KSP) was screened in 56 yeast species belonging to 23 genera (previously isolated from nectar of different plants) by cross reactions. Spectrum of killing activity and sensitivity (species vise) is presented respectively in table 1 and their percentages in table 2. *Bensingtonia miscanthi* and *Bullera megalospora* with 41.07% killing activity were found as the most killer strains. However, significant killer activity was also observed in strains of *Bullera pyricola*, *B. pseudoalba*, *Candida friedrichii*, *C. gropengiesseri*, *C. magnoliae*, *C. membranifaciens*, *C. rhagii*, *C. sake*, *C. succiphila*, *C. versatilis*, *C. xestobii*, *Cryptococcus albidus*, *Phaffia rhodozyma* and *Pseudozyma fusiformata* (Tables 1 and 2). Similarly some of the yeast species appeared as the most sensitive strains. These included *Candida valdiviana*, *Cryptococcus laurentii*, *Mrakia frigida*, *Pichia fabianii*, *Pichia jadinii*, *Saccharomyces kluyveri* and *Williopsis californica* (Tables 1 and 2).

The killer strains *Bensingtonia miscanthi* and *Bullera megalospora*, as well as, other killer strains produced strong ($K^{+3}$) and very strong ($K^{+4}$) killing zones specially against most sensitive strains and in some cases also against other species like *Bullera pseudoalba*, *Candida xestobii*, *Cryptococcus albidus*, *C. laurentii*, *Pichia mississippiensis* and *Rhodotorula toruloides* (Tables 1 and 2).

The Killer-Sensitive-Pattern (KSP) was also screened in 23 yeast species belonging to 13 genera previously isolated from slime fluxes of trees. Spectrum of killing activity and sensitivity, and their percentages are respectively presented in table 3 and 4. During these cross reactions *Pichia strasburgensis* (77.27%) appeared to be the most sensitive strain, whereas, *Bullera pseudoalba* (63.64%), *Pichia anomala* (77.27%) and *Sporidiobolus ruineniae* (59.10%) were found to be the most killer strains (Tables 3 and 4).

All the killer strains obtained by cross reactions from slime fluxes showed strong killing action ($K^{+3}$) against *Pichia strasburgensis* as well as against *Candida valdiviana*, *Cryptococcus albidus*, *C. gastricus*, *Debaryomyces hansenii*, *Phaffia rhodozyma*, *Pichia angusta*, *Saitoella complicata* and *Williopsis californica*. *Candida succiphila*, *Cryptococcus albidus*, *C. gastricus*, *Pichia angusta*, *P. rabaulensis* and *Rhodosporidium toruloides* also showed significant killing activity against species of their own community (Tables 3 and 4). Apart from super killer strains, *Candida succiphila*, *Cryptococcus albidus*, *C. gastricus*, *Pichia angusta*, *P. rabaulensis*, *Rhodosporidium toruloides* also showed various degree of killing activity (Tables 3 and 4).

It is important to note that a number of yeasts appeared neutral i.e., showed no reaction even against the supper killer strains. This phenomenon of insensitivity towards killer yeasts generally occurs at the cell wall level. Resistant (neutral) yeasts lack receptors necessary for the formation of the link and thus for the action of the killer toxin (Marquina *et al*., 2002; Golubev, 2006). Taxonomically, different cell wall compositions are used for classification of organisms. As a result, if different cell wall chemical compositions are taxon-associated, then resistance causing insensitivity could be a taxon-related property as well (Golubev, 1998, 2006).

In few studies Golubev (Golubev, 1992; Golubev *et al*., 1997) inferred that killer toxin effectiveness is inversely related to phylogenetic affinity (e.g., ascomycetous yeasts are usually insensitive to toxins produced by basidiomycetous species and vice versa). However, in the present studies, we observed a mixed effectiveness of killer yeasts against the neutral (insensitive) yeasts (Table 1). In this context, we emphasize which Golubev (2006) also emphasized in his studies that the use of killer toxins as a taxonomic tool should be preceded by a careful study of their KSP. Broad-spectrum killer toxins should be used for overall phylogenetic evaluation, while those characterized by a narrow range of activity may be used for clarifying relationships between more closely related species, or for grouping phenotypically similar strains before using molecular techniques e.g., nucleotide composition in the D1/D2 domains and ITS regions of the ribosomal DNA (r-DNA).

On the other hand a number of yeast strains showed strong killing activity sensitive yeasts by cross reactions. In nature certain strains of killer yeasts dominate only in particular niches (Zorg *et al*., 1988). Killer activity is one of the mechanisms of antagonism among yeasts during spontaneous fermentations and because of this mechanism killer strains may be used to avoid contamination by sensitive spoilage yeasts (Starmer *et al*., 1987; Bussey *et al*., 1988; Jacobs *et al*., 1988; Longo *et al*., 1990; Vagnoli *et al*., 1993; Hidalgo and Flores, 1994).

**Table 2. Percentages of killer, sensitive and neutral phenotypes in yeasts' species isolated from nectar.**

| No. | Yeast species | Phenotypes of seeded yeast strains (%) | | |
|---|---|---|---|---|
| | | Killer | Sensitive | Neutral |
| 1. | *Bensingtonia miscanthi* | 40.00 | 5.45 | 54.55 |
| 2. | *Bullera megalospora* | 41.81 | 1.82 | 56.36 |
| 3. | *B. pseudoalba* | 32.73 | 5.45 | 61.82 |
| 4. | *B. pyricola* | 25.45 | 3.64 | 70.91 |
| 5. | *Candida friedrichii* | 21.82 | 0.00 | 78.18 |
| 6. | *C. gropengiesseri* | 29.09 | 3.64 | 67.27 |
| 7. | *C. magnoliae* | 27.27 | 9.09 | 63.64 |
| 8. | *C. membranifaciens* | 25.45 | 9.09 | 65.45 |
| 9. | *C. rhagii* | 20.00 | 0.00 | 80.00 |
| 10. | *C. sake* | 21.82 | 16.36 | 61.82 |
| 11. | *C. succiphila* | 20.00 | 1.82 | 78.18 |
| 12. | *C. valdiviana* | 18.18 | 41.82 | 40.00 |
| 13. | *C. versatilis* | 29.09 | 10.91 | 60.00 |
| 14. | *C. xestobii* | 25.45 | 3.64 | 70.91 |
| 15. | *Cryptococcus albidus* | 20.00 | 21.82 | 58.18 |
| 16. | *C. curvatus* | 10.91 | 3.64 | 85.45 |
| 17. | *C. flavus* | 16.36 | 7.27 | 76.36 |
| 18. | *C. heveanensis* | 16.36 | 10.91 | 74.55 |
| 19. | *C. humicolus* | 9.09 | 5.45 | 85.45 |
| 20. | *C. hungaricus* | 14.55 | 10.91 | 74.55 |
| 21. | *C. laurentii* | 14.55 | 41.82 | 43.64 |
| 22. | *C. macerans* | 9.09 | 14.55 | 76.36 |
| 23. | *Cystofilobasidium bisporidii* | 18.18 | 5.45 | 76.36 |
| 24. | *Debaryomyces castellii* | 5.45 | 16.36 | 78.18 |
| 25. | *D. hansenii* | 7.27 | 5.45 | 87.27 |
| 26. | *D. vanrijii* | 3.64 | 16.36 | 78.18 |
| 27. | *Exophila salmonis* | 3.64 | 9.09 | 87.27 |
| 28. | *Fibulobasidium inconspicuum* | 9.09 | 23.64 | 67.27 |
| 29. | *Filobasidiella neoformans* | 7.27 | 1.82 | 90.91 |
| 30. | *Issatchenkia occidentalis* | 3.64 | 23.64 | 72.73 |
| 31. | *Lipomyces starkeyi* | 7.27 | 1.82 | 90.91 |
| 32. | *Mrakia frigida* | 1.82 | 45.45 | 52.73 |
| 33. | *Phaffia rhodozyma* | 21.82 | 7.27 | 70.91 |
| 34. | *Pichia angusta* | 12.73 | 20.00 | 67.27 |
| 35. | *P. castillae* | 9.09 | 29.09 | 61.82 |
| 36. | *P. dryadoides* | 5.45 | 7.27 | 87.27 |
| 37. | *P. fabianii* | 5.45 | 54.55 | 40.00 |
| 38. | *P. guilliermondii* | 7.27 | 18.18 | 74.55 |
| 39. | *P. jadinii* | 14.55 | 41.82 | 45.45 |
| 40. | *P. lynferdii* | 16.36 | 3.64 | 80.00 |
| 41. | *P. methanolica* | 7.27 | 0.00 | 92.73 |
| 42. | *P. mississippiensis* | 12.73 | 20.00 | 67.27 |
| 43. | *P. ofunaensis* | 14.55 | 0.00 | 85.45 |
| 44. | *P. ohmeri* | 10.91 | 34.55 | 58.18 |
| 45. | *Pseudozyma antarctica* | 16.36 | 16.36 | 69.09 |
| 46. | *P. fusiformata* | 21.82 | 1.82 | 76.36 |
| 47. | *Rhodosporidium toruloides* | 14.55 | 29.09 | 60.00 |
| 48. | *Rhodotorula fragaria* | 16.36 | 9.09 | 74.55 |
| 49. | *R. hinnulea* | 20.00 | 25.45 | 60.00 |
| 50. | *Saccharomyces kluyveri* | 7.27 | 60.00 | 34.55 |
| 51. | *Sporidiobolus ruineniae* | 10.91 | 21.82 | 69.09 |
| 52. | *Stephanoascus ciferii* | 12.73 | 25.45 | 60.82 |
| 53. | *Tremella aurentia* | 10.91 | 5.45 | 85.45 |
| 54. | *Williopsis californica* | 7.27 | 52.73 | 41.82 |
| 55. | *W. pratensis* | 7.27 | 9.09 | 83.64 |
| 56. | *Zygoascus helinicus* | 7.41 | 18.18 | 74.55 |

**Table 4. Percentages of killer, sensitive and neutral phenotypes in yeast species isolated from gum.**

| No. | Yeast species | Phenotypes of seeded yeast strains (%) | | |
|---|---|---|---|---|
| | | Killer | Sensitive | Neutral |
| 1. | *Bullera pseudoalba* | 63.64 | 4.54 | 36.36 |
| 2. | *Candida lyxosophila* | 18.18 | --- | 81.81 |
| 3. | *C. succiphila* | 36.36 | 27.27 | 36.36 |
| 4. | *C. valdiviana* | 13.64 | 40.71 | 50.00 |
| 5. | *Cryptococcus albidus* | 36.36 | 36.36 | 36.36 |
| 6. | *C. gastricus* | 36.36 | 4.54 | 59.10 |
| 7. | *Debaryomyces castellii* | 9.09 | 36.36 | 54.54 |
| 8. | *D. hansenii* | 4.54 | --- | 95.44 |
| 9. | *D. yamadae* | 22.72 | 31.81 | 45.45 |
| 10. | *Fibulobasidium inconspicuum* | 27.27 | 27.27 | 45.45 |
| 11. | *Mrakia frigida* | 18.18 | --- | 81.81 |
| 12. | *Phaffia rhodozyma* | 18.18 | 36.36 | 45.45 |
| 13. | *Pichia angusta* | 31.81 | 18.18 | 50.00 |
| 14. | *P. anomala* | 77.27 | --- | 22.72 |
| 15. | *P. lynferdii* | 22.72 | 22.72 | 59.01 |
| 16. | *P. methanolica* | 4.54 | --- | 95.45 |
| 17. | *P. rabaulensis* | 36.36 | 13.64 | 50.00 |
| 18. | *P. strasburgensis* | --- | 77.27 | 22.72 |
| 19. | *Rhodosporidium toruloides* | 22.72 | 18.18 | 59.10 |
| 20. | *Rhodotorula bacarum* | 4.54 | --- | 95.45 |
| 21. | *Saitoella complicata* | 18.18 | 36.36 | 45.45 |
| 22. | *Sporidiobolus ruineniae* | 59.10 | 22.72 | 27.27 |
| 23. | *Williopsis californica* | 13.64 | 36.36 | 50.00 |

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